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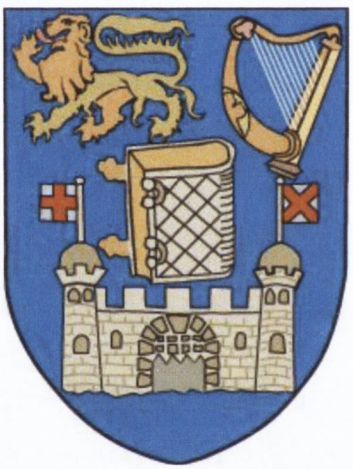
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Characterisation of T cell responses during *Staphylococcus aureus* infection

Alison Murphy

B.A (Mod) Biochemistry with Immunology



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School of Biochemistry and Immunology

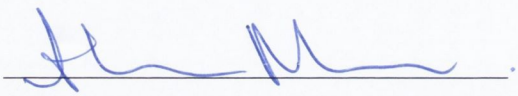
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Publications

Staphylococcus aureus infection of mice expands a population of memory γδ T cells that are protective against subsequent infection.

Murphy AG, O'Keeffe KM, Lalor SJ, Maher BM, Mills KHG, McLoughlin RM

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Targeted Nasal Vaccination Provides Antibody-Independent Protection Against *Staphylococcus aureus*.

Misstear K*, McNeela EA*, **Murphy AG***, Geoghegan JA, O'Keeffe KM, Fox J, Chan K, Heuking S, Collin N, Foster TJ, McLoughlin RM, Lavelle EC.

J Infect Dis. 2013 Dec 29. doi: 10.1093/infdis/jit636

*denotes equal contribution.

Nlrp-3-driven interleukin 17 production by γδT cells controls infection outcomes during Staphylococcus aureus surgical site infection.

Maher BM, Mulcahy ME, **Murphy AG**, Wilk M, O'Keeffe KM, Geoghegan JA, Lavelle EC, McLoughlin RM.

Infect Immun. 2013 Dec; 81(12):4478-89.

Murphy AG. & McLoughlin RM. Chapter 10: The importance of cell mediated immunity for bacterial vaccines. *Vaccine Design: Innovative Approaches and Novel Strategies.* Horizon Press. Publication date 2015.

Abbreviations

αβ	Alpha beta
Ahr	Aryl Hyrdocarbon Receptor
AIDS	Acquired Immunodeficiency Syndrome
AMPs	Antimicrobial peptides
APC	Antigen Presenting Cell
BFA	Brefeldin A
CA	Community Acquired
CD	Cluster of Differentiation
CFA	Complete Freund's Adjuvant
CFU	Colony Forming Unit
CGD	Chronic Granulomatous Disease
ClfA	Clumping Factor A
CLR	C type Lectin Receptors
CP	Capsular Polysaccharide
CpG	CpG oligodeoxynucleotide
CSA	Columbia Soy Agar
CSF	Colony Stimulating Factor
CTL	Cytotoxic Lymphocyte
d	Day
D	Diversity (TCR gene segment)
DAMP	Danger Associated Molecular Pattern
DC	Dendritic Cell
123-DHR	123-Dihyrdorhodemine
DNA	Deoxyribonucleic Acid
Eap	Extracellular Adhesion Protein
ELISA	Enzyme Linked Immunosorbent Assay
FACS	Flouresence Activated Cell Sorting
GM-CSF	Granulocyte macrophage colony stimulating factor
G-CSF	Granulocyte colony stimulating factor
γδ	Gamma delta
h	Hour
HA	Hospital Acquired
HIES	Hyper IgE Syndrome
HIV	Human Immunodeficiency Virus
HK	Heat Killed
HMBPP	Hydroxy-3-methyl-but-2-enyl pyrophosphate
ICAM-1	Intercellular Adhesion Molecule 1
Ig	Immunoglobulin
IL	Interleukin
IL-1R$^{-/-}$	IL-1 receptor deficient
IELs	Intraepithelial lymphocytes
IFNγ	Interferon gamma
ILC	Innate lymphoid cells

Ion	Ionomycin
I.p.	Intraperitoneal
IPP	Isopentenyl pyrophosphate
IsdB	Iron regulated surface determinant B
J	Junctional (TCR gene segment)
KC	Keratinocyte-derived chemokine
LPS	Lipopolysaccharides
LTi	Lymphoid Tissue inducer cells
MCP-1	Monocyte Chemotactic Protein 1
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MIC A/B	MHC class I-related chains A/B
MIP-2	Macrophage Inflammatory Protein 2
mins	Minutes
ml	Millilitre
µl	Microlitre
MLN	Mediastinal Lymph Node
µM	Micromolar
MRSA	Methicillin resistant *S. aureus*
NOD	Nucleotide-binding Oligomerisation Domain
NET	Neutrophil Extracellular Traps
NK	Natural Killer
NLR	NOD-like Receptor
NO	Nitric Oxide
NOS2	Nitric oxide synthase 2
OD	Optical Density
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEC	Peritoneal Exudate Cells
PMA	Phorbol 12-myristate 13-acetate
PNAG	Poly-N-acetyl glucosamine
PRRs	Pathogen Recognition Receptors
PVL	Panton Valentine Leukotoxin
QS21	*Quillaja saponaria* fraction 21
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted
RLR	RIG-like Receptor
RNA	Ribonucleic acid
RNS	Reactive Nitrogen Species
RORγt	Retinoid-related orphan receptor γt
ROS	Reactive Oxygen Species
RPM	Rotations per minute
RT	Real Time
s.c.	Subcutaneous
SCID	Severe combined immunodeficiency

SEA	Staphylococcal Enterotoxin A
STAT	Signal Transducers and Activators of Transcription
TAP	Transporter associated with Antigen Processing
T-bet	T box Transcription Factor
TCR	T cell Receptor
TGFβ	Transforming Growth Factor beta
Th	T helper cell
TLRs	Toll Like Receptor
TNFα	Tumour Necrosis Factor alpha
Treg	Regulatory T cell
TSA	Tryptic Soy Agar
TSST-1	Toxic shock syndrome toxin 1
UEA-1	*Ulex europaeus* agglutinin 1
V	Variable (TCR gene segment)
WT	Wild Type

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Abstract

The development of anti-*S. aureus* vaccines has consistently failed in clinical trials, likely due to inefficient induction of cellular immunity. A comprehensive understanding of the discrete T cell subsets critical for site-specific bacterial clearance will be necessary to inform the development of vaccines that efficiently target adaptive cellular immunity. To investigate how exposure to *S. aureus* impacts cellular immunity, a novel murine model of recurrent *S. aureus* peritonitis was developed. Prior exposure to *S. aureus* protected against subsequent infection through increased IFNγ production by CD4 and CD8 T cells, elevated IL-17 secretion by γδ T cells and enhanced phagocyte recruitment and activation.

Further characterisation of γδ T cell responses revealed that prior exposure expanded a population of memory γδ T cells, with a $V\gamma4^{+}CD44^{+}CD27^{-}$ phenotype, that produce increased levels of IL-17 and mediate enhanced bacterial clearance upon re-infection with *S. aureus*. In contrast to its critical requirement for naive γδ T cell activation, IL-1 signalling was dispensable for activation and expansion of memory γδ T cells upon re-exposure to *S. aureus*. Preliminary work indicated that these memory γδ T cells were *S. aureus*-specific and partially relied on MHC II signalling for activation.

The importance of Th1 cells in anti-staphylococcal immunity was confirmed, as adoptive transfer of *S. aureus*-specific Th1 cells protected naive mice against infection through enhanced macrophage responses. With the identification of protective Th1 cells, a model vaccine was designed utilising the Th1 inducing adjuvant CpG combined with the staphylococcal antigen ClfA. Vaccine induced type 1 immunity protected against *S. aureus* infection through enhanced phagocyte activation. Given that prior exposure and subcutaneous vaccination also induced humoral immunity, antibody responses could potentially be contributing to protection in these models. The importance of anti-staphylococcal cellular immunity was definitively proven, through musocal vaccination with UEA-1m targeted, ClfA loaded nanoparticles, which drove a purely protective cellular response, independently of an antibodies, against systemic *S. aureus* infection.

The identification of protective T cell subsets contributes to rational anti-*S. aureus* vaccine design, as these specific T cell phenotypes could potentially be generated by immunisation strategies to induce full prophylactic protection. These findings demonstrate that memory T cell responses can be induced upon *S. aureus* exposure, and suggest that both IFNγ and IL-17 expressing T cells are important correlates of anti-staphylococcal immunity.

Chapter 1

Introduction

1.1. Core functions of the immune system

The immune system comprises a myriad of cells and molecules which fulfil a series of core functions. Initially, immune recognition facilitates the distinction of infectious non-self from self. This recognition then triggers the immune system to mount an appropriate effector immune response against the invading pathogen. This complex multi-component process translates initial recognition into a response that specifically eliminates or neutralises the invasive pathogen. Concurrently, the immune response is under tight control, to limit collateral damage to the host. Immune regulation, or the ability of the immune response to self-regulate, is an essential feature of immunity, the loss of which can lead to the development of allergic and autoimmune diseases. Finally, the immune system strives to protect against the reoccurrence of the same infection by generating immunological memory.

Though referred to as a single entity the immune response can be broadly divided into two systems, the innate and the adaptive immune system. It is important to note that innate and adaptive immune responses do not function independently of one another but rather act in concert to clear pathogenic threats and protect the host. Innate immune recognition is executed via a series of constitutively expressed receptors, known as pathogen recognition receptors (PRRs), which efficiently recognise pathogen associated molecular patterns (PAMPs) conserved amongst different microbial species. However, they are not equipped to distinguish between subtle differences in molecules expressed by individual species. The second arm of immunity, known as the adaptive immune response, develops and adapts to recognise and eliminate pathogens in a specific manner and is capable of distinguishing antigens from different bacterial species. Once activated it provides a comprehensive line of defence to eliminate pathogens that have overcome innate immunity. A critical aspect of the adaptive immune response is the generation of immunological memory which is the fundamental basis of vaccination.

1.2. The Innate Immune Response to Bacterial Infection

1.2.1. Pathogen Recognition Receptors

The innate immune system comprises antigen presenting cells (APCs) such as Dendritic cells (DCs) and macrophages which express multiple PRRs capable of recognising PAMPs. PRRs are germline encoded receptors responsible for the detection of microbially derived PAMPs and host derived danger associated molecular patterns (DAMPs). PAMPs are evolutionarily conserved across a wide range of microorganisms, due to their vital role in microbial viability. The triggering of several PRRs simultaneously by bacterial PAMPS induces diverse innate immune responses, the downstream effects of which provide the necessary signals to generate an effective adaptive immune response. PRR ligation can also result in the production of a range of defensive chemicals, such as cytokines and chemokines, capable of recruiting other effector cell types (1). Different pathogenic bacteria express distinct PAMPs, which function as a bacterial "fingerprint" recognised by PRRs, leading to the signalling events that tailor the immune response to particular bacteria.

There are several families of PRRs including Toll-like receptors (TLRs), retinoic acid-inducible gene -1 (RIG)-like receptors (RLRs), nucleotide binding oligomerisation domain (NOD)-like receptors (NLRs) and C type lectin receptors (CLRs). TLRs 1, 2, 4, 5 and 6 are found primarily on the surface of innate cells where they sample the surrounding environment for bacterial PAMPs (2, 3). For example TLR4 recognises lipopolysaccharide (LPS), a component of gram negative bacteria, whereas TLR2 recognises lipoteichoic acid, a component of gram positive bacteria. TLRs 3, 7, 8 and 9 are exclusively expressed in endocytic compartments within the cytoplasm and are responsible for sensing nucleic acids from viruses or intracellular bacteria (2, 3). The RLR family is primarily responsible for the recognition of ribonucleic acid (RNA) from RNA viruses (4, 5). NOD1 and NOD2 are two cardinal members of the NLR family which recognise and respond to the degradation products of bacterial peptidoglycan, a cell wall component of gram positive bacteria (5, 6). CLRs recognise mannose, fucose and glucan carbohydrate structures which have been shown to be important in the recognition of mycobacteria. Recognition by CLRs leads to internalisation, degradation and subsequent presentation of the invading bacteria (7).

1.2.2. Neutrophils

Neutrophils are mononuclear phagocytic cells that are capable of recognising, engulfing and killing bacteria. Neutrophils are short-lived cells which are abundant in the circulation but not normally present in healthy tissues. They are released from the bone marrow into the bloodstream as mature or nearly mature cells lacking proliferative potential. Neutrophils can be characterised morphologically through identification of their polylobed nucleus or by their expression of the surface molecule Ly6G in mice and CD16 in humans, and myeloperoxidase (an enzyme involved in respiratory burst). Upon infection or injury, neutrophils in the peripheral blood are rapidly recruited to infection sites. Local cells in the infected or inflamed tissue release a range of cytokines such as interleukin 1β (IL-1β) and tumour necrosis factor α (TNFα), which upregulate adhesion molecules on vascular endothelial cells. Neutrophil receptors then interact with these adhesion molecules initiating a series of events that leads to extravasion, a process whereby the neutrophil squeezes between adjacent vascular endothelial cells. Upon entry into the tissues neutrophils migrate along a chemokine (chemotaxis signalling proteins) concentration gradient secreted by cells at the site of infection (8).

At the site of infection neutrophils recognise pathogens via a series of cell surface receptors including PRRs. Neutrophils express Fc receptors and complement receptors, which allow them to recognise antibody or complement opsonified bacteria. Neutrophils are also capable of inducing further recruitment of immune cells via the release of chemokines, cytokines and lipid mediators. The production of cytokines such as IL-1β (9), IL-17 (10) TNFα, CC and CXC chemokines as well as angiogenic factors (11) by neutrophils has been reported, highlighting neutrophils' modulatory role in inflammation.

The key role of neutrophils at the site of infection however, lies in their function as phagocytic cells. Recognition of a bacterium leads to phagocytosis. During this process, a phagocytic membrane extruded from the neutrophil engulfs the bacterium internalising it into a membrane enclosed vesicle, known as the phagosome. This phagosome becomes acidified which kills most pathogens. In addition to this, neutrophils contain lysosomes which are membrane enclosed granules containing enzymes, proteins and peptides that attack the microbe. The lysosome fuses with the phagosome, which contains the bacteria, to form the phagolysosome. Upon formation of the phagolysosome, the lysosomal contents are released allowing the bacteria to be killed safely in a contained compartment; a process referred to as oxygen independent killing. In addition to bactericidal effects of the

lysosomal contents and the acidic pH within the phagosome, the enzyme NADPH oxidase assembles on the phagosomal membrane and generates antimicrobial reactive oxygen species (ROS) by a process known as respiratory burst. NADPH oxidase transfers electrons from intracellular NADPH to molecular oxygen to produce the reactive free radical superoxide. Superoxide is capable of spontaneously forming hydrogen peroxide which can be converted to hypochlorous acid by the enzyme myeloperoxidase (MPO). The ROS generated as a result of oxidative burst kill the bacteria within the phagosome; this is referred to as oxygen dependent killing (8, 12-14).

Neutrophils can also impede bacterial growth through the extracellular release of antimicrobial proteins or peptides (AMPs) into their surrounding environment, which antagonise bacterial growth and compromise bacterial integrity (14). Neutrophils are also capable of killing non-phagocytosed bacteria through the formation of neutrophil extracellular traps (NETs) (15). NETs are comprised of tangled chromatin and granule proteins released upon rupture of the neutrophil cell membrane. These structures ensnare bacteria resulting in their exposure to high local concentrations of antimicrobial molecules. This process of NET release represents an alternative death to necrosis or apoptosis and is often referred to as NETosis. Net inducing stimuli include whole bacteria such as *Staphylococcus aureus* (16, 17), *Klebsiella pneumoniae* (18), *Listeria monocytogenes* (19) and *Mycobacterium tuberculosis* (20) as well cell surface components such as LPS.

Upon bacterial phagocytosis and destruction, neutrophils undergo apoptosis (programmed cell death) (21). Apoptotic neutrophils express phosphatidylserine on their outer cell membranes which act as "eat me" signals enabling their recognition and ingestion by resident macrophages. Apoptosis of inflammatory neutrophils and their subsequent clearance by macrophages prevents the lytic release of noxious components within the neutrophil into the microenvironment (22), which can drive the persistence of inflammation and impair bacterial clearance (23). Additionally, ingestion of apoptotic neutrophils by macrophages triggers the production of anti-inflammatory mediators by the macrophages (24, 25), initiating the resolution of inflammation.

1.2.3. Macrophages

Macrophages are mononuclear phagocytic cells which mature continuously from monocytes and migrate from the circulation to various tissues throughout the body. Tissue resident macrophages are highly specified depending on their anatomical location, for

example microglia in the brain and Kupffer cells in the liver. Tissue resident macrophages are the first cells encountered by the invading bacteria and are responsible for the initial recognition of the bacterium, but are soon reinforced by recruited neutrophils. Macrophages express PRRs such as CD14 and TLRs that bind PAMPS on the invading bacteria. Recognition of a bacterium leads to phagocytosis as well as secretion of pro-inflammatory cytokines by the macrophage, which initiate the recruitment of other effector immune cells (13).

Like neutrophils, macrophages are capable of phagocytosing bacteria into a phagosome which then fuses with the lysosome. Within this phagolysosome toxic enzymes digest the bacteria. Macrophages predominantly produce reactive nitrogen species (RNS) which are antimicrobial molecules derived from nitric oxide (NO) and superoxide, produced by the enzymes nitric oxidase synthethase 2 (NOS2) and NADPH oxidase respectively. NOS2 is expressed primarily by macrophages following induction via cytokines and microbial products, notably Interferon γ (IFNγ) or LPS. These reactive nitrogen species are toxic for microorganisms and also promote pro-inflammatory pathways (8, 14).

Activation of macrophages increases their ability to clear intracellular bacteria (26). IFNγ stimulated macrophages demonstrate increased bacterial clearance (27) which is associated with maturation of the phagosome and fusion with lysosomes to form phagolysosomes (28). This response can be further enhanced via engagement of TLRs (29). IL-1β was also shown to play a role in macrophage mediated intracellular killing as IL-1β signalling enables macrophages to overcome the arrested maturation of phagosomes containing *M. tuberculosis* (30, 31).

Macrophages help maintain tissue homeostasis by serving various house-keeping functions e.g. removal of necrotic tissues. These functions are mediated through the constitutive expression of scavenger receptors. As discussed in Section 1.2.2., upon ingestion and killing of phagocytosed bacteria, neutrophils undergo apoptosis. Macrophages are then recruited to phagocytose dying neutrophils, minimising collateral tissue damage and aiding the resolution of inflammation. Beyond ingesting already apoptotic neutrophils, macrophages can actively induce apoptosis within neutrophils (32-36). These regulatory functions are an important step towards achieving immune homeostasis.

Macrophages are also capable of initiating the adaptive immune response by acting as non professional APCs capable of presenting antigen loaded onto major histocompatibility complex (MHC) II molecules, to lymphocytes thereby turning on adaptive immunity. In

particular, macrophages play an important role in shaping adaptive immunity through their secretion of large amounts of pro-inflammatory cytokines such as TNFα, IL-12 and IL-23 (37) which contribute to the polarisation of specific T cell phenotypes.

1.2.4. Dendritic Cells

DCs are derived from bone marrow resident multipotent haematopoietic stem cells. Two main types of DCs that exist are myeloid DCs (or conventional DCs) and plasmacytoid DCs. Conventional DCs can then be further divided in migratory DCs and lymphoid resident DCs (Figure 1.1). Migratory DCs sample antigens at peripheral sites and migrate through the lymph to the nodes to activate antigen-specific T cells and initiate adaptive immune responses. Lymphoid resident DCs on the other hand do not migrate but rather collect and present antigens within the lymphoid organ itself (38-40). Plasmacytoid DCs are found in the circulation and peripheral lymphoid organs, and are characterised by their production of large quantities of type I IFN upon microbial stimulation (41, 42).

DCs are pivotal in translating initial innate information to adaptive immunity via their capacity to capture, process and present antigens (summarised in Figure 1.2). Antigen presentation can be carried out by either MHC I or MHC II expressed by the DC. MHC I molecules are constitutively expressed by all nucleated cells and are involved in the presentation of endogenous antigens, such as intracellular bacteria, to CD8 cytotoxic T cells. Intracellular peptides destined for MHC I presentation are labelled with ubitquitin for degradation by the proteasome. Following degradation the resulting peptides are transported to the endoplasmic reticulum by the transporter associated with antigen processing (TAP). The antigenic peptides are then loaded onto MHC I molecules and transported to the cell membrane via the Golgi complex. DCs are also capable of cross presentation, which involves the ability to load peptides derived from exogenous antigens onto MHC I molecules (43, 44).

MHC II molecules are only expressed by APCs, mainly DCs and macrophages, and are involved in the presentation of exogenous antigens, e.g. antigens derived from extracellular bacteria, to CD4 helper T cells. Exogenous antigens are endocytosed from the extracellular environment delivering antigens to the cytosol within a membrane bound vesicle or phagosome. This phagosomal compartment undergoes a series of modifications, transforming from an early phagosome to a late or mature phagosome and finally fusing with the lysosome forming a phagolysosome. Inside the phagolysosome MHC II molecules

contained within the lysosome are loaded with peptides from the phagosome. Antigen loaded MHC II molecules are then transported to the cell surface where then engage antigen-specific CD4 T cells. Both MHC I and MHC II antigen processing and presentation are important in the initiation and activation of adaptive cellular immunity, namely CD8 and CD4 T cells respectively, the downstream effects of which lead to amplification of the immune response and the eventual resolution of infection (43, 44).

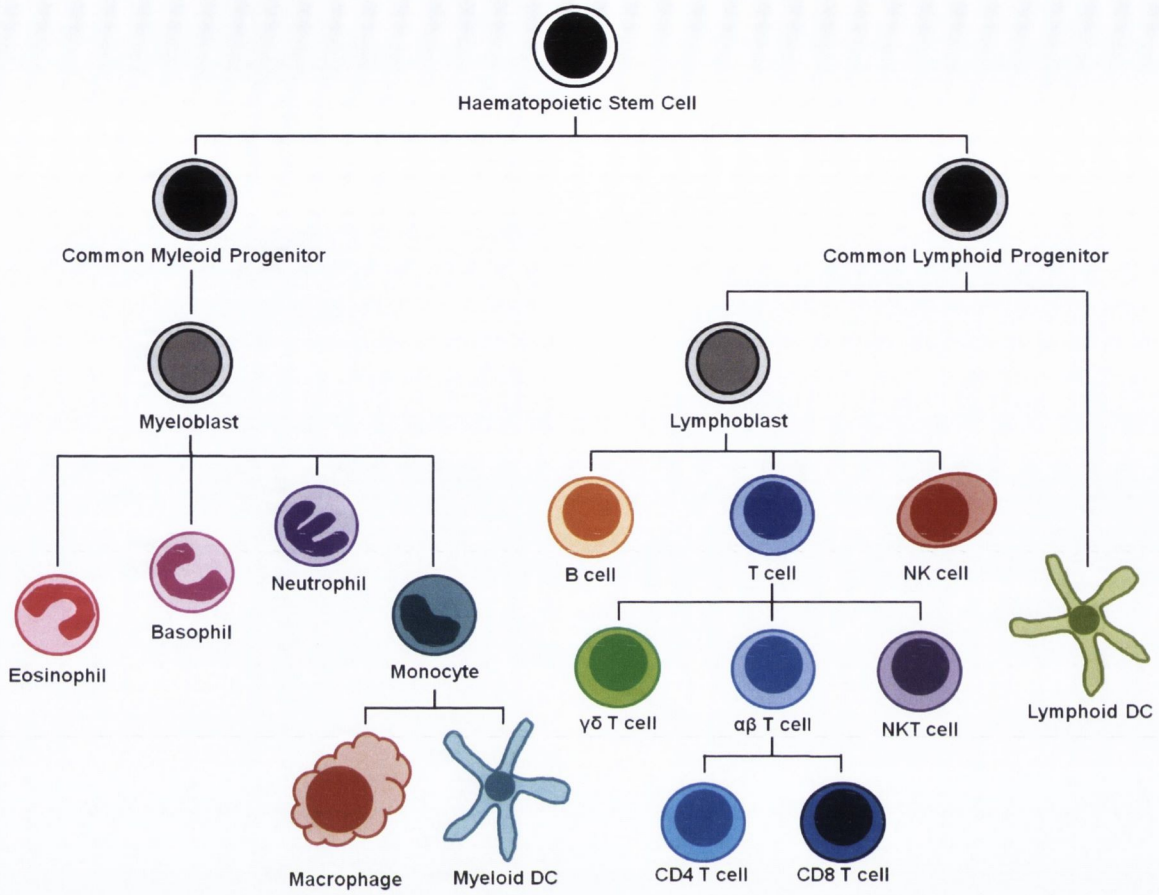


Figure 1.1: Myeloid and Lymphoid cell lineages

Hematopoietic stem cells are the blood cells from which all other blood cells are derived, namely the myeloid (phagocytes and APCs) and lymphoid lineages. Eosinophils, basophils, neutrophils and monocytes are derived from a common myeloid progenitor. Monocytes can further differentiate into either macrophages or myeloid DCs. Lymphocytes can be broadly categorised into B cells, which mature in the bone marrow, T cells, which mature in the thymus and NK cells which can mature in the bone marrow, lymph nodes, spleen or thymus. Once stimulated, B cells differentiate into effector cells known as plasma cells, responsible for antibody production. T cells can be further differentiated into αβ T cells, γδ T cells and NKT cells, all of which carry out a diverse array of effector functions within the immune system. NK cells are large granular lymphocytes important in anti-tumour and anti-viral immunity.

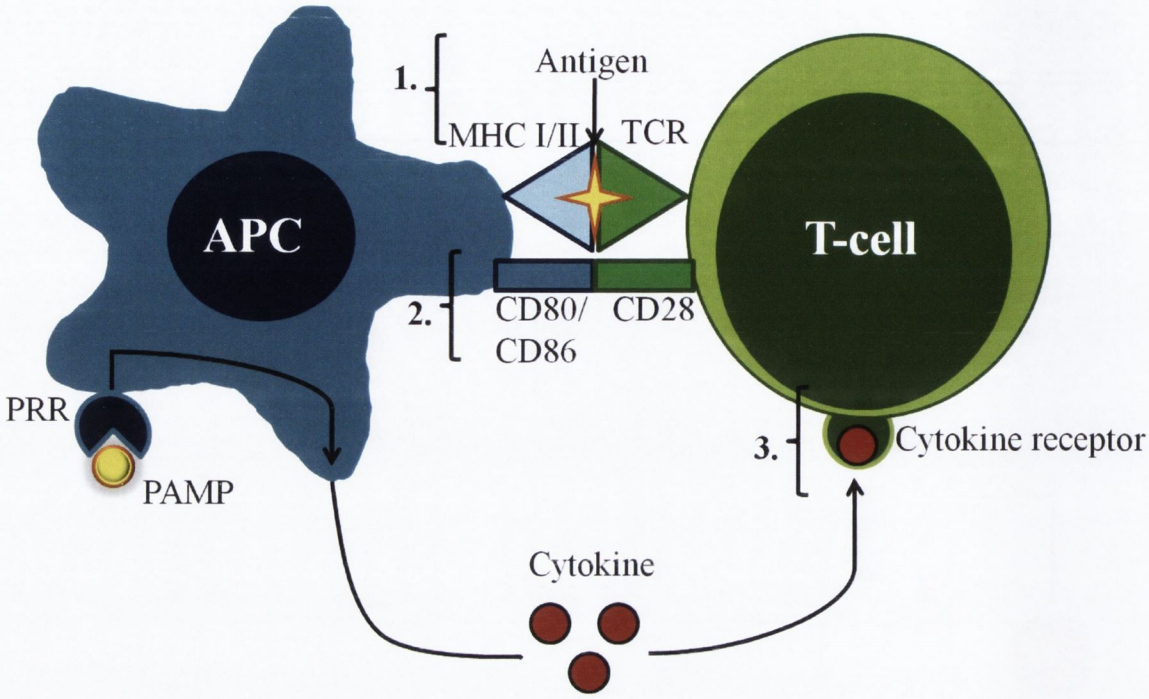


Figure 1.2: APC: T cell interactions

Upon encountering a bacterial pathogen, DCs upregulate their expression of MHC II molecules loaded with pathogen derived antigenic peptides. The interaction of peptide-loaded MHC II with specific T cell receptors (TCRs), referred to signal **1**, is the first activating stimulus received by the T cell. DC pathogen interactions (e.g. TLR ligation) also induce the expression of co-stimulatory molecules such as CD80 and CD86. These molecules engage their counter-receptors on the T cell, particularly CD28, transmitting the signals required for T cell proliferation and survival. This constitutes signal **2**. Finally, signal **3** involves cytokine secretion by DCs, which is needed for effector T cell differentiation and the inhibition of T cell anergy (45). These specific activation steps prevent unnecessary T cell activation which could lead to tissue damage and autoimmunity, but also allow for activation of a specific immune response against particular pathogens.

1.3. T cell responses to bacterial infection

Antigen-specific T lymphocytes are at the core of the adaptive cellular immune response. Due to the expression of highly variable antigen receptors on their surface, these cells enable the body to respond specifically to a wide range of foreign antigens. Upon antigen recognition, naive T cells become activated and differentiate into effector lymphocytes which mediate an array of protective functions culminating in efficient clearance of the invading pathogen and the generation of immunological memory against subsequent re-exposure.

Haematopoietic stem cells differentiate in the bone marrow into putative common lymphocyte progenitors, from which Natural Killer (NK), T, and B lymphocytes originate (Figure 1.1). When T cell precursors egress to the thymus from the bone marrow these progenitor cells lack most of the surface receptors characteristic of mature T cells. Interaction with the thymic stroma initiates sequential phases of differentiation and maturation involving ordered rearrangement of antigen receptor genes leading to the eventual formation of the complete heterodimeric antigen receptor. Two distinct lineages of T cells are produced during thymic development and can be distinguished based on their distinct T cell Receptors (TCRs); α:β and γ:δ. Another lineage of cells can develop, known as NKT cells, bearing an αβTCR of limited diversity that also express the NK1.1 receptor.

In addition, there are subsets of innate lymphoid cells (ILCs), which produce many adaptive T cell cytokines but do not express the cell surface markers associated with classical lineages. These cells, which include NK cells and lymphoid tissue inducer cells (LTi's), develop in the bone marrow like classical T cells but do not necessarily require localisation to the thymus for maturation. NK cells are large, granular, non-specific lymphocytes that play an important role in immunity against tumours and virally infected cells. NK cells are capable of spontaneously killing infected cells and producing high levels of cytokines, particularly IFNγ (46). LTi cells are involved in the development of lymph nodes (47-49) and have also been linked to the maintenance of T cell memory (50). LTi cells were shown to play an important role in the immune response to bacterial enteric infections due to their propensity for rapid IL-17A and IL-22 production (51, 52).

1.3.1. αβ T cells

αβ T cells express a TCR composed of disulfide linked heterodimer between a TCRα chain and a TCRβ chain. The TCR is encoded by homologous genes and its enormous diversity

is generated by somatic recombination of variable (V), diversity (D) and joining (J) gene segments (53, 54). The αβ TCR facilitates recognition of antigenic peptides displayed by MHC molecules on APCs (Figure 1.2). The variable regions of the peptide binding groove in TCRs are specific for a wide array of possible antigens, thus facilitating recognition of the diversity of antigens expressed by potential pathogens.

Upon activation by antigen presentation, antigen-specific naive T cells undergo clonal expansion, which involves repeated T cell proliferation of differentiated effector cells bearing a TCR identical to the specificity of the parental cell. As part of their differentiation these effector cells upregulate specific adhesion molecules and chemoattractant receptors that allow for interactions with the vascular endothelia, directing migration of the antigen-specific T cells to particular tissues where they can execute their effector functions (55). Resolution of infection is followed by a contraction phase where approximately 90% of the effector T cells die, leaving behind a population of long lived memory T cells. Under steady-state conditions, memory T cells slowly turn-over in the absence of antigen. Upon antigen re-exposure memory T cells have a higher sensitivity to antigenic stimulation and are less dependent on co-stimulation, allowing for rapid activation and amplification of the antigen-specific T cell effector response.

αβ T cells are further classified as either $CD4^+$ helper T cells or $CD8^+$ cytotoxic T cells. Both CD molecules function as co-receptors aiding in the process of antigen presentation by APCs. During antigen presentation CD4 or CD8 molecules associate with the TCR on the T cell surface and bind to invariant sites on MHC II or MHC I molecules respectively. Binding of CD4 and CD8 molecules increases the antigen sensitivity of the TCR and the TCR signal (56).

1.3.1.1. $CD4^+$ T cells

$CD4^+$ T helper cells function by activating and directing or "helping" the function of other effector immune cells primarily via cytokine release. The downstream effects of these cytokines enhance phagocytic and bactericidal activity of innate effector cells, induce antibody class switching, and promote activation and expansion of CD8 T cells. CD4 T cells can be divided into different effector subsets that are characterised by their expression of specific transcription factors, signal transduction pathways and cytokine secretion profiles (Figure 1.3).

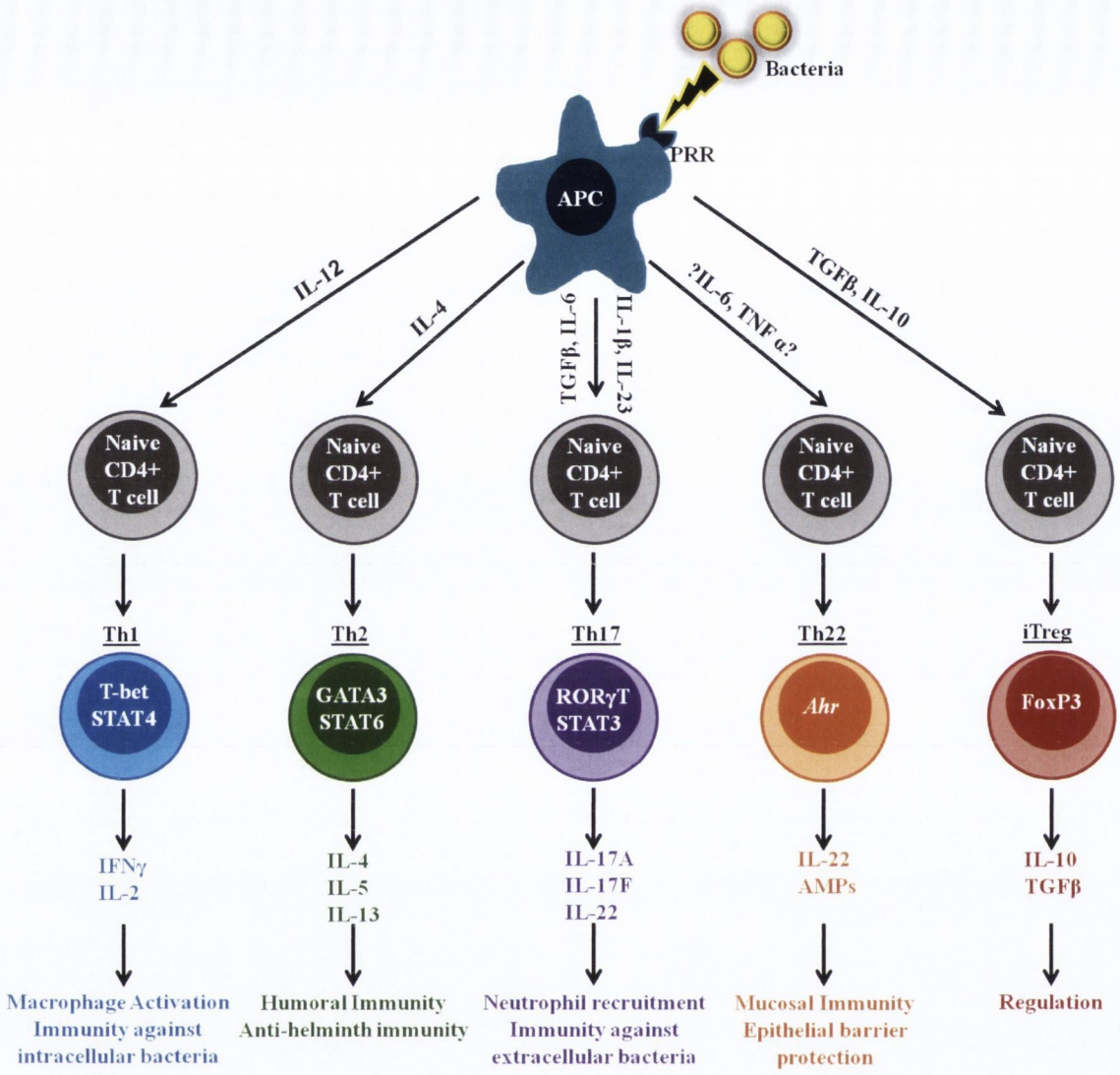


Figure 1.3: CD4$^+$ helper T cell differentiation depends on the cytokine milieu

Following antigen presentation by the APC naive CD4$^+$ T cell precursor cells can differentiate into subsets of effector T cells (Th1, Th2 and Th17, and Th22 cells) and a subset of inducible T regulatory cells (iTregs). Subset differentiation is driven by selective cytokines and transcription factors. Each subset carries out specialised functions within the immune response to pathogens and the eventual resolution of inflammation.

1.3.1.1.1. T helper 1 (Th1) cells

Th1 cells are characterised by their secretion of IFNγ and are vital for the cellular immune response to infections with intracellular-pathogens such as *L. monocytogenes* (57) and *Mycobacterium spp.* (58, 59). IL-12 produced by innate immune cells upon PRR activation combined with IFNγ produced by NK cells, polarise $CD4^+$ T cells towards Th1 differentiation through the activation of signal transducer and activator of transcription (STAT) 4 and STAT 1 and T box transcription factor (T-bet) (57, 60).

In the context of a bacterial infection, antigen-specific Th1 responses are vital for enhancing the effector functions of macrophages and transforming them into potent anti-bacterial effector cells. IFNγ has been shown to augment macrophage activation in response to bacterial products and pro-inflammatory cytokines (61). In addition, IFNγ enhances the production of reactive NO (62) by upregulation of NOS which is involved in the formation of microbicidal NO by macrophages (63). IFNγ signalling also enhances respiratory burst and the formation of ROS by upregulating gp91phox, a subunit of NADPH oxidase in both macrophages and neutrophils (64). Macrophages also upregulate their expression of FcRγ1, a receptor involved in binding of opsonised bacteria or their components, in response to IFNγ signalling (65). In addition, IFNγ promotes expression of co-stimulatory molecules and MHC complex expression, enabling more efficient antigen presentation by the macrophage (59). IFNγ can also regulate B cell functions, namely immunoglobulin production and isotype switching to IgG2a, thus promoting opsonophagocytic killing (66). Th1 cells have also been shown to promote the recruitment of leukocytes to infection sites through induction of monocyte chemoattractant protein-1 (MCP-1), a chemoattractant for monocytes/macrophages, and macrophage inflammatory protein 1α (MIP-1α) and MIP-1β, chemoattractants for $CD4^+$ and $CD8^+$ cells (67). Furthermore, it can increase intercellular adhesion molecule 1 (ICAM-1) expression by endothelial cells thus facilitating immune cell migration (68).

The critical role played by Th1 cells in protective immunity to intracellular pathogens is evidenced by the fact that patients with defects in IFNγ signalling, including defects in IFNγ receptor expression, IL-12 signalling pathways or mutations in STAT1, suffer from severe intracellular bacterial infections (69-72). In murine models, infection with *L. monocytogenes* induces a strong Th1 response (57). Neutralisation of IFNγ production in mice increased susceptibility to infection and impaired the generation of protective T cells (73). Mice deficient in IFNγ are also highly susceptible to infections with *Mycobacterium*

spp. due to impaired macrophage activation and reduced macrophage expression of MHC II (58, 59). Th1 cells have also been shown to a play a protective role during extracellular bacterial infections, as mice deficient in IL-12 display increased susceptibility to infections with *K. pneumoniae* (74). This effect is likely due to inefficient macrophage activation. These studies demonstrate the importance of Th1 protective immunity against both intracellular and extracellular bacterial infections through their abilities to enhance phagocytic cell (particularly macrophage) responses.

1.3.1.1.2. T helper (Th) 2 cells

Th2 cells are characterised by their secretion of IL-4, IL-5 and IL-13 and are primarily involved in the induction of humoral immunity and immunity against helminth infections through the activation of basophils and mast cells (75). IL-4 produced by innate cells upon PRR ligation polarises naive $CD4^+$ T cells towards Th2 differentiation through the activation of signal transduction pathways involving GATA3, a master gene regulator, and STAT6 (76, 77). Downstream effects of Th2 cells involve stimulation of B cell proliferation, induction of B cell antibody class switching to IgE (which binds to FCεR1 on the surface of basophils and mast cells) and augmentation of neutralising antibody (e.g. IgG1) production (78). IL-4 signalling also induces the production of thymic stromal lymphopoietin by epithelial or stromal cells (79), which enhances the maturation of DCs (80).

1.3.1.1.3. T helper (Th) 17 cells

Th17 cells are characterised by their secretion of IL-17A, IL-17F and IL-22 and are vital for protection against extracellular bacterial infection, particularly at mucosal surfaces through their abilities to regulate CXC chemokine induced neutrophil recruitment to sites of infection. More recently Th17 cells have been associated with the induction of IgA, an antibody important in mucosal defences (81). Production of TGF-β, IL-6, IL-21 and IL-23 by activated innate immune cells polarises naive $CD4^+$ T cells towards Th17 differentiation through activation of STAT3 signalling and the transcription factor retinoid-related orphan receptor (ROR) γt (82-85).

Th17 cells are fundamentally important for controlling neutrophil responses. IL-17A promotes granulopoiesis through the induction of CSF and G-CSF by epithelial cells which in turn signal to bone marrow stimulating the proliferation of hemopoietic stem cells. In addition, IL-17A is a potent activator of CXC chemokine secretion by endothelial cells,

epithelial cells and keratinocytes which attracts neutrophils to sites of infection (86-89). Leukocyte migration is further facilitated by IL-17A induced upregulation of ICAM-1 expression by keratinocytes (90). Not only does IL-17A aid in the recruitment of neutrophils, it also enhances bacterial killing by neutrophils (91) and can stimulate other innate immune responses to induce the production of AMPs (e.g. β-defensin) by epithelial cells (92, 93).

In humans, patients with Hyper IgE syndrome (HIES) have genetic mutations in their STAT3 genes leading to impaired Th17 cell function and decreased IL-17 levels. As a result these patients suffer from recurrent and often severe *Candida albicans* and *S. aureus* infections (94, 95). The importance of IL-17 in protection against extracellular bacterial infection was first described in murine models of *K. pneumoniae* infection. IL-17R deficient mice had impaired clearance of the infection compared to wild-type mice and this correlated with defective G-CSF and CXC chemokine production and consequently reduced neutrophil recruitment to the infection site (86, 96). IL-23 deficient mice also demonstrated substantial mortality following a sublethal dose of *K. pneumoniae* and this was directly associated with decreased IL-17 production by T cells. Administration of IL-17 restored bacterial control in the IL-23 deficient mice (74). Immunity to *Streptococcus pneumoniae* also appears to be mediated by Th17 cells, as adoptive transfer of CD4 T cells isolated from mice immunised with killed or live pneumococci, protected T cell deficient mice against pulmonary infection via IL-17A production and neutrophil recruitment (91). It has also been shown that IL-17A could increase pneumococcal killing by human neutrophils suggesting that in the *in vivo* model of *S. pneumoniae* infection IL-17 may contribute directly to bactericidal effects of neutrophils (91). During *Bordetella pertussis* infection, pertussis toxin has been shown to induce a Th17 response, the inhibition of which leads to reduced chemokine expression locally in the lung corresponding to impaired neutrophil recruitment and consequently reduced bacterial clearance (88). Immunisation with whole cell pertussis vaccine has been shown to evoke protective Th17 responses in a murine model. In this model the authors noted the novel effect of IL-17 in promoting macrophage killing of *B. pertussis* (97).

1.3.1.1.4. T helper (Th) 22 cells

Th22 cells are a recently described lineage of T helper cells. They are characterised by their propensity to express the skin homing chemokine receptors CCR4 and CCR10 in conjunction with CCR6 (characteristic of Th17 cells) and to selectively produce large

amounts of IL-22 but little to no IL-17 or IFNγ (98, 99). In these Th22 cells, IL-22 production was associated with the expression of the transcription factor for the aryl hydrocarbon receptor (AhR), which interacts with environmental toxins and metabolites. *In vitro* studies revealed that the differentiation of Th22 cells can occur in the presence of IL-6, TNF, AhR agonists, Vitamin D3 metabolites and that interaction with plasmacytoid DCs favoured Th22 development in comparison to interaction with conventional DCs (98, 99).

Given the propensity of Th22 cells to home to the skin, IL-22 has unsurprisingly been shown to be important for protection against infections at barrier sites (e.g. skin, respiratory tract) where it promotes AMP production in the epithelium (100, 101). In the skin, IL-22 induces AMPs, such as defensins, but also promotes keratinocyte proliferation and inhibits differentiation, demonstrating IL-22's role in wound healing and tissue remodelling (102, 103). IL-22 was shown to play an important role in protection against cutaneous infection with *C. albicans* through AMP production and maintenance of the epithelia (104). IL-22 also plays a role in immunity to pulmonary *K. pnuemoniae* infection (100) and enteric *Citrobacter rodentium* infection (101). During murine enteric *C. rodentium* infection, which is analogous to human enterohaemorrhagic *Escherichia coli* infection, researchers demonstrated that IL-22 deficient animals were more susceptible to the infection than their wild-type (WT) counterparts and that adoptive transfer of Th22 cells differentiated *in vitro* conferred complete protection against the infection in IL-22 deficient mice whereas transfer of Th17 cells did not (105).

1.3.1.1.5. T regulatory cells (Treg)

Homeostasis within the immune system depends on a balance between pro-inflammatory and regulatory responses. Treg cells are a diverse population of lymphocytes with the critical role of maintaining this balance. They are required to suppress immune responses to self antigens which could lead to autoimmunity and regulate the pro-inflammatory environment initiated during an infection thus limiting collateral host tissue damage. Mice deficient in the immunosuppressive cytokine IL-10 demonstrated multiple organ failure and increased mortality during *E. coli* infection. This was observed despite an accelerated bacterial clearance in comparison to WT mice (106). Comparable observations have been made using IL-10 deficient mice infected with *L. monocytogenes* (107), *Helicobacter pylori* (108) and *Helicobacter hepaticus* (109). Similar results were observed during pneumococcal infection with the gram positive bacterium *S. pneumoniae*. Here, researchers demonstrated that increased levels of TGF-β correlated with a rapid influx of

Tregs into the lung. These infiltrating Tregs were necessary for protecting the host against increased bacterial dissemination to the periphery (110). Additionally, adoptive transfer of these Tregs to naive mice prolonged survival and decreased dissemination from the lung to the blood upon streptococcal challenge (110).

Little is known about the role of Tregs during *S. aureus* infection. Using a murine model of biofilm-mediated prosthetic implant infection, C57/BL6 mice developed chronic implant infection, whereas Balb/c mice cleared the infection. Clearance in the Balb/c mice was associated with a more robust Th2/Treg response, suggesting regulatory responses are required for the resolution of infection (111). Following intravenous challenge with S. aureus IL-10 deficient mice developed more frequent and destructive arthritis. Furthermore, IL-10 deficiency resulted in increased bacterial loads in the blood and kidneys compared to immunocompetent controls, indicating that IL-10 is essential for efficient elimination of bacteria and protection against sepsis in this model of septic arthritis (112).

Thus in the absence of Tregs an exaggerated immune response takes place which initially deals with the bacterial infection but the lack of inflammatory control damages host tissues leading to increased mortality. Efficient clearance of infection therefore, depends upon an appropriate balance of effector and regulatory T cells present during the infection (113).

1.3.1.2. $CD8^+$ T cells

The primary function of $CD8^+$ T cells known as cytotoxic T lymphocytes (CTLs) is to kill intracellularly infected, cancerous or abnormal host cells. CD8 T cells interact with MHC class I molecules on the surface of nucleated cells which can present cytosolic antigens (i.e. antigens derived from viruses and intracellular bacteria). To minimise damage to the surrounding tissues CD8 T cells kill in a specific manner by stimulating target cells to undergo apoptosis, or programmed cell death. Upon the detection of an infected cell the CTLs release special lytic granules which contain perforins and granzymes among other protein. The perforin released by the CTL polymerises the target cell membrane creating a pore through which granzyme can gain entry to the intracellular space (114-117). Once inside, granzymes A and B, cleave and activate multiple protein substrates, primarily pro-apoptotic caspases, which eventually results in the initiation of apoptosis (118-120). CTLs can also induce killing of target cells by the engagement and aggregation of target cell death receptors (e.g. Fas) with their cognate ligands (e.g. Fas ligand) on the CTL

membrane. These interactions result in classical caspase dependant apoptosis of the target cell (121, 122). CD8 T cells are also capable of modulating other effectors of the immune response through cytokine secretion (123-125).

In the context of bacterial infections, CD8 T cells play well defined roles in protection against mycobacterium infections (126, 127). During *M. tuberculosis* infection, mice deficient in TAP rapidly die and display severe tissue pathology and increased bacterial burden in comparison to their WT counterparts (128). Additionally, mice lacking CD8 expression on the T cell surface and mice deficient for MHC I molecules also show increased susceptibility to *M. tuberculosis* infection (129). CD8 T cells have also been shown to play a protective role during *L. monocytogenes* infections (130, 131). Highly specific CD8 T cells contributed more substantially to long term protective immunity against *L. monocytogenes* in comparison to CD4 T cells, as mice with impaired MHC I presentation succumbed to both primary and secondary infection more readily than mice deficient in MHC II presentation (131, 132).

Evidence also exists for the importance of CD8 T cells in immunity against extracellular bacterial infections. Mice deficient in CD8 T cells were found to be more susceptible to infection with *Yersinia pseudotuberculosis*. CD8 T cell derived perforin mediated cytolytic killing of *Y. pseudotuberculosis*-associated cells, resulting in phagocytosis of both bacteria and cells by neighbouring phagocytes (133). CD8 T cells were also shown to play a protective role during infection with *S. pneumoniae* serotype 3, a serotype not covered by the pneumococcal vaccine. This study demonstrated that CD8 deficient mice succumbed to infection more readily than their immunocompetent counterparts. This effect was associated with increased bacterial burden and inflammatory infiltrate to infected lungs. Using perforin deficient and IFNγ deficient mice the authors concluded that CD8 T cell protection was mediated by the effects of these molecules (134).

1.3.2 Innate-like Lymphocytes.

1.3.2.1 Gamma delta (γδ) T cells

γδ T cells are a distinct subset of T cells that mature in the thymus like classical αβ T cells, however unlike classical T cells they express a TCR comprised of a γ and a δ chain. Like αβ TCRs, γδTCRs are generated through somatic rearrangement of V, D and J gene segments. γδ T cells comprise 1-5% of circulating lymphocytes but are present in greater numbers at epithelial surfaces such as the skin or the intestines. Upon microbial infection,

human γδ T cell numbers have been shown to increase dramatically within the circulation, in some cases reaching more than 50% of peripheral T cells within days (135). γδ T cell expansion was observed in infections with bacteria such as *M. tuberculosis* (136), *S. pneumoniae* (137) and *L. monocytogenes* (138).

Individual γδ T cell subsets resident in particular locations are biased towards the expression of particular TCR V gene segments and in some cases express invariant TCRs. Expression of particular Vγ chains and/or Vδ chains can be used to categorise γδ subsets with specific Vγ subsets associated with particular locations and cytokine profiles (139). Recent studies are beginning to fully characterise the complexity of these γδ subsets in both mice and humans, revealing that it can be difficult to draw comparisons between the two species. For example, certain subsets are species-specific, such as human Vγ9Vδ2 and murine Vγ5Vδ1. In addition, surface markers such as CD2, CD5, CD6 (140-142) and CD27 (143-145) show interspecies variation. Notably, there is no unequivocal species-wide γδ cell-specific surface marker other than the γδTCR itself.

Two different nomenclature systems for murine Vγ subsets have arisen which are summarised in Table 1.1. Of note, the Garman labelling system is used throughout this report (146). Traditionally, γδ T cells of the Vγ1.1 subset are thought to be anti-inflammatory with depletion studies revealing exacerbated inflammatory responses upon infection with *L. monocytogenes* (147) and coxsackievirus B3 infection (148). Conversely, Vγ2 cell depletion attenuated inflammatory responses in both of these models (147, 148). Further studies revealed that Vγ2 cells were pathogenic in murine models of autoimmunity (149, 150). However, these pro-inflammatory Vγ2 cells were shown to be important sources of IL-17 during infection with *L. monocytogenes* (151). Vγ4 cells contribute to the clearance of bacterial infections such as *L. monocytogenes* (152), *Bacillus subtilis* (153) and *E. coli* (154-157). A sizeable number of γδ T cells, predominantly of both theVγ1.1 and Vγ5 lineage, reside in the epithelial layers of the gastrointestinal tract, and are commonly referred to as intraepithelial lymphocytes (IELs) (158, 159). γδ IELS play an immunoprotective role in the gut through their cytolytic activity (158, 160) and secretion of cytokines such as IFNγ (161).

Table 1.1: Murine Vγ subset nomenclature

Heilig & Tonegawa (162)	Garman (146)	Associated Locations
Vγ1	Vγ1.1	Lymphoid Tissues (163, 164), Intestinal Mucosa (158, 159)
Vγ2	Vγ1.2	Not well characterised; genes have been amplified from fetal thymocytes and adult lymphoid tissues (146, 162)
Vγ3	Vγ1.3	Not well characterised; genes have been amplified from fetal thymocytes and adult lymphoid tissues (146, 162)
Vγ4	Vγ2	Lung (165-167), Lymphoid Tissues (150, 151), Spleen (168)
Vγ5	Vγ3	Skin (169-172)
Vγ6	Vγ4	Peritoneal Cavity (154-157), Lymphoid Tissues (173, 174), Reproductive tract (175)
Vγ7	Vγ5	Intestinal mucosa (158, 159)

Two major lineages of γδ T cells have been characterised in humans (Table 1.2). Vγ9Vδ2 are the predominant γδ subset in human blood and are capable of recognising conserved self and foreign non-peptide antigens, such as phosphoantigen metabolites from isoprenoid synthesis pathways (135). The most potent human γδ T cell activating phosphoantigen documented to date is hydroxymethyl-but-2-enyl-pryophosphate (HMBPP) which is an intermediate metabolite of the non-melavonate pathway of cholesterol synthesis (135). HMBPP molecules are produced exclusively by prokaryotes e.g. *E. coli, L. monocytogenes* and *M. tuberculosis* (176, 177). Vγ9Vδ2 cells are also activated by isopentenyl pyrophosphate (IPP) an intermediate of the melavonate pathway which is conserved in both prokaryotes and eukaryotes. Thus IPP represents a self phosphoantigen induced under condition of stress (178) . Human Vδ1 cells are the major γδ T cells present at epithelial sites such as the skin and intestine (179, 180).

Table 1.2: Human Vγ subset nomenclature

Subset (142)	Associated Locations
Vδ1	Epithelial tissues; Skin and intestines (179, 180)
Vδ2	Blood (178)

Murine γδ T cells can respond rapidly to cytokine activation. In the absence of antigen γδ T cells were shown to secrete IL-17 in response to IL-1 and IL-23 stimulation (149). Recently, antigen activation of γδ T cells in conjunction with inflammatory cytokine stimulation was shown to stimulate increased γδ T cell activation compared to either stimulus alone, suggesting that γδ T cells are capable of synergising both activation mechanisms enabling increased effector function (181). γδ T cells are also capable of responding to pathogen products directly via expression of their PRRs and environmental signals via their Ahr (182). Additionally, γδ T cells differ from classical T cells in that they exhibit a pre-activated phenotype, with high expression of the activation marker CD44, and the chemokine receptors CCR5 and CCR6 (183, 184), enabling rapid recruitment and induction of effector functions upon stimulation. Activated murine γδ T cells contribute to host resistance through production of effector cytokines. γδ T cell derived IL-17 contributes to protection against bacterial infections such as *S. aureus* (165, 169), *E. coli* (154, 155, 157), and *L. Monocytogenes* (151, 185), through induction of neutrophil recruitment. γδ T cell derived IFNγ plays an important role in anti-tumour immunity (186) and anti-viral immunity (148, 187-190) via regulation of type 1 responses.

In vitro phosphoantigen activated Vγ9Vδ2 T cells are capable of producing IFNγ, TNFα and of lysing infected cells via the secretion of perforin (191-197). Activated human Vγ9Vδ2 T cells can also display cytotoxic effector functions (197, 198) and produce bacteriostatic or lytic molecules (197, 199). Unlike the more pre-committed fate of murine γδ T cells (144), phosphoantigen activated Vγ9Vδ2 cells display remarkable plasticity, comparable to CD4 T cells, depending on the cytokine milieu, with Th1-like, Th2-like (200), Treg-like (201) and Th17-like (202) phenotypes reported. Mucosal Vδ1 cells respond to stress associated factors, (e.g. MHC class I-related molecule;MICA) expressed by transformed or virally infected cells (203), and are thought to be important for tumour surveillance in epithelial tissues (180). Similar to murine γδ IELs, activated Vδ1 cells often display a cytotoxic Th1-like phenotype characterised by IFNγ secretion (179).

Like classical adaptive T cells, human γδ T cells are capable of antigen recall where they recognise a bacterial antigen upon re-exposure to the organism allowing for a more rapid and efficient immune response (181, 192, 204). Antigen recall by murine γδ T cells has only recently been identified in a murine model of *L. monocytogenes* infection (173). In this study, murine memory γδ T cells were of the Vγ4 subset and displayed a $CD27^{-}CD44^{hi}$ phenotype. These *Listeria*-specific γδ T cells contributed to protection upon re-exposure to *L. monocytogenes* through increased secretion of IL-17 and IFNγ. With the identification of protective memory γδ T cells in murine models, pre-clinical experiments can now be carried out to fully characterise the potential of generating these cells via vaccination.

1.3.2.2. Natural Killer T (NKT) cells

NKT cells are a heterogeneous group of T cells that share properties of both T cells and NK cells. NKT cells were originally named because of their co-expression of a αβTCR along with typical NK cell surface receptors, such as NK1.1 (205). Similar to innate cells, NKT cells exist in a poised effector state (46) and can swiftly respond to infection by rapid cytokine secretion and cell lysis. However, like conventional T cells, they are also capable of responding to antigen presentation. NKT cells in particular recognise lipid antigens, such as glycolipids, presented by CD1d molecules on APCs (206, 207). The best known subset of CD1d-dependent NKT cells expresses an invariant TCRα chain and are referred to as invariant NKT cells (iNKT) cells (205). Mature iNKT cells are widely distributed and can be found in the blood, gastrointestinal tract, liver, lymph nodes and spleen (208, 209).

In the absence of microbial glycolipid iNKT cells can be indirectly activated via cytokines (210), endogenous stress ligands (211) or a combination of the two (212, 213), initiating a rapid response to a wide range of pathogens. iNKT cells can also be activated directly through the interactions of their TCR with lipid antigen presented by CD1d molecules. It also important to note that ligation of the NK cell like receptors expressed by iNKT cells, such as NK1.1 (a receptor with no known ligand) and the stress receptor NKG2D, may also contribute to iNKT cell activation (214, 215). Upon activation, iNKT cells rapidly produce effector cytokines, leading to neutrophil recruitment (216, 217) and macrophage activation (218, 219), which contribute to protection against infections with *S. pneumoniae* (216), *Pseudomonas aeruginosa* (218) and *M. tuberculosis* (219, 220).

1.4. *Staphylococcus aureus*

1.4.1 Epidemiology

Bacteria of the genus *Staphylococcus* are non-motile, Gram-positive spheres which appear as grape like clusters under a microscope. This genus encompasses at least 45 species and 21 subspecies (221, 222). *S. aureus* is a facultative anaerobe distinguishable from other staphylococci due its gold pigmentation, ability to ferment mannitol and its production of a thermostable DNase. The *S. aureus* genome consists of a circular chromosome of approximately 2800 base pairs encoding 2700 proteins, which are either cytoplasmic, membrane associated, cell wall associated or secreted. The production of extracellular coagulase differentiates *S. aureus* from other coagulase negative staphylococci (223). *S. aureus* is generally considered more virulent than other coagulase negative staphylococci. Molecular and sequence based typing methods are used to categorise *S. aureus* into various lineages, known as clonal complexes (CC), by examining allelic variation in seven housekeeping genes (224, 225). Ten major lineages of *S. aureus* are responsible for the majority of infections in humans. These are referred to by their clonal complex number and consist of CC1, CC5, CC8, CC12, CC15, CC22, CC25, CC30, CC45 and CC51. In addition, most clinical isolates of *S. aureus* express a capsule to aid in immune evasion, the most dominant serotypes being capsule type 5 (CP5) or 8 (CP8). However, 20–25% of clinical isolates produce no capsule (226, 227).

S. aureus causes a wide range of illnesses from minor skin infections, such as abscesses, cellulitis, folliculitis and impetigo, to more life threatening diseases upon invasive entry, such as bacteraemia and septicaemia (228). The mortality of *S. aureus* bacteraemia is approximately 10-30% despite the availability of anti-microbials (229). These infections can potentially result in dissemination and infection of other tissues such as bone (osteomyelitis), heart valves (endocarditis), joints (septic arthritis) and lungs (pneumonia) (228). *S. aureus* is one of the leading causes of nosocomial infections worldwide, in particular causing infections in hospitalised patients with indwelling medical devices or immunodeficiencies (228).

The treatment of staphylococcal infections is becoming increasingly difficult with the emergence of antibiotic resistant strains in health care associated (HA) settings, the most notable of which is Methicillin-resistant *S. aureus* (MRSA) (Figure 1.4 A). The MRSA epidemic is an escalating problem in Western Europe and North America. In the U.S. it has been reported that invasive HA-MRSA infections result in more deaths annually (>18,500)

than HIV/AIDS, viral hepatitis and influenza combined (230, 231). In Ireland, the number of reported HA-MRSA bloodstream infections has decreased in recent years (Figure 1.4 B). The incidence, however, is still relatively high and compares with UK and Southern European countries. With an antibiotic resistance rate of 21.3% amongst all *S. aureus* bloodstream isolates in Ireland, the country is ranked 9th among 28 countries reporting to the European Antimicrobial Resistance Surveillance Network (EARS-Net) (Figure 1.4 C) (232).

Of growing concern is the emergence of MRSA infections in young, immunocompetent individuals outside of the healthcare system. These community acquired (CA) MRSA infections are characterised by distinct genotypes and distinct determinants of virulence (e.g. expression of Panton Valentine Leukotoxin). CA-MRSA strains are considered more virulent than their representative health care associated strains (233, 234) and are also associated with rapid dissemination amongst particular groups of individuals such as prisoners, athletes and the military (235). Skin and soft tissue infections represent 90% of the infections caused by CA-MRSA (236). Although many clinical manifestations of CA-MRSA are mild, severe infections leading to necrotising fasciitis and necrotising pneumonia have been associated with mortality rates of 20% and 75% respectively (237-239).

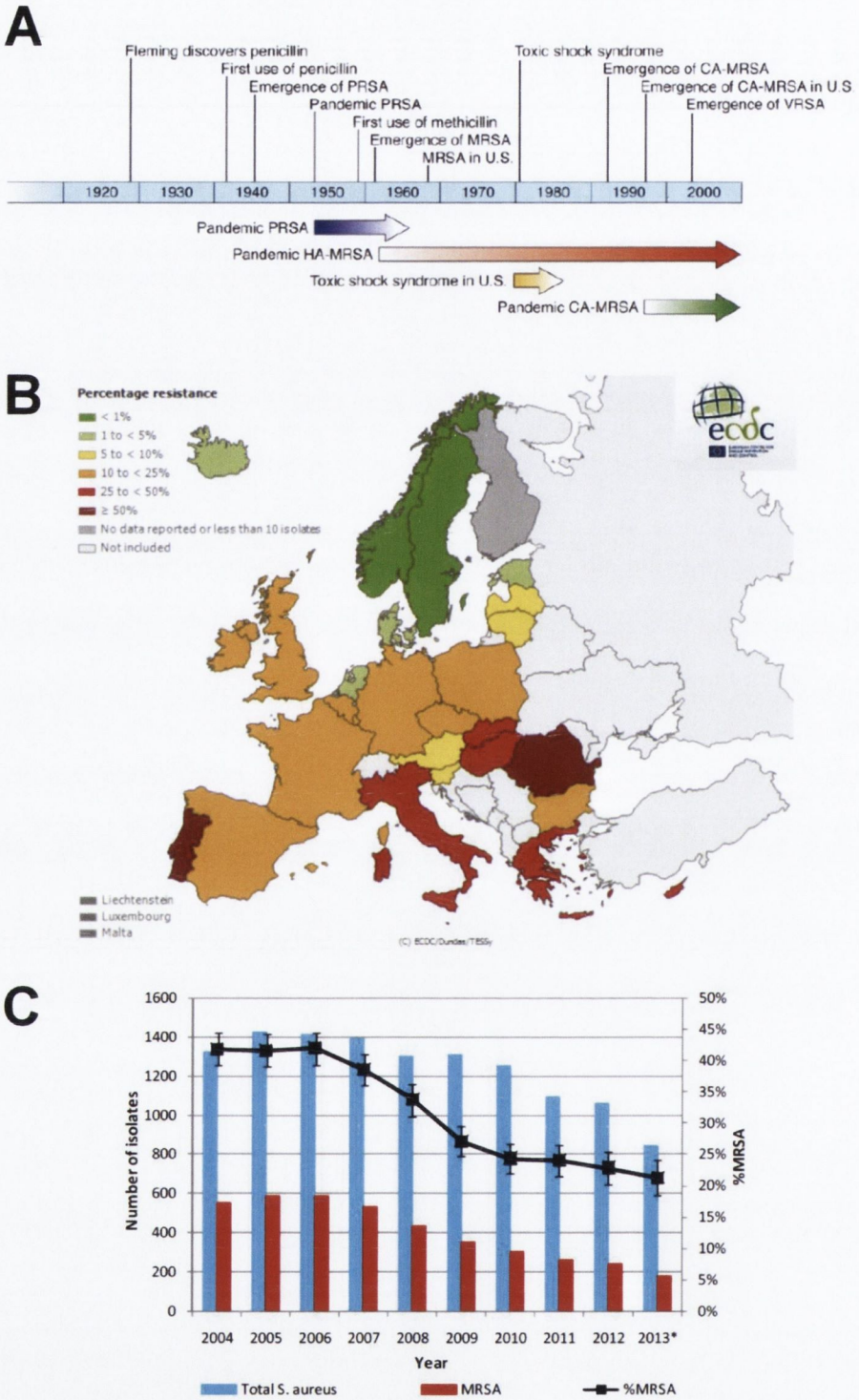


Figure 1.4: Staphylococcal Antibiotic resistance

Emergence of antibiotic resistance over the past century (240). The timeline indicates the year in which an event occurred or was reported. Arrows indicate approximate length of time for each pandemic/epidemic (A). Distribution of MRSA in EARS-Net countries as of 2011 (B). Total numbers and percentages of *S. aureus*/MRSA isolates in Ireland from 2004 to Q3 of 2013. Data correct as of 12.12.13. (www.hspc.ie) (C).

4.2. S. aureus Colonisation

In contrast to its invasive infective potential, *S. aureus* comprises part of the normal microbiota, permanently colonising the moist squamous epithelium of the anterior nares of approximately 20% of the population, with the remainder colonised intermittently (241, 242). *S. aureus* has also been found to colonise oropharyngeal and inguinal areas in individuals irrespective of their nasal colonisation status (243). The host factors involved in staphylococcal nasal carriage are poorly understood. Genetic studies revealed that single nucleotide polymorphisms in C-reactive proteins, IL-4 and complement cascade inhibitor proteins are associated with persistent *S. aureus* colonisation (244-246). Reduced AMP expression in nasal secretions such as human β-defensin-3 (247) and alterations of PRRs such as TLR2 on the nasal epithelium (248) have also been shown to affect carriage. In addition, commensal flora can inhibit pathogen colonisation and bacterial interference has been reported to negatively influence *S. aureus* nasal colonisation (249-251).

Little is known about how colonisation affects the immune system. Most studies to date have focused on profiling anti-*S. aureus* antibodies in colonised individuals and suggest that exposure to the bacteria via colonisation does impact the immune system, as increased anti-*S. aureus* antibody titres were detected in colonised individuals in comparison to non-carriers (252). However, even in the absence of *S. aureus* nasal carriage, significant levels of serum antibodies against *S. aureus* are still detected suggesting the majority of the population has been exposed to the organism at some point as a consequence of transient colonisation or sub-clinical infection (252, 253). Clinical studies have demonstrated that *S. aureus* carriers are at an increased risk of acquiring nosocomial infections (254). Genotyping revealed that 80% of strains that cause bacteraemia in a patient are actually endogenous (254), indicating that their colonising staphylococcal strain breached mucosal barriers and was responsible for the resulting infection even in the presence of anti *S. aureus* antibodies. Although *S. aureus* colonised patients are at an increased risk of contracting staphylococcal bacteraemia, upon invasive infection these individual were associated with decreased mortality compared to non-carriers, suggesting that colonisation may have some beneficial impact upon host immunity (254).

1.4.2. S. aureus Virulence Factors

As humans are colonised with *S. aureus* this allows for consistent interaction between the bacterium and the host immune system. This sustained exposure to the immune response

has enabled *S. aureus* to evolve a diverse number of strategies to evade host anti-bacterial immune responses and highlighting the complexity of treating staphylococcal disease. In addition, the evasive capacity of *S. aureus* has also contributed to the difficulties in understanding correlates of immunity and selection of antigens for vaccine design.

In order to evade anti-bacterial immune responses, *S. aureus* expresses a wide array of secreted and cell surface associated virulence factors which aid in immune evasion and modulation. *S. aureus* is capable of inhibiting neutrophil chemotaxis through the use of several virulence factors. For example, Extracellular adhesion protein (Eap) inhibits neutrophil adhesion, diapedesis and extravasion (255). The secretion of toxins capable of forming β-barrel pores in leukocyte membranes is one of the cardinal features of *S. aureus*. Recently, staphylococcal leukotoxin ED was demonstrated to lyse leukocytes expressing CCR5 (256), CXCR1 and CXCR2 (257). Staphylococcal virulence factors also aid in the inhibition of phagocytosis. Surface expressed Staphylococcal protein A inhibits opsonisation by binding to the Fc region of IgG and interfering with recognition of IgG by Fc receptors on the surface of phagocytes (258, 259). Most clinical *S. aureus* isolates express a capsule comprised of serotype 5, serotype 8 or serotype 336 capsular polysaccharide (CP), which is anti-opsonic (260, 261). Additionally, staphylococcal virulence factors can inhibit AMP mediated killing of the bacteria, for example staphylokinase binds α-defensins enhancing bacterial survival *in vivo* (262). If *S. aureus* is successfully engulfed by a neutrophil it can survive intracellularly within the neutrophil by several mechanisms, such as interference with endosome fusion and AMP release (263), as well as expression of virulence factors that scavenge oxygen free radicals (264).

Finally, *S. aureus* virulence factors are also capable of not only evading but also modulating the immune response. For example the expression of superantigens leads to non-specific proliferation of T cells preventing the development of a normal immune response and leading to T cell anergy (265, 266). Eap is also capable of immune modulation by shifting the cellular immunity from a Th1 response to a Th2 response resulting in enhanced bacterial survival (267).

1.5. Anti-*S. aureus* Vaccines

Recently the emergence of *S. aureus* strains resistant to vancomycin (268), linezolid (269) and daptomycin (270) (last viable antibiotic treatment options for severe MRSA infections) has been reported revealing the rapid ability of this bacterium to become resistant to the newest forms of antibiotics. Alternative approaches to standard antibiotic therapies are therefore urgently required. It is likely that future success in the treatment of severe MRSA infection will rely upon the use of immunomodulatory therapies and the development of anti-*S. aureus* vaccines which would circumvent the ability of the bacteria to develop direct resistance.

1.5.1 Successful anti-bacterial Vaccines

Vaccination is the process by which an individual is intentionally exposed to a modified version of a pathogen or part thereof resulting in the establishment of long-term immunological memory. Vaccines are one of the most effective and sustainable ways of preventing infectious disease. Most successful anti-bacterial vaccination strategies are based on the induction of memory B cells capable of producing antigen-specific antibodies. A pathogen invading a vaccinated host is then rapidly targeted by pre-existing antibodies allowing for swift resolution of infection (271).

Historically, anti-bacterial vaccines consisted of live, attenuated (e.g. *Bacillus anthracis*) or killed, whole cell bacteria (e.g. *B. pertussis*) (272). Toxoid vaccines induce protective immunity by generating antibodies against bacterial exotoxins alone, as is the case for vaccination against Tetanus and Diphtheria. Acellular vaccines were developed in the late 20th century, using purified bacterial components, often in combination with an adjuvant to increase their immunogenicity, e.g. acellular *B. pertussis* vaccine (273). The development of reverse vaccinology in the early 21st century allowed for the rational selection of antigens by screening the entire pathogenic genome for potential immunogenic targets and was first implemented in a vaccine against Serogroup B meningococcus (274). Anti-bacterial vaccine strategies were then further expanded by the development of conjugate vaccines. These vaccines involved the conjugation of bacterial polysaccharides to a protein carrier, enabling peptide recognition by T cells leading to the generation of a T cell dependent anti-polysaccharide antibody response. Vaccination against encapsulated bacteria such as meningococci and pneumococci use conjugate vaccines to generate antibodies specific for carbohydrates expressed on the surface of the pathogen (275, 276).

In conclusion, most licensed anti-bacterial vaccine strategies strive to generate antibody mediated protection and this form of vaccination has been incredibly successful in eradicating or controlling a number of infectious diseases (Table 1.3). However, these vaccination strategies are not without limitations as substantial efforts have been employed to develop anti-*S. aureus* vaccines with no success to date (277).

Table 1.3: Examples of past and present licensed anti-bacterial vaccines adapted from (272)

Vaccine Type	Disease	Immunogenic Components	Approx time of availability
Live, attenuated	*Bacillus anthracis*	Chemically attenuated whole organism	1881
Killed	*B. pertussis*	Inactivated whole organism	1926
Toxoids	*Clostridium tetani*	Tetanus Toxin	1927
Purified	*B. pertussis*	Pertactin, pertussis toxin, fimbriae, filamentous hemaglutinin	1996
Conjugate Vaccines	*S. pneumoniae*	Capsular polysaccharide conjugated to a protein carrier	2000

1.5.2. S. aureus Vaccines

Due to previous successful vaccination strategies against other encapsulated bacteria anti-*S. aureus* vaccine strategies originally focused on the induction of antibodies against capsular polysaccharides. StaphVAX®, a bivalent vaccine, was one of the first vaccines developed against *S. aureus* by NABI pharmaceuticals. The vaccine comprised of CP5 and CP8 conjugated to the protein carrier *Pseudomonas* exotoxoid A. Initially, Phase II clinical trials were promising as the vaccine induced high antibody titres that were maintained for approximately 6 months (278). However this vaccine did not reduce the incidence of invasive *S. aureus* infections in a large phase III trial. This lack of efficacy occurred despite the induction of opsonophagocytic, anti-capsular antibodies in immunised patients (279).

Prevention of iron uptake was also targeted as a strategy, as interfering with iron uptake by *S. aureus* results in reduced bacterial growth and death (280). The leading vaccine strategy that adopted this approach was developed by Merck and targeted the surface-exposed haem-binding protein Iron-regulated Surface determinant B (IsdB). In a murine model of *S. aureus* induced sepsis, for all strains tested, mice immunised with IsdB adjuvanted with alum, had better survival rates than mice immunised with adjuvant alone (281). However when this IsdB based vaccine underwent clinical trials it failed to elicit desired responses and Merck terminated the vaccine trial during phase IIb in June of 2011 (282). A press release by Merck stated that the vaccine was "unlikely to demonstrate a statistically significant clinical benefit, plus there were safety concerns regarding overall mortality and multi-organ dysfunction that occurred with greater frequency in vaccine recipients, compared with placebo" (283).

In addition to active immunisation, passive vaccination strategies targeting staphylococcal surface proteins have also been attempted. The Aurexis® vaccine consisted of anti-staphylococcal monoclonal antibody raised against the ligand-binding domain of staphylococcal ClfA. Administration of this antibody resulted in a non-significant trend towards improved outcomes for *S. aureus* infected patients (284). However another study showed that high titres of anti-ClfA polyclonal antibody did not correlate with protection among high risk neonates (285). These trials were then halted. The vaccine Veronate® also targeted ClfA as well as the *Staphylococcus epidermidis* adhesin SdrG and consisted of an anti-ClfA and anti-SdrG immunoglobulin preparation. Elevated antibody levels against both proteins were observed, however it was unsuccessful in Phase III trials due to insufficient protection (286).

Vaccinations targeting *S. aureus* nasal carriage have also been attempted as a means of reducing the incidence of *S. aureus* infection. Mucosal immunisation with killed *S. aureus* significantly reduced the bacterial burden present in murine nares after staphylococcal challenge (287). Moreover, systemic and intranasal vaccination with Clumping factor B (ClfB), a staphylococcal surface protein that plays a role in nasal colonisation (288), significantly reduced the CFU present in the mouse nares after subsequent challenge (287). A preclinical trial using a CP conjugate vaccine to protect against nasal colonisation in humans was attempted. Immunisation did not result in a statistically significant reduction in colonisation compared to pre-colonisation rates. This was observed despite an increase in antibody concentrations against CP5 and CP8 (289). Moreover, natural antibody

responses detected in infants against staphylococcal surface proteins have been reported to provide no protection against subsequent carriage a year later (290).

1.5.3. Problems with S. aureus Vaccine Development

Numerous challenges have been encountered to date with the development of anti-*S. aureus* vaccines (Table 1.4). Many of the vaccines which have undergone clinical trials demonstrated significant efficacy in murine models, but failed to elicit protection in human clinical trials. This may be linked to the fact that rodents are not natural hosts for *S. aureus* and are therefore naturally more resistant to the bacteria than humans. It also means that mice are immunologically naive to *S. aureus* and unlike humans do not possess pre-existing antibodies. The redundancy of *S. aureus* virulence factors capable of interacting with the immune response also hinders vaccine development, as it makes the selection of specific antigens increasingly difficult. In addition, the capacity of *S. aureus* to infect numerous tissue types presents a challenge not only in the selection of target immune components but also in determining the optimal route of vaccination. Finally, one of the major issues impeding progress in this field is an overall lack of understanding of the correlates of immunity required to confer robust protection against staphylococcal infection.

Table 1.4: *S. aureus* vaccine design challenges

	Problems Facing *S. aureus* Vaccine Design
1.	No individual is immunologically naive as *S. aureus* is part of the normal microbiota.
2.	*S. aureus* is capable of infecting a wide range of tissues.
3.	*S. aureus* expresses multiple virulence factors capable of evading the host immune response.
4.	Protection induced by vaccination in animal models does not translate to protection in humans.
5.	Lack of knowledge as what constitutes to natural immunity against *S. aureus* infections.

1.6. Correlates of immunity to *S. aureus* infection

The immune response induced as a consequence of invasive *S. aureus* infection is typified by an elevation of *S. aureus*-specific antibody titres in the serum post infection (291, 292). It is not clear however if this response is sufficient to protect against subsequent re-infection as recurrence is often a hallmark of *S. aureus* pathologies (293, 294). Furthermore, it appears that the majority of the population already possess antibodies against *S. aureus* given the prevalence of this organism as a commensal coloniser (242, 243, 252, 253). Consequently, most people who succumb to invasive *S. aureus* infection will likely already have antibodies against this organism, supporting the notion that possession of antibodies alone is not sufficient to protect against subsequent infection. In a recent study it was revealed that anti-*S. aureus* antibodies may actually have the potential to neutralise one another's opsonophagocytic activity when mixed together (295). The authors postulated that these antibodies could bind together impeding one another's effector functions.

Although *S. aureus* has not traditionally been classified as an intracellular organism, its ability to enter and survive within both phagocytic (neutrophils (263), macrophages (296)) and non-phagocytic cells (endothelial cells (297), epithelial cells (298, 299) and osteoblasts (300)) has been well established. This intracellular survival may contribute towards chronic infection (263). Considering this ability of *S. aureus* to survive intracellularly, it is likely that humoral responses alone would be unable to eliminate the bacteria and may explain, in part, the lack of effective protection induced by humoral immunity during *S. aureus* infections in humans.

The absolute requirement of humoral immunity for protection against *S. aureus* infections is further brought into question as patients with B cell or antibody deficiencies do display increased susceptibility to severe *S. aureus* infections (301). In contrast however, individuals with cellular defects suffer from exacerbated *S. aureus* infections. Patients with impaired neutrophil responses have a higher incidence of *S. aureus* disease. Examples of neutrophil disorders include frank neutropenia (302), chronic granulomatous disease (CGD) where the neutrophils are unable to form ROS (303), and Chediak-Higashi Syndrome where neutrophils display reduced chemotaxis and phagolysosome function (304). Defects in adaptive cellular responses are also associated with enhanced susceptibility to *S. aureus* infection, as subjects with defects in T cells (305), particularly Th17 cells (94, 95) are at increased risk of developing staphylococcal infections. These

data from the clinic highlight the importance of cellular immunity in host resistance to *S. aureus* in humans.

1.6.1. Role of phagocytes in S. aureus infection

In the context of neutrophils, mice lacking the gp91phox subunit of NADPH oxidase have decreased survival rates upon *S. aureus* infection (306-308). Biochemical inhibition of neutrophils (treatment with neutrophil blocking antibodies) also results in decreased survival rates upon *S. aureus* infection (307). These studies demonstrate that an effective neutrophil response is absolutely necessary for resistance to *S. aureus* infections. Macrophages have also been demonstrated to play a role in anti-staphylococcal immunity. TLR2 and MyD88 (a signalling molecule downstream of the TLR pathway) deficient macrophages displayed reduced pro-inflammatory cytokine production which was associated with decreased survival in *S. aureus* infected TLR2 and MyD88 deficient mice (309). Biochemical inhibition of NOS increased the severity of *S. aureus* septicaemia and septic arthritis and was also associated with decreased intracellular bacterial killing by macrophages (310, 311).

Conflicting reports are however emerging which suggest that neutrophils may also have a negative effect during staphylococcal infections. Gresham *et al* demonstrated that limiting but not ablating neutrophil numbers actually enhanced bacterial clearance during *S. aureus* induced peritonitis, whereas increasing neutrophil influx by administration of recombinant CXC chemokines decreased survival rates and increased bacterial burden. The authors postulated that this was due to the intracellular survival of *S. aureus* within the neutrophil (263). Studies using a model of *S. aureus* surgical wound infection also demonstrated that suppression of neutrophil recruitment, through the use of chemokine receptor blocking antibodies, was associated with improved bacterial clearance at the site of infection (312). In addition, a clinical study has demonstrated reduced incidence of *S. aureus* bacteraemia in neutropenic patients compared to non-neutropenic bacteraemic patients (313, 314). Some researchers have postulated that *S. aureus* uses its ability to survive within the neutrophil as a means of protecting itself from the host immune response and a means of dissemination to other tissues (315). These studies suggest that whilst neutrophils are an essential component of anti-staphylococcal immunity, an "appropriate" neutrophil response is needed to control *S. aureus* infection, in which neutrophils are rapidly recruited for phagocytosis and after sufficient clearance of the bacterium undergo apoptosis allowing for a timely resolution of the inflammatory response.

Other studies have highlighted contrasting roles for macrophages during *S. aureus* infection. Using a model of murine surgical intratracheal infection it was revealed that depletion of alveolar macrophages did not alter the clinical outcome of *S. aureus* infection (308). More recently another study was published using a model of *S. aureus* pneumonia which demonstrated that depletion of neutrophils was detrimental to the host, whereas depletion of macrophages was actually associated with increased survival (307). However this increased survival rate had no effect on bacterial burden, and the authors postulated that this observation was linked to less macrophage mediated inflammatory damage facilitating bacterial dissemination. This suggests that whilst macrophages are capable of killing *S. aureus* via induction of RNS, a more important role for the macrophage during *S. aureus* infection might involve immunomodulatory functions, such as enhancing differentiation of particular T cell phenotypes (37) or clearance of apoptotic neutrophils (32-36), as opposed to direct bacterial killing.

1.6.3. Role of T cell responses in S. aureus infection

Historically, the majority of research into T cells responses during *S. aureus* infections focused on superantigen mediated T cell activation by staphylococci, e.g. toxic shock syndrome toxin-1 (TSST-1). In recent years developments in the field have identified super-antigen independent mechanisms of CD4 T cell activation by *S. aureus*. CD4 T cells activation can occur through presentation of staphylococcal antigens. Zwitterionic polysaccharides expressed by *S. aureus* have been shown to activate T cells. These molecules contain repeated units of both positive and negative charges which are necessary for driving cell mediated immunity (316). For example, CP8 expressed by *S. aureus* has been shown to activate CD4 cells in an MHC II dependant mechanism which was dependent upon the unique charge structure of this molecule (317). In addition, other zwitterionic polysaccharides such as wall teichoic acid can also induce CD4 T cell proliferation in a MHC II dependant manner (318). *S. aureus* is also capable of indirectly activating CD4 T cells through activation of TLR2 and MyD88 signalling pathways in APCs leading to IL-1, IL-12 and IL-23 production by the APC, which enhances the downstream production of cytokines by CD4 T cells (319).

Similar to patients with T cell deficiencies, T lymphocyte deficient mice demonstrate increased susceptibility to *S. aureus* infection compared to WT mice (320). Studies using mice deficient in T cell derived cytokines have revealed further insights into immune mechanisms important in *S. aureus* infections. Subepithelial infections of IL-17A$^{-/-}$ IL-

$17F^{-/-}$ double knock-out mice (321) and subcutaneous (169) or surgical site (322) infections of IL-17R deficient mice resulted in increased bacterial burden and disease pathology. However, IL-$17A^{-/-}$IL-$17F^{-/-}$ mice were not significantly impaired in their ability to clear systemic *S. aureus* infection suggesting a site-specific role for IL-17 in anti-*S. aureus* immunity (321). In contrast to IL-17, the definitive role of IFNγ in host resistance to *S. aureus* infection is less clear. Some studies have demonstrated that IFNγ signalling is dispensable for protection against *S. aureus*, with $IFN\gamma R^{-/-}$ and WT mice displaying similar pathologies upon cutaneous staphylococcal infection (169). Other reports have demonstrated that IFNγ signalling is detrimental to the host, as deficiency in IFNγ was associated with protection in a model of surgical site infection (323), and increased bacterial clearance upon intravenous challenge (324). Conversely, researchers have demonstrated that administration of recombinant IFNγ led to increased phagocyte recruitment and bacterial clearance during intravenous infection, suggesting a protective function for IFNγ in this model (325). Alternatively, studies have documented differential roles for IFNγ signalling during *S. aureus* infection. Upon intravenous infection with *S. aureus*, $IFN\gamma R^{-/-}$ mice developed significantly more severe sepsis and increased mortality early after inoculation compared to WT controls. However, during the later stages of infection the inverse of this was observed, with WT mice displaying increased mortality compared to $IFN\gamma R^{-/-}$ mice (326). Similarly, treatment with anti-IFNγ monoclonal antibody resulted in increased bacterial burden 24 hours after lethal infection with *S. aureus*. By day 4 however, the antibody treated group displayed enhanced clearance of the infection compared to controls (327). These studies highlight the differential role for IFNγ in natural immunity to *S. aureus* infection, depending on the localisation of the infection and on the stage of disease. T cell derived IL-22 has been demonstrated to be important in protection against mucosal bacterial infection (105). However, IL-22 appears to play no role in anti-staphylococcal immunity, as $IL\text{-}22^{-/-}$ animals displayed comparable susceptibility to *S. aureus* cutaneous infection as their immunocompetent counterparts (328).

Recently, γδ T cells have been identified as a potent source of innate IL-17 during *S. aureus* infection and as such mice with deficiencies in γδ T cells succumb to *S. aureus* infection more rapidly than their WT counterparts. In a cutaneous infection model, γδ T cell deficient mice were found to have reduced neutrophil recruitment to the infection site and impaired bacterial clearance, compared to WT controls (169). γδ T cells were also shown to have a protective role in *S. aureus* induced pneumonia through their capacity to produce IL-17, with increased bacterial loads detected in $\delta TCR^{-/-}$ mice compared to WT

controls (165). Recently, $\delta TCR^{-/-}$ mice have also been reported to display increased susceptibility to *S. aureus* surgical site infection (322).

The cellular immune response is also important for acquired immunity against *S. aureus* infection. Adoptive transfer of antigen-specific $CD3^+$ T cells but not B cells or serum containing antibodies from mice vaccinated with a model *S. aureus* vaccine, mediated protection against subsequent *S. aureus* infection in naive recipient mice (320). Lin *et al* demonstrated that both Th1 and Th17 cells are important in mediating vaccine induced protective immunity against intravenous *S. aureus* infections (329). In this study researchers vaccinated mice with Candidal rAls3p-N adhesion molecule, which is similar in structure to staphylococcal ClfA, prior to intravenous challenge with *S. aureus*. Vaccine induced protective immunity required CD4 cell derived IFNγ and IL-17 both of which enhanced effective phagocyte recruitment (329), demonstrating the powerful upstream control of CD4 T cells on downstream effector cells. Intramammary vaccination with live attenuated *S. aureus* protected against infection through induction of IFNγ producing CD4 and CD8 T cells (330).

Together, these studies highlight the importance of effective cellular responses in both natural and acquired immunity in protection against *S. aureus* infections. It is likely that this cellular immune response acts in concert with antibody responses, leading to efficient clearance of the bacterium via opsonophagocytic killing. An effective vaccine against *S. aureus* would ideally target both the cellular and humoral arms of immunity to induce full prophylactic protection. However further study is require to understand the specific types of cellular immunity that should be targeted.

1.7. Project Aims

With the treatment of *S. aureus* infections becoming increasingly difficult due to the emergence of antibiotic resistant strains and the lack of an effective vaccine, more information on the correlates of protection against *S. aureus* infection are required to inform anti-*S. aureus* vaccine development. Reports suggest that adaptive humoral responses alone may not be sufficient to induce full prophylactic protection against *S. aureus* infection. Cellular immunity has been shown to be important in host resistance to *S. aureus* infection, and the development of strategies targeting adaptive T cells should be explored as a means to advance anti-*S. aureus* vaccine design. Precise understanding of what drives "appropriate" cellular immunity to staphylococcal infection will provide the immunological framework for rational anti-*S. aureus* vaccine design. Before particular T cell phenotypes can be targeted by vaccination, the discrete T cell populations capable of inducing site specific protection and natural immunity to *S. aureus* need to be identified.

Nasal colonisation with *S. aureus* was associated with protection upon invasive infection (254), suggesting that sustained exposure to the entire bacterium can impact upon host immunity. This thesis aims to characterise the specific T cell phenotypes expanded as a consequence of long-term exposure to *S. aureus,* in order to identify the specific T cell subsets capable of recalling antigen and conferring protection upon re-exposure, which may represent potential vaccine targets for future immunisation strategies.

Specifically this project will address the following:

1. Investigate the ability long-term exposure to *S. aureus* to confer protection against subsequent infection through expansion of protective T cells.
2. Characterise γδ T cell responses upon a single *S. aureus* exposure and during long-term exposure, with particular focus on the ability of these non-classical lymphocytes to recall antigen and confer protection.
3. Establish the relative importance of Th1 versus Th17 cells in mediating protective immunity against *S. aureus* infection.
4. Identify the significance of type 1 immunity in host resistance to *S. aureus* via vaccination with a staphylococcal antigen combined with a Th1 polarising adjuvant.
5. Determine the relative importance of humoral versus cellular immunity against *S. aureus*, using a mucosal vaccination strategy consisting of a staphylococcal antigen in combination with novel adjuvants.

Chapter 2

Materials & Methods

2.1. Materials

Table 2.1: General Reagents

General Reagents	
Tryptic Soy Agar (TSA)	30g Tryptic Soy (TS) Broth (Sigma Aldrich) 15g Agar (Sigma Aldrich) 1L dH_2O
Columbia Soy Agar (CSA)	35g Columbia Soy (CS) Broth (Sigma Aldrich) 15g Agar (Sigma Aldrich) 1L dH_2O
5% Blood Agar	25mls Horse Blood Defribinated (Fischer) 1L of TSA
cRPMI Media	500 ml RPMI (Sigma Aldrich) 10% (v/v) Fetal Calf Serum (FCS; Biosera) 100mM L-Glutamine (Gibco) 100µg/ml penicillin/streptomycin (Gibco)
Ammonium Chloride Lysis Buffer	0.77g NaH_4Cl (Sigma Aldrich) 1L dH_2O
10X PBS	80g NaCl (Sigma Aldrich) 11.6g Na_2HPO_4 (Sigma Aldrich) 2g KH_2PO_4 (Sigma Aldrich) 2g KCl (Sigma Aldrich) pH 7.2-7.4
FACS Buffer	1% (w/v) BSA (Sigma Aldrich) 1X PBS
MACS Buffer	0.5% (w/v) BSA 2mM EDTA (Sigma Aldrich) 1X PBS pH 7.2
1M NaOH	40g NaOH (Sigma Aldrich) 1L ddH_2O
1.5% (w/v) Agarose Gel	1.5g Agarose (Sigma Aldrich) 100mls 1X TAE buffer 9µl Ethidium Bromide (Sigma Aldrich)
Tracking Dye for Agarose Gel	0.0125g Bromophenol Blue (Sigma Aldrich) 0.0125g Xylene Cyanol (Sigma Aldrich) 15ml Glycerol (Sigma Aldrich) 35ml dH_2O
Tris Buffered Saline (TBS)	2.5g Trizma Base (Sigma Aldrich) 8.8g NaCl (Sigma Aldrich) 1L dH_2O pH 7.2-7.4

Table 2.2: Other Reagents

Reagent	Supplier
CpG oligodeoxynucleotides (CpG)	Oligos Etc.
PBS	Sigma Aldrich
TRIzol	Invitrogen
Brefeldin A	Sigma Aldrich
123-Dihydrorhodemine (DHR)	Sigma Aldrich

Table 2.3: ELISA Reagents

ELISA Reagents	
Wash Buffer	1L 10X PBS 9L dH_20 5ml Tween 20 (Sigma Aldrich)
Reagent Diluent Solutions	Depending on the cytokine investigated, one of the following was used; a) 1% (w/v) BSA in 1X PBS b) 5% (v/v) Tween 20 in 1X PBS c) 0.01% (w/v) BSA, 5% (v/v) Tween 20 in TBS pH 7.2-7.4
Phosphate Citrate Buffer	14.6g Na_2HPO_4 (Sigma Aldrich) 10.1g Anhydrous Citric Acid (Sigma Aldrich) 1 L 1X PBS pH 5
Substrate Solution	25ml Phosphate Citrate Buffer 10mg o-Phenylenediamine dihydrochlorideTablet (Sigma Aldrich) 7µl 30% H_2O_2 (Sigma Aldrich)
Stop Reagent	1M H_2SO_4 (Sigma Aldrich)

Table 2.4: Murine ELISA kits

Cytokine	Supplier	Capture Antibody Dilution factor	Detection Antibody Dilution factor
IFNγ	R&D	1:180	1:180
IL-1α	R&D	1:180	1:180
IL-1β	R&D	1:180	1:180
IL-4	R&D	1:180	1:180
IL-10	R&D	1:180	1:180
IL-12p70	R&D	1:120	1:60
IL-17A	R&D	1:180	1:180
IL-17F	R&D	1:180	1:180
IL-18	MBL	1:500	1:2000
IL-22	R&D	1:180	1:180
IL-23	R&D	1:180	1:180
KC	R&D	1:180	1:180
MIP-2	R&D	1:180	1:180
MCP-1	R&D	1:900	1:180
RANTES	R&D	1:180	1:180

Table 2.5: Dilution factors for Detection Antibodies used in Antibody Titre Assays

Biotinylated Antibody	Dilution
Total IgG (BD biosciences)	1:5000
IgG2a (BD biosciences)	1:4000

Table 2.6: Vγ subset Primers

Gene	Forward Primer	Reverse Primer
Vγ1.1	5' TTCTGCTGCCTCTGGGTTTTT 3'	5' TCCCTCCTAAGGGTCGTTGAT 3'
Vγ2	5' TTGGTACCGGCAAAAAACAAATCA 3'	5' CAATACACCCTTATGACATCG 3'
Vγ3	5' TTGCAAGCTCTCTGGGGTTC 3'	5' GGCACAGTAGTACGTGGCTT 3'
Vγ4	5' GGAAGCAGTCTCACGTCACC 3'	5' CTGCCATGTCCTTGCCTCATA 3'
Vγ5	5' GATCCAACTTCGTCAGTTCCACAAC 3'	5' AAGGAGACAAAGGTAGGTCCCAGC 3'
18S*	5' CCTGCGGCTTAATTTGACTC 3'	5' AACTAAGAACGGCCATGCAC 3'

* Endogenous control gene

Table 2.7: Recombinant Cytokines

Cytokine	Supplier	*In vitro* Concentration
IL-1β	R&D	10ng/ml
IL-12	R&D	10ng/ml
IL-23	R&D	10ng/ml

Table 2.8: Monoclonal Antibodies

Cytokine	Supplier	Clone	*In vitro* Concentration
Anti-IL-1R1	R&D	JAMA147	1-4μg/ml
Anti-IL-23R	R&D	258010	1μg/ml
Anti-CD3	BD Pharmingen	145-2C11	1μg/ml
Anti-MHCII	R&D	114.15.2	5-50μg/ml

Table 2.9: FACS Antibodies

Location	Antigen	Clone	Supplier
Extracellular	CD3	500A2	BD Biosciences
	CD4	RM4-5	BD Biosciences
	CD8	53-6-7	eBioscience
	γδ	GL3	Biolegend
	Vγ1.1	2.11	Biolegend
	Vγ2	UC3-10Ab	eBioscience
	Vγ3	536	Biolegend
	IL-1R (CD121)	Java47	Biolegend
	CD11b	M1/70	Biolegend
	F480	BM8	eBioscience
	Ly6G	RB6-8C5	BD Biosciences
	MHCII	M5/114.15.2	eBioscience
	CD44	IM7	eBioscience
	CD62L	MEL-14	eBioscience
	CXCR2	TG11	Biolegend
Intracellular	IFNγ	XMG1.2	eBioscience
	IL-17A	17B7	eBioscience
Viability Dyes	PI		eBioscience
	Aqua Live Dead		Invitrogen

2.2. Mice

Wild-type (WT) male and female C57BL6/J mice were purchased from Harlan, UK. IFNγ deficient ($IFN\gamma^{-/-}$) and IL-1 receptor deficient ($IL\text{-}1R^{-/-}$) mice on a C57BL6/J background were obtained from the Jackson Laboratory and bred in house. Mice used in experiments were 6-12 weeks of age and were housed in groups of 5 per cage under specific pathogen free (SPF) conditions. In all experiments, mice in each treatment group were age and gender matched. All experiments were performed under license (#281109) from the Department of Health and Children and license (#AE19136/P006) under the Irish Medicines Board, and with approval from the Trinity College Dublin BioResources Ethics Committee.

2.3 Bacteria

2.3.1. Strains

S. aureus PS80 is a clinically derived strain that expresses the capsular polysaccharide serotype 8, and has been previously described (317). *S. aureus* SH1000 is an acapsular strain which is a derivative of strain 8325-4, and has been previously described (331, 332). Staphylococci were cultivated from frozen stocks for 24 hours (h) at 37°C on Columbia agar supplemented with 2% (w/v) NaCl. *E. coli* strain CFT073 is a clinical isolate from St. James' Hospital Dublin in July 2012. *E. coli* were cultivated from frozen stocks for 24 h at 37°C on 5% blood agar. All bacterial work was carried out in a Class II biological safety hood.

2.3.2. Preparation of bacterial inoculum for in vivo challenge studies

Following overnight growth on CSA agar, a turbid solution of bacteria (1ml) was prepared by scraping bacterial colonies using a sterile loop and agitating in sterile PBS. It was mixed thoroughly to ensure the bacteria were in solution. Appropriate dilutions were prepared and the optical density (OD) of the bacterial solution was determined at 600nm using a spectrophotometer. The OD_{600nm} of the solution was adjusted to 0.45, which had previously been established to represent ~$4x10^8$ Colony Forming Units (CFU)/ml (312, 323). The amount of bacteria in the turbid solution was then adjusted to $5x10^9$ CFU/ml, with 100µl of this solution ($5x10^8$ CFU) used as the inoculum for all *in vivo* experiments. To confirm the inoculum concentration, serial dilutions of the bacterial suspension were spread onto Tryptic Soy Agar (TSA) plates and left to grow for 24 h at 37°C. The following day the number of colonies visible on the plates was counted which represented the CFU/100 µl.

Stocks of *S. aureus* ($5x10^9$ CFU/ml) in PBS were stored at -80°C for use during the course of a single *in vivo* experiment. Bacterial viability was confirmed after freezing and prior to injection by plating inoculum onto TSA.

PS80 was grown to stationary phase for neutralisation antibody assays. Liquid cultures of PS80 were set up to achieve this. A single PS80 colony from an overnight plate was transferred to 20mls of TSB and incubated at 37°C whilst shaking overnight.

2.3.3 Preparation of heat killed S. aureus

A bacterial suspension of OD_{600nm} equal to 0.45 in sterile PBS, which represented ~$4x10^8$ CFU/ml, was prepared from an overnight culture grown on TSA. The bacterial suspension (500µl) was transferred to 1.5 ml tubes and was incubated at 70°C for 30 minutes (mins). The resulting heat killed bacteria were incubated for 24 h at 37°C on TSA to confirm no bacterial growth. Heat killed stocks of *S. aureus* were stored at -20°C for use in *in vitro* cell culture experiments.

2.4. *In vivo* model of *S. aureus* recurrent peritonitis

2.4.1 In vivo challenge

To establish if prior exposure to *S. aureus* can modulate cellular immune responses to subsequent re-exposure, a novel murine model of recurrent peritonitis was developed. In this model, groups of mice were exposed to *S. aureus* via 3 intra-peritoneal (i.p.) injections ($5x10^8$ CFU) on days (d) 0, 7 and 14. Preliminary experiments allowed mice to recover for 7 d or 21 d to establish when residual inflammatory responses had resided. Having established that 21 d recovery was sufficient for this, on d 35, the prior exposed mice received a further *S. aureus* i.p. challenge ($5x10^8$ CFU) alongside a control group of naïve mice. Prior to, and at specific time points post challenge, mice were sacrificed and systemic infection levels and immune responses assessed (Figure 2.1).

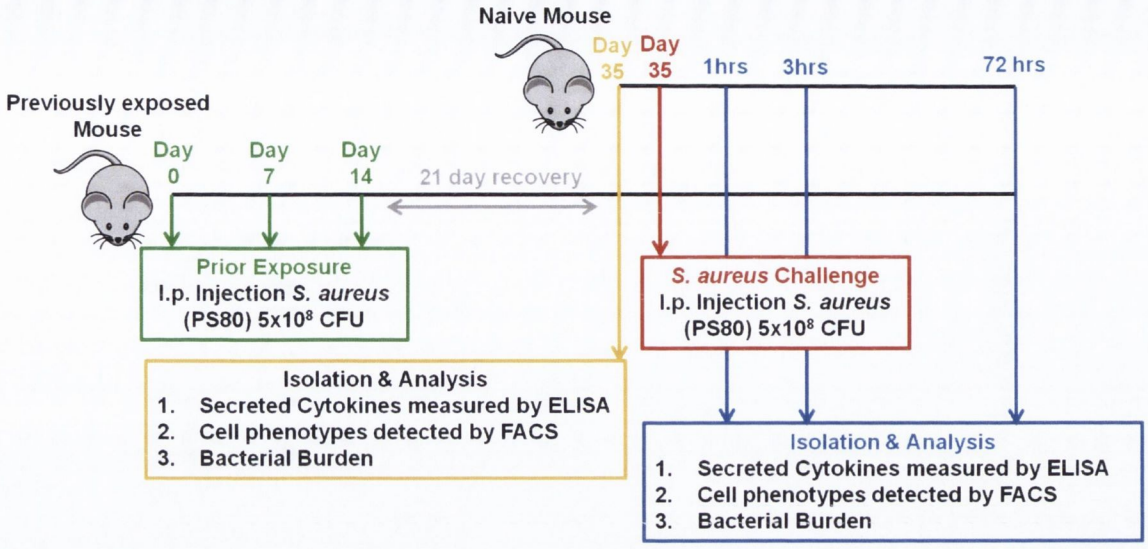


Figure 2.1: Prior exposure to *S. aureus*

Groups of mice were exposed to *S. aureus* via i.p. injection ($5x10^8$ CFU) on d 0, 7 and 14. On d 35 prior exposed mice were then re-challenged with *S. aureus* ($5x10^8$ CFU) alongside a control group of naïve mice. Prior to and at specific time points post challenge the bacterial burden, local cytokine production, T cell responses and phagocyte influx were assessed.

2.4.2 Harvesting of tissues

The peritoneal cavity was lavaged with sterile PBS (2 ml) in order to collect the peritoneal exudates cells (PECs). Mediastinal lymph nodes (MLN) and inguinal lymph nodes (ILN) were collected for analysis of cells. Spleens were also collected for analysis of both cells and bacterial burden. Livers and kidneys were isolated to assess bacterial burden. Blood was collected by cardiac puncture.

2.4.3. Isolation of cells and sera

The peritoneal cavity was lavaged with sterile PBS (2 ml) in order to collect the PECs. Lavage samples were centrifuged at 300x*g* for 10 mins. Cell-free supernatants were then removed and stored at -20°C for cytokine analysis by Enzyme Linked Immunosorbent Assay (ELISA). MLN, ILN and spleen cells were disrupted in cRPMI using 40µm filters to obtain single cell suspensions. Any red blood cells present in samples were lysed using lysis buffer (Table 2.1). All cell samples were re-suspended in cRPMI. Collected blood was allowed to settle at room temperature (RT) for 1 h, centrifuged at 750xg for 5 mins and sera contained in the supernatant transferred to a fresh 1.5ml tube and frozen at -20°C until further analysis.

2.4.4. Quantification of Bacterial Burden

Serial dilutions of the peritoneal lavage fluid and disrupted spleen solution were prepared in sterile PBS and 100µl spread onto TSA plates. Plates were incubated for 24 h at 37°C. The following day the number of colonies visible on the plates was counted which represented the CFU/100µl. Organs (liver and kidneys) were homogenized in 3 ml of sterile PBS using a homogenizer (Kinematica Polytron PT 2500E) with a 7mm blade. The homogenizer was cleaned between samples using 1M NaOH, sterile H_2O, 70% (v/v) EtOH and sterile PBS. Serial dilutions of the homogenate were then prepared in sterile PBS and plated out as previously described for the peritoneal fluid. All plates were kept at RT for a further 48 h to confirm a lack of contaminating bacterial species.

2.4.5. Total cell counts/cytospins

Total cell counts of PECs, MLN and spleen cells were determined using trypan blue staining to differentiate between live and dead cells on a haemocytometer under a light microscope. To establish differential cell counts, cytospins were prepared using Shandon Cytospin 3. Cell suspension (100 µl) was loaded into cytospin funnels attached to

microfrost slides and centrifuged at 300x*g* for 3 mins. After spinning slides were allowed to air dry. They were then stained using a Kwik Diff kit (Thermo Scientific). Stained slides were counted on a haemocytometer using a light microscope to visually distinguish macrophages, neutrophils and lymphocytes.

2.5. Investigation of *S. aureus* antigen-specific recall responses

To investigate if cells exposed *in vivo* to *S. aureus* could respond to *in vitro* stimulation with *S. aureus* antigen, antigen recall experiments were set up. Mice were exposed to *S. aureus* ($5x10^8$ CFU) *in vivo* via a single i.p. injection and cells were isolated from the peritoneal cavity, MLN and spleen at specific time points post challenge. Cells were re-suspended at a $2x10^6$ cells/ml in cRPMI and 200µl was transferred into wells of a 96 well round bottomed plate. Cells were stimulated with heat killed bacteria (10^6 CFU/well) or ClfA (10µg/ml). Cells were also stimulated with anti CD3 (1µg/ml) + phorbol 12-myristate 13-acetate (PMA) (10ng/ml) or IL-1β+IL-23 (10ng/ml each) as positive controls. At 6 h post stimulation cells were collected for intracellular cytokine staining analysis by flow cytometry. After 72 h incubation at 37°C and 5% CO_2 the cell supernatants were collected for cytokine analysis by ELISA.

2.6. Purification of T cells

2.6.1 Purification of T cells by Magnetic activated cell sorting (MACS)

$CD3^+$ T cells were isolated from naive spleens, as well as the peritoneal cavity, draining MLN and spleen of *S. aureus* infected mice, using magnetic activated cell sorting. A murine Pan T cell isolation Kit II (Miltenyi Biotec) was used which allows for negative selection of $CD3^+$ T cells based on a cocktail of biotin-conjugated antibodies against CD11b, CD11c, CD19, CD45R (B220), CD49b (DX5), CD105, anti-MHC class II, and Ter-119. Thus isolation of T cells is achieved by depletion of magnetically labelled cells. Total cell counts post isolation were determined using trypan blue staining. Cells were then centrifuged at 300x*g* for 10 m and re-suspended in MACS buffer (Table 2.1, 40µl/10^7cells). Biotin-Antibody Cocktail (10µl/10^7cells) was added. The cell suspension was mixed thoroughly and incubated at 4°C for 10 mins. After incubation, MACS buffer (30µl/10^7 cells) and Anti-Biotin Microbeads (20µl/10^7cells) were added. The cell suspension was mixed thoroughly and incubated at 4°C for 15 mins. After incubation the cells were washed in MACS buffer (1-2ml/10^7cells) and centrifuged at 300xg for 10 mins. The cells were then re-suspended in 500µl of MACS buffer and added to an LS column, (a

column suitable for the depletion of strongly magnetically labelled cells) before being placed in the magnetic field of the MACS Separator. The column was first prepared by washing it 3 times with 3ml of MACS buffer. The cell suspension was applied to the column and the flow-through containing the unlabelled cells was collected. Cells were then washed in sterile cRPMI before use in *in vivo* and *in vitro* experiments.

2.6.2 Cell sorting of γδ T cells

γδ T cells and $V\gamma1.1^{-}V\gamma2^{-}$ cells were sorted from samples that had previously been purified using PAN T cell isolation kit II. Purified $CD3^{+}$ cells were surface stained for expression of γδTCR, Vγ1.1 and Vγ2 as described below and live cells were sorted using Beckmann Coulter (Dako) MoFlo Cell Sorter. Sorted cells were collected into sterile tubes and stored on ice for use in *in vivo* and *in vitro* experiments.

2.7. Polarisation and adoptive transfer of *S. aureus antigen*-specific Th1 and Th17 cells

To generate *S. aureus*-specific Th1 cells WT mice underwent prior exposure to *S. aureus* as described above (Section 2.4.1). To generate *S. aureus*-specific Th17 cells $IFN\gamma^{-/-}$ mice underwent prior exposure to ensure no reversion by $CD4^{+}$ cells to a Th1 phenotype *ex vivo*. At 7 d post 3rd exposure, total cells were isolated from the peritoneal cavity, mediastinal lymph and spleen as previously described (Section 2.4.3). Cells were isolated 7 d post exposure to minimise the length of experiments. Cells were re-suspended in cRPMI at $4x10^6$ cells/ml and 4ml of cell suspension was transferred to 6-well flat bottomed plates. Cells were stimulated with heat killed *S. aureus* (10^5 CFU/ml). Cytokines were added to the wells to polarise cells as follows: IL-12 (10ng/ml) was used to drive Th1 cells and IL-1β + IL-23 (10ng/ml of each) were used to drive Th17 cells. Cells were incubated at 37°C and 5% CO_2 for 96 h. After incubation the cell supernatants were removed for cytokine analysis by ELISA. cRPMI was used to wash wells and remove dead cells. Cells were purified using Pan T cell Isolation Kit as previously described (Section 2.6.1). After purification cells were counted using trypan blue staining and re-suspended in cRPMI at $5x10^7$ cells/ml. Polarised cells ($5x10^6$ Th1 or Th17 cells) were transferred via i.p. injection into naive mice. As a control, groups of mice received $5x10^6$ naive $CD3^{+}$ cells which had been isolated from the spleen of a naive mouse and purified using a PAN T cell isolation kit II. At 3 h post cell transfer recipient mice received an i.p. challenge of *S. aureus* ($5x10^8$

CFU) (Figure 2.2). At specific time points post *S. aureus* challenge the bacterial burden and immune responses of recipient mice were assessed.

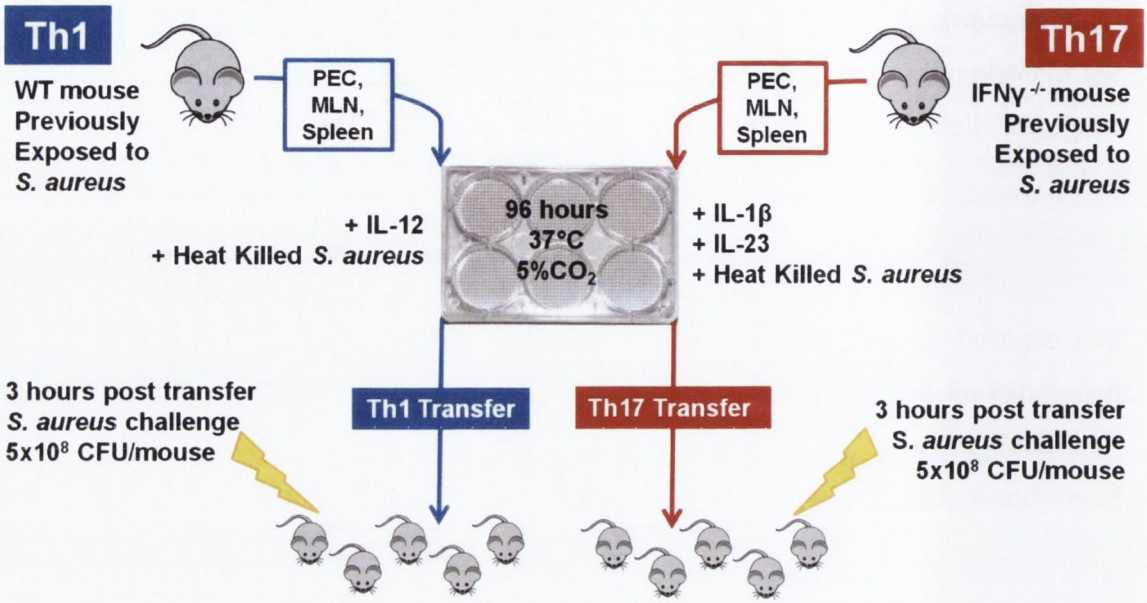


Figure 2.2: Adoptive Transfer of Th1 and Th17 cells

WT or IFNγ$^{-/-}$ mice were exposed to *S. aureus* via i.p. injection (5x10^8 CFU) on d 0, 7 and 14. On d 21 the PEC, MLN and spleen cells were isolated and stimulated *in vitro* in the presence of heat killed *S. aureus* (10^5 CFU/ml) and either rIL-12 (10ng/ml) for polarisation of Th1 cells, or rIL-1β and rIL-23 (10ng/ml of each) for polarisation of Th17 cells. Cells were stimulated for 96 h at 37°C. After incubation cells were isolated and being purified for CD3$^+$ T cells using the PAN T cell isolation kit. Upon purification of polarised CD3$^+$ T cells, 5x10^6 Th1 or Th17 cells were transferred into naive mice via i.p. injection. 5x10^6 naive splenic CD3$^+$ T cells were transferred as a control. At 3 h post cell transfer, mice were challenged with *S. aureus* via i.p. injection (5x10^8 CFU).

2.8. Murine Vaccine studies

2.8.1. ClfA synthesis and construction of UEA-1m targeted, ClfA loaded nanoparticles

ClfA was synthesised by Prof. Joan Geoghegan of the Staphylococcal Pathogenesis Group at TCD, as previously described (333). The ClfA protein used consisted of the recombinant His-tagged A domain of ClfA (amino-acids 40-559), which was purified by Ni^{2+} chelate chromatography. LPS was removed from the recombinant protein by Dr. Karen Misstear using the Detoxi-Gel Endotoxin Removing Gel (Thermo Scientific).

UEA1-m targeted, ClfA loaded nanoparticles were constructed by Dr. Karen Misstear of the Adjuvant Research Group at TCD (Figure 2.3). Streptavidin-coated polystyrene nanoparticles with diameters of 300-390nm were supplied by Spherotech Inc. and Corpuscular Inc. Biotinylated UEA-1m was synthesised by Polypeptide and provided as a lyphophilised powder which was reconstituted in endotoxin-free PBS. ClfA was biotinylated to using the EZ-Link Sulfo-NHS-LC biotinylation kit (Thermo Scientific).

QS21 was kindly provided by the Vaccine Formulation Laboratory in Lausanne, Switzerland.

2.8.2. Subcutaneous vaccinations with ClfA+CpG or CpG alone

To investigate if a vaccine was capable of driving protective Th1 cells against *S. aureus,* naive mice were vaccinated via subcutaneous (s.c.) injection with CpG alone (50μg/mouse) or CpG (50μg/mouse) in conjunction with ClfA (1μg/mouse) on d 0, 14 and 28. At 5 weeks post final boost (d 63), and prior to challenge with *S. aureus,* mice were sacrificed to analyse immune responses. Vaccinated mice were then challenged with *S. aureus* via i.p. injection ($5x10^8$ CFU) alongside a control group of naive mice (Figure 2.4). At specific time points post challenge, mice were sacrificed and bacterial burdens and immune responses were assessed.

Preliminary work established that vaccination with ClfA alone was not capable of driving a protective immunity. Mice were vaccinated with ClfA alone (1μg/mouse) via s.c. injection, on d 0, 14 and 28. On d 35 mice were scarified and bacterial burdens assessed.

2.8.3. Intranasal vaccination with ClfA combined with novel adjuvants

In collaboration with the Adjuvant Research Group in the School of Biochemistry and Immunology, mice were vaccinated intranasally with either UEA-1m targeted, ClfA loaded nanoparticles or ClfA and saponin QS21 on d 0, 14 and 28.On d 35 vaccinated mice were

challenged with *S. aureus* via i.p. injection ($5x10^8$ CFU) alongside a control group of naive mice (Figure 2.5). At specific time points post challenge mice were sacrificed and systemic infection levels and immune responses were assessed.

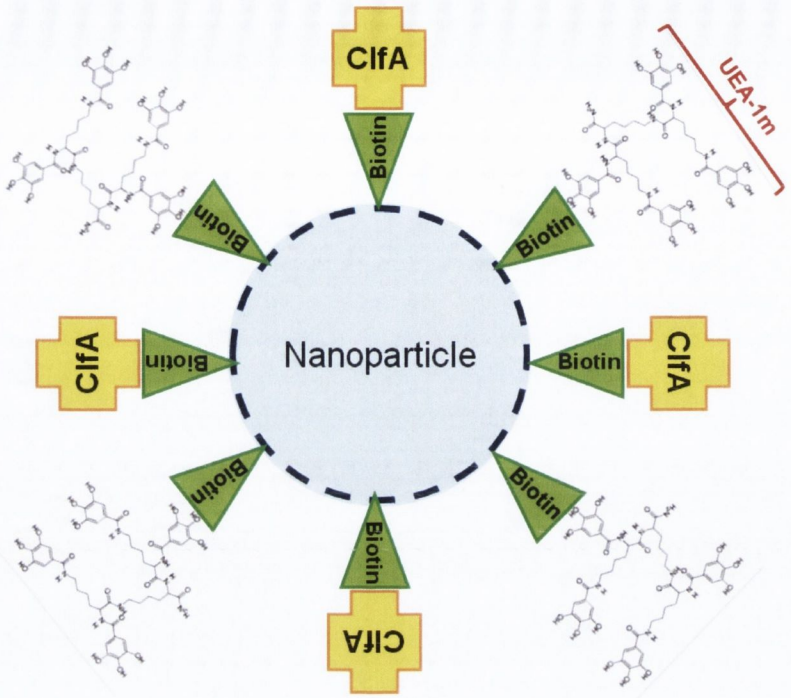


Figure 2.3: Schematic of UEA-1m targeted, ClfA loaded nanoparticles

UEA-1m and ClfA were biotinylated to the strepavdin layer of nanoparticles. ***Work carried out by Dr. Karen Misstear, Adjuvant Research Group, School of Biochemistry and Immunology, TCD.***

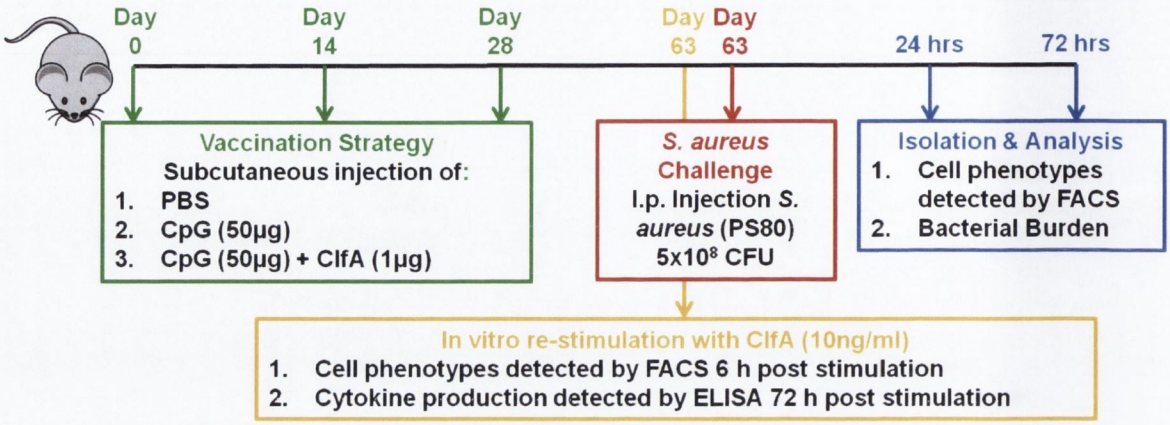


Figure 2.4: Strategy of subcutaneous vaccination with ClfA+CpG

Mice were vaccinated via s.c. injection with CpG (50µg/mouse) with or without ClfA (1µg/mouse) on d 0, 14 and 28. On d 63 cells from ClfA+CpG vaccinated, CpG treated and non-immunised mice were stimulated *in vitro* with ClfA for 72 h to assess T cell responses via cytokine production. On d 63 another group of ClfA+CpG vaccinated, CpG treated or non-immunised mice were challenged with *S. aureus* ($5x10^8$ CFU) via i.p injection. At 24 and 72 h post infection the bacterial burden, local cytokine production, T cell responses and phagocyte influx were assessed and compared between the groups.

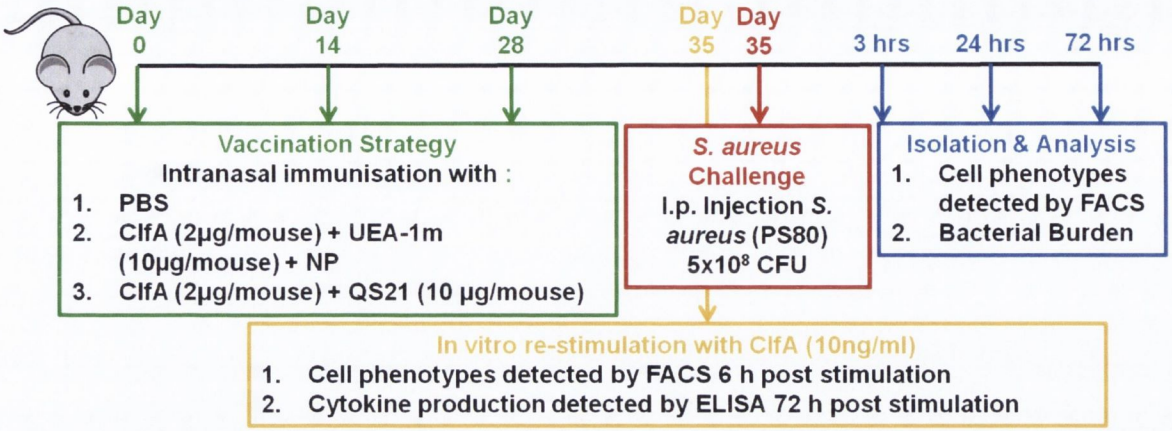


Figure 2.5: Strategy of intranasal vaccination with UEA-1m targeted, ClfA loaded nanoparticles or ClfA+QS21

Mice were immunized intranasally with on d 0, 14, and 28 with ClfA (2µg/mouse) and UEA-1m (10µg/mouse) attached to nanoparticles (NP) or with ClfA (2µg/mouse) and QS21 (10µg/mouse). On d 35 cells from vaccinated and non-immunised mice were stimulated *in vitro* with ClfA for 72 h to assess T cell responses via cytokine production. On d 35 another group of vaccinate mice were challenged with *S. aureus* ($5x10^8$ CFU) via i.p injection. At 3, 24 and 72 h post infection the bacterial burden, local cytokine production, T cell responses and phagocyte influx were assessed and compared between the groups.

2.9. Flow Cytometry

Isolated cells were incubated at 37°C and 5% CO_2 for 4 h with Brefeldin A (BFA, 5μg/ml) in the presence or absence of PMA (10ng/ml) and Ionomycin (1μg/ml). After incubation, cells were centrifuged and the cell pellets re-suspended in Aqua Live Dead stain diluted to a working concentration of 1:1000 in PBS for 20 mins at 4°C. Cells were then washed in PBS and centrifuged at 300xg for 5 mins. Cells were re-suspended in Fcγ block (1 μg/ml) to block non-specific antibody binding before extracellular staining with antibodies against specific cell surface antibodies (Table 2.9). Cells were suspended in a final concentration of 1:100 of surface antibody solution diluted in FACS buffer (Table 2.1) and were incubated for 20 mins at 4°C after which they were centrifuged at 300xg for 5 mins. Using the Dakocytomation Intrastain Kit (Alere) to stain for intracellular cytokines, cells were re-suspended in 60μl of Intrastain Reagent A, a fixative reagent (contains 5-10% formaldehdye) and incubated at RT for 15 mins. Samples were then washed in FACS buffer and centrifuged at 300xg for 5 mins. Next, 60μl of Intrastain Reagent B, a permeabilisation reagent, containing fluorescence conjugated antibodies specific for intracellular cytokines (Table 2.9) was added to each sample. Cells were re-suspended in a final concentration of 1:100 intracellular antibody solution diluted in Reagent B and were incubated for 15 mins at RT in the dark. Samples were washed again and re-suspended in FACS Buffer. Flow cytometry analysis was performed with BD FACSCanto II using FACS DIVA and FloJo software (Treestar, Inc). Gating for analysis was set on respective Fluorescence Minus One (FMOs).

To investigate reactive oxygen species (ROS) activity within phagocytes 123-dihydrorhodemine assays were carried out, as previously published by Richardson *et al* (334). ROS breaks down 123-DHR to rhodemine within the cell. Rhodemine fluoresces on the FL1 channel allowing rhodemine positive cells to be identified by flow cytometry. After surface staining, cells were re-suspended in 100μl cRPMI containing 25μl of 5μM 123-DHR solution. Cells were incubated for 10 mins at 37°C, after which cells were washed in PBS, spun down and re-suspended in 1% paraformaldehdye. Stained cells were then run on flow cytometry straightaway.

2.10. ELISA

ELISA assays were carried out on cell supernatants to determine various cytokine levels (Table 2.4). A high binding 96-well flat bottomed plate was coated with capture antibody (diluted to a working concentration in PBS, 50μl/well) and was incubated at RT overnight.

Wells were washed in ELISA wash buffer (Table 2.3) 3 times and were incubated with 1% (w/v) BSA (200µl/well) for 1 h at RT to block non-specific binding. Wells were again washed and cytokine standards, made up in reagent diluent (RD, Table 2.3), and samples (50µl) were added to the appropriate wells for 2 h at RT or overnight at 4°C. Following sample incubation wells were washed and detection antibody solution, diluted to a working concentration in RD (50µl/well), was added for 2 h at RT. After washing, 50µl streptavidin-horse radish peroxidase (HRP) solution, diluted to a working concentration (1:200) in RD, was added to the wells for 20 mins at RT in the dark. After washing, substrate solution (Table 2.3, 50µl/well) was added to the wells for 20 mins at RT in the dark. The reaction was stopped using ELISA stop reagent (Table 2.3, 25µl/well). Absorbance was measured at 450nm using a micro-plate reader and Softmax Pro software.

2.11. Antibody Assays

2.11.1. Antibody end point titre

Medium-binding 96-well plates were coated with of heat killed PS80 (10^8 CFU/ml, 50µl/well) or ClfA (1µg/ml, 50µl/well) and incubated for 2 h at 37°C. Plates were washed 3 times in ELISA wash buffer (Table 2.3) and non-specific binding was blocked by coating the plates with 10% (w/v) milk powder (Sigma Aldrich) in PBS (100µl/well) for 2 h at RT. After blocking, plates were washed and coated with doubling dilutions of serum samples in PBS (50µl/well). Samples were incubated over night at 4°C. The following day plates were washed and biotinylated antibody was added at the appropriate concentration (Table 2.5, 50µl/well) and incubated at 37°C for 1 h. Following this, plates were washed and incubated for 20 mins at RT in the dark with HRP diluted to a working concentration (1:200) in PBS (50µl/well). The plates were again washed and incubated at RT in the dark with ELISA substrate solution (Table 2.3). The enzymatic reaction was stopped by the addition of 25µl of 1M H_2SO_4 to each well. Absorbance was then measured at 450nm on a microplate reader.

To determine the end point antibody titres, best fit linear regression curves were calculated for the absorbance values obtained for each serum titration. These curves were extrapolated against titrations of naive sera to find the intersection point between the two. These intersection points were used to find the corresponding dilution at which the absorbance values reached PBS control levels. This intersection revealed the Log_{10} value of the antibody titres (Figure 2.6).

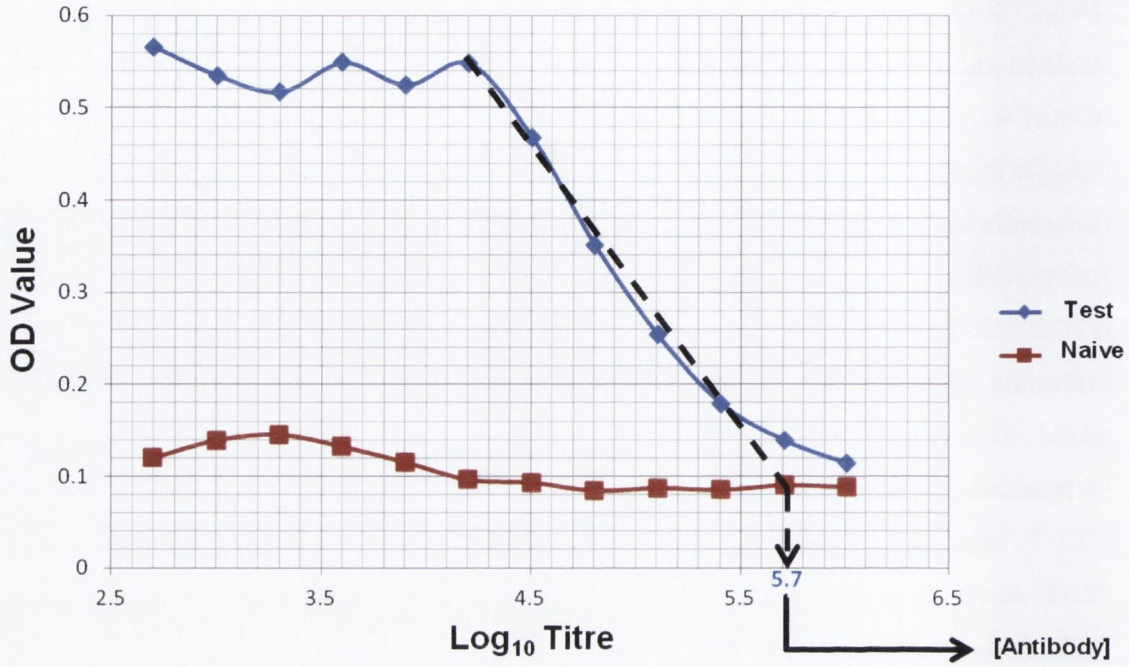


Figure 2.6: Determining antibody end point titres

The OD values for each serum titration were plotted on a curve and a best fit linear regression curves was calculated for both the naive sera (control) and the experimental (test) sera. A standard curve was calculated for the test sample and where this curve intersected the naive linear regression curve was used to find the corresponding dilution at which the absorbance values reached naive control levels. This intersection revealed the Log_{10} value of the antibody titres.

2.11.2. Neutralising antibody assay

A flat bottomed 96-well plate was coated with 2µg/ml fibrinogen (Sigma, 100µl/well) in PBS and incubated at 4°C overnight. The next day plates were washed with ELISA wash buffer and blocked with sterile 5% (w/v) BSA (100µl/well) for 2 h at 37°C. Stationary phase liquid cultures of PS80 were centrifuged at 3000xg for 10 mins and the bacterial pellet was washed once in PBS to remove any proteases. The suspension was pelleted and was re-suspended in 1ml PBS, and adjusted to an OD_{600nm} of 1 by dilution in PBS to give the bacterial working solution. Into the wells of a separate 96 well round bottomed plate, 2µl of undiluted serum was added to 118µl of bacterial working solution. 'Bacteria only' and 'PBS only' wells were included as positive and negative controls, respectively. Sera were incubated with bacteria on a shaker for 30 mins at RT.

After blocking for 2 h in 5% BSA, the fibrinogen coated plate was washed 3 times in ELISA wash buffer (Table 2.3) and 100µl of sera: bacteria solution was transferred to the fibrinogen coated plate. This plate was then incubated for 90 mins at 37°C. Following incubation the plate was washed 3 times in ELISA wash buffer and wells were incubated with 100µl of 25% (v/v) formaldehyde for 20 mins at RT to fix any fibrinogen bound bacteria. Plates were then washed and stained with 100µl of crystal violet for 1 min which stains the bound and fixed staphylococci. Following washing, 100µl of 5% (v/v) acetic acid was added to the wells and incubated for 5 mins with shaking to solubilise the crystal violet. Absorbance was measured at 570nm on a microplate reader (Labsystems).

The presence of neutralising antibodies was determined by testing the ability of serum to inhibit the adherence of *S. aureus* to fibrinogen. Adherence was expressed as a percentage of bacterial adherence in the absence of serum. Percentage inhibition was determined by subtracting the percentage adherence values from 100.

% Adherence= (OD of sample wells/ OD of Bacteria only wells) x 100
% Inhibition= 100 - % Adherence

2.12. Amplification of the Vγ4 gene within purified $V\gamma1.1^{-}V\gamma2^{-}$ cells

2.12.1. RNA extraction

Cells were isolated from the PEC and MLN of mice 3 h post *S. aureus* infection. $V\gamma1.1^{-}$ $V\gamma2^{-}$ γδ T cells were purified using cell sorting and were re-suspended in 1ml of TRIzol reagent (Invitrogen) in an RNase-free tube and immediately stored at -80°C until RNA

extraction was ready to be preformed. Cells were thawed at RT for 5 mins before addition of 200µl of chloroform (Sigma Aldrich). Samples were then shaken vigorously for 15 sec before being incubated at RT for 3 mins. Samples were then centrifuged at 12,000xg for 15 mins at 4°C. After centrifugation, the colourless upper aqueous layer was aspirated into new RNase free tube and 500µl Iso-2-propanol (Sigma Aldrich) was added. Samples were left to stand at RT for 15 mins followed by centrifugation at 12,000xg for 10 mins at 4°C. Supernatants were then aspirated and RNA pellets were re-suspended in 1ml of 75% ethanol and vortexed. Following this, samples were centrifuged at 7,500xg for 5 mins at RT. The supernatants were aspirated and the pellet left to dry for 10 mins. Next, the RNA pellet was dissolved in 30µl RNase free H_2O (Invitrogen) and incubated at 60°C for 7-10 mins. The extracted RNA was immediately put on ice to avoid degradation. Total RNA yield was determined using Nanodrop ND 1000 spectrophotometer.

2.12.2. cDNA synthesis

RNA was stored at -80°C until cDNA synthesis was performed. cDNA was generated from RNA using the iScript cDNA Synthesis Kit (Bio-rad). Reactions were typically carried out in 20 µl volumes using RNA (1µg) made up to 13µl in nuclease free H_2O, 5µl iScript select reaction mix, 2µl $Oligo(dT)_{20}$ primers and 1µl iScript reverse transcriptase. The samples were placed in PTC-200 Peltier Thermal Cycler using the following conditions: 25°C for 10 mins, 37°C for 2 h, 85°C for 5 mins.

2.12.3. Primer Design

Primers for the specific genes of interest were designed using the NCBI Primer-BLAST primer designing tool. mRNA sequences for the genes of interest were obtained using NCBI GenBank and the sequence of each variable region used to design the specific primers. Primers were tested with the NCBI Primer Blast to ensure specificity for the target gene. Forward and reverse primer pairs are listed in Table 2.6. Primers were supplied in lyophilised form by Integrated DNA technologies and reconstituted at 20mM in RNase-free H_2O and Tris-EDTA. Primers specific for the Vγ2 subset and the endogenous control gene 18S rRNA were designed based on previously published primers (335).

2.12.4. Polymerase Chain Reaction (PCR)

Working primer solutions were made up using H_20 at a final concentration of 600nM. cDNA was used at a concentration of 250ng/µl. Sybr green (Bio-Rad) was used in the PCR

reaction according to manufacturers' instructions. PCR was carried out on a CFX96 touch real-time PCR detection system (Bio-Rad) using the following cycle parameters: 95°C for 1 min, 62°C for 30 sec and 72°C for 1 min, repeated for 30 cycles. The presence of amplified target DNA was determined by agarose electrophoresis using 1.5% agarose gels (Table 2.1). Briefly, 1.5g agarose was dissolved by boiling in TAE buffer (100ml), and cooled to 65°C. Ethidium bromide was added and gels were cast in mini trays. DNA samples in loading buffer (Tracking dye, Table 2.1) were pipetted into wells along with DNA size markers (DNA 100 base pair ladder, Invitrogen). Electrophoresis of samples was routinely performed at 90-100 V. Gels were viewed under UV light to capture images. Target DNA was extracted from the agarose using High Pure PCR Product Purification Kit (Roche) according to the manufacturers' instructions and identified by sequencing (Source Biosciences, Dublin). Sequence alignment to the translated reference gene (NG 007033.1) was carried out using ClustalW software

2.13. Statistical analysis

Statistical analyses were performed using GraphPad Prism statistical analysis software. Differences between two groups at a single time point were analysed using an unpaired student's t-test. Differences between multiple groups at a single time point were analysed using a one way ANOVA with Tukey's comparison post test. Differences between multiple groups at multiple different time points were analysed using a two way ANOVA with Bonferroni correction post test. A p value < 0.05 was considered significant.

Chapter 3

Effects of prior exposure to *S. aureus* on the cellular immune response

3.1. Introduction

The development of novel immunotherapies and effective vaccines against *S. aureus* has been hindered by a number of factors, most pressing of which is the lack of understanding of what actually constitutes protective immunity against *S. aureus,* i.e. which form of adaptive immunity, cellular versus humoral, should be targeted to induce prophylactic protection. Emerging evidence suggests that inefficient induction of cellular immunity against the invading bacteria is a contributing factor to the failure of anti-*S. aureus* vaccine strategies to date (277, 336).

Although the majority of these vaccination strategies have focused on the induction of humoral responses, B cell deficiencies in patients (301) or mice (320) are not associated with increased susceptibility to *S. aureus* infection. In addition, numerous studies have shown that *S. aureus*-specific antibodies are produced upon exposure to the bacterium during infection or colonisation (252, 291). However, these antibodies do not necessarily protect against *S. aureus* infection (291, 337) and recurrence of infection is often observed in the clinic (338, 339). Furthermore, transfer of sera that contained anti-staphylococcal antibodies or B cells isolated from mice vaccinated with rAls3p-N, a candidal molecule similar in structure to the staphylococcal surface protein ClfA, did not protect against subsequent infection in a murine model of bacteraemia (320). These studies suggest that induction of an antibody response alone is incapable of mediating full protection against *S. aureus* infections.

Recent reports have suggested an important protective role for T cells during *S. aureus* infections. T cell deficiencies in patients (94, 95, 305) and mice (320) are associated with an increased susceptibility to *S. aureus* infection. A vaccine inducing robust anti-*S. aureus* T cell responses was shown to protect against infection through the ability of the T cells to regulate downstream phagocytic cell effector functions that facilitate efficient clearance of the infection (329). Indeed, using novel adjuvant technology in combination with the staphylococcal antigen ClfA, vaccine induced protection against acute systemic *S. aureus* infection in mice was recently demonstrated in the complete absence of an antibody response (Section 5.2.12) (340).

Specific T cell subsets are associated with protection against particular pathogens, for example Th1 derived IFNγ is vital for cellular immunity against intracellular infections such as *L. monocytogenes* (57) and *Mycobacterium spp.* (58, 59), whereas Th17 cells are essential for protection against extracellular bacteria, particularly at mucosal sites, through

their ability to regulate neutrophil recruitment (74, 86, 88, 91). Conflicting evidence currently exists on the importance of IFNγ and Th1 responses in natural anti-staphylococcal immunity, with some reports demonstrating no role for IFNγ responses (169) and others revealing both protective (325) and detrimental effects (323, 324) as well as additional studies documenting differential roles for IFNγ during the course of *S. aureus* infection (326, 327). Exploiting the genetic backgrounds of C57Bl/6 (Th1 skewed) and Balb/c (Th2 skewed) mice, researchers have demonstrated that C57Bl/6 mice were more resistant to *S. aureus* induced pneumonia (307), bacteraemia (341) and keratitis (342), suggesting that Th1 responses contribute to anti-staphylococcal immunity in these models. Th1 derived IFNγ is also necessary for vaccine induced immunity against systemic (329) and intramammary (330) *S. aureus* infection. Alternatively, due to defects in their Th17 cells HIES patients suffer from exacerbated *S. aureus* infection (94, 95). Additionally, patients with atopic dermatitis are more susceptible to *S. aureus* colonisation (343), due in part to impaired IL-17 responses (344). Furthermore, studies in murine models demonstrated that IL-17A$^{-/-}$IL-17F$^{-/-}$ double knock-out mice displayed increased susceptibility to sub-epithelial, but not systemic *S. aureus* infection (321). These studies support a strong role for IL-17 responses in anti-staphylococcal immunity at mucosal surfaces. Th17 cells are important in acquired immunity against systemic infection (329), whereas γδ T cell derived IL-17 is essential for protection against primary *S. aureus* infections in mucosal tissues (165, 169) and in surgical site models (322). A recent report in the field of pertussis demonstrated not only the importance of inducing cellular immunity, but also the induction of appropriate T cell responses, for optimal vaccine induced immunity (345). Therefore, a more comprehensive understanding of the role played by particular T cell subsets critical for site-specific staphylococcal clearance is required to inform the development of vaccines that will efficiently target anti-*S. aureus* cellular immunity.

Although the correlates of natural immunity to *S. aureus* infection remain unclear, one study suggests that nasal colonisation with *S. aureus* can modulate immune responses to generate protection upon invasive infection. This clinical study revealed that even though *S. aureus* colonised patients had an increased risk of contracting bacteraemia they actually had a better survival rate upon infection than non-carriers (254). As previously mentioned, exposure to *S. aureus* through colonisation induces anti-staphylococcal antibodies (252), however these antibodies do not necessarily protect against re-infection (291, 337). Therefore it is tempting to speculate that the protection observed upon invasive

staphylococcal infection in this colonised patient cohort was T cell mediated, however this was not investigated in the study and no immune mechanism responsible for protection was identified. This report does suggest that prolonged or sustained exposure to the whole bacterium modulates the immune response in such a way that it is beneficial to the host upon *S. aureus* infection.

T cells are important in immunity against *S. aureus* infection in humans and are capable of inducing site-specific protection in murine models, however the existence of *S. aureus*-specific memory T cells suitable for vaccine targets remains to be established. The overall aim of this study is to characterise the T cell phenotypes expanded upon long-term exposure to *S. aureus*. Given the fact that *S. aureus* is a common asymptomatic coloniser in humans, it is important to understand how the immune system reacts to this bacterium as a consequence of continued exposure. Mice are naturally resistant to *S. aureus* infection and typically clear the bacterium from the peritoneal cavity within 7 d. Repeated injections on d 0, 7 and 14 therefore provided a mechanism to maintain exposure to *S. aureus* for a prolonged period. On the basis of this, a novel murine model of recurrent peritonitis was developed to assess whether prior exposure to *S. aureus* could mediate protection against subsequent infection. Specifically, the impact of prior exposure to *S. aureus* on T cell recall responses and downstream phagocytes during subsequent infection was established.

3.2. Results

3.1.1. *S. aureus* induced peritonitis is cleared within 7 d post infection

Initial experiments established the time course of primary *S. aureus* infection. Groups of WT mice received an i.p injection of *S. aureus* ($5x10^8$ CFU). By 7 d post *S. aureus* exposure mice had cleared the bacteria from the peritoneal cavity (Figure 3.1 A), kidneys (Figure 3.1 B) and spleen (Figure 3.1 D) with <10 CFU/ml detected in these sites. Residual bacteria was however still present in the liver on d 7 (Figure 3.1 C). These levels represented a 1.75 log reduction from d 5, suggesting that the bacteria were actively and rapidly being cleared from the liver at these later time points.

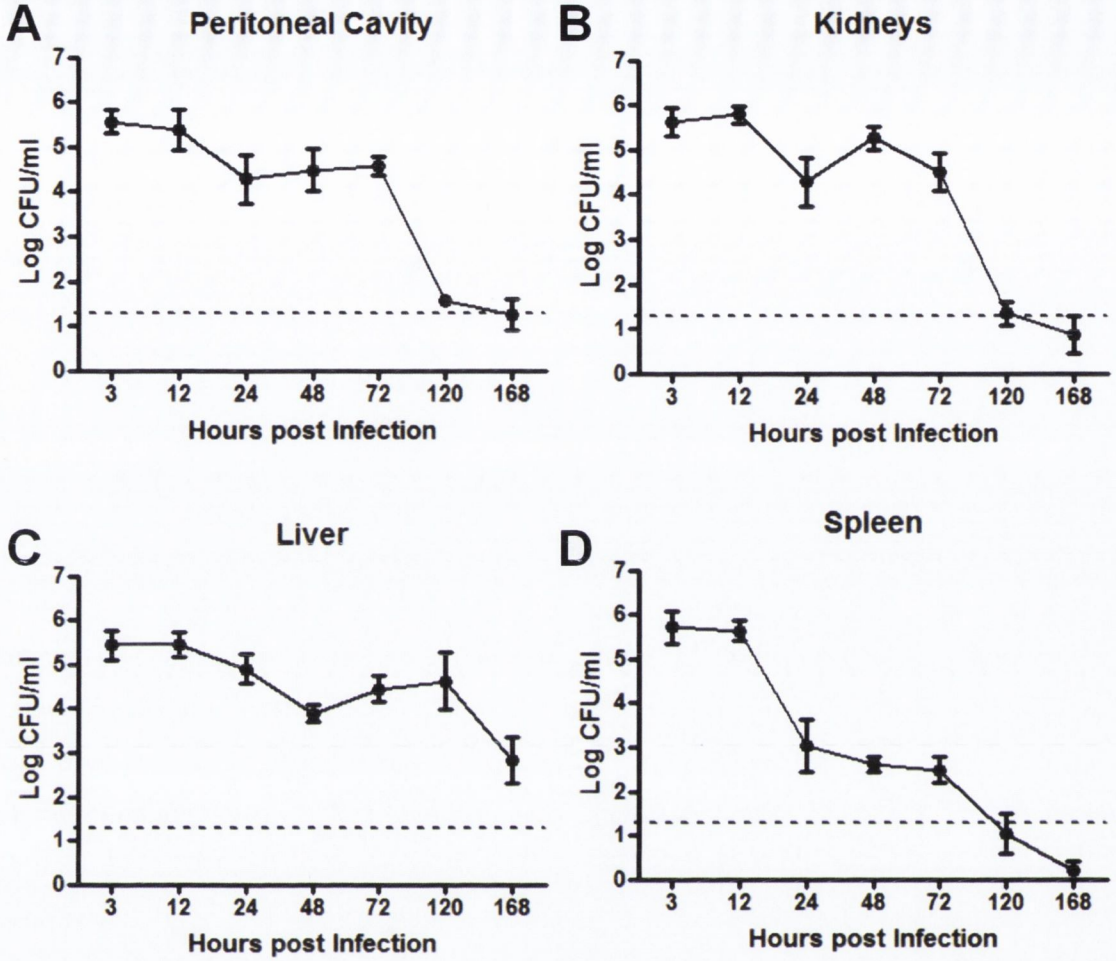


Figure 3.1: *S. aureus* induced peritonitis is cleared within 7 d post infection.

Groups of mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injection. At indicated time points post challenge a lavage of the peritoneal cavity (A) was carried out in order to assess the local bacterial burden. The kidneys (B), liver (C) and spleen (D) were also isolated to assess the dissemination of the infection. Results expressed as Log CFU/ml of mean±SEM. Dashed lines indicate limit of detection. n=5 per group.

3.2.2. Prior exposure to S. aureus results in the expansion of S. aureus antigen-specific T cells

To investigate if prior exposure to *S. aureus* results in the expansion of specific T cell populations capable of responding to subsequent re-exposure to staphylococcal antigens, *S. aureus* antigen-specific recall response assays were established. Groups of mice were exposed to a single i.p. injection of *S. aureus* ($5x10^8$ CFU). At 7 and 21 d post exposure, total cells were isolated from the peritoneal cavity, draining mediastinal lymph (MLN) and spleen. The cells were then cultured *in vitro* and re-exposed to heat-killed (HK) S. *aureus* (10^6 CFU) for 72 h. Cells isolated from naive mice were used as a control. Cytokine secretion by T cells was measured by ELISA as a read out of *S. aureus* antigen-specific responses.

Cells isolated from the peritoneal cavity, MLN or spleen of naive mice did not produce IFNγ and IL-17 in response to *in vitro* stimulation with HK *S. aureus*, suggesting that naive T cells are unable to respond to staphylococcal antigens *in vitro* through production of IFNγ and IL-17. In contrast peritoneal cells from mice that had been exposed to *S. aureus in vivo* for 7 and 21 d were capable of responding to *S. aureus* stimulation *in vitro* through production of significant amounts of IFNγ (Figure 3.2 A) and IL-17 (Figure 3.2 B). A similar trend was observed in cells isolated from the MLN of *S. aureus* exposed mice, with increased levels of IFNγ (Figure 3.2 C) and IL-17 (Figure 3.2 D) production detected in response to *in vitro* antigen stimulation, compared to naïve MLN cells. Only splenic cells isolated from mice exposed to *S. aureus in vivo* for 21 d were capable of responding to antigen re-stimulation through production of IFNγ (Figure 3.2 E), but not IL-17 (Figure 3.2 F). Splenic IFNγ producing cells may only be observed during longer exposure (i.e. 21 d) to *S. aureus* as migration of IFNγ producing cells to the spleen may require this extra time. The lack of an IL-17 response may reflect the lower frequencies of IL-17 producing cells present within the spleen, or the preference of these cells to be maintained at the infection site or translocate to the MLN. However taken together these data suggest that upon *in vivo* exposure to *S. aureus* there is an expansion of IFNγ and IL-17 producing T cells, both locally at the site of infection and in the periphery, which are capable of responding to *in vitro* antigen stimulation. This effect was observed 7 d post challenge and maintained until d 21, suggesting the maintenance of antigen-specific T cells.

To confirm that T cells were the sources of these cytokines, $CD3^+$ cells were purified from the peritoneal cavity and MLN of naïve mice or mice exposed to *S. aureus* for 7 d,. Purified $CD3^+$ cells were then stimulated *in vitro* with irradiated APCs and HK *S. aureus* 10^6 CFU. Naïve $CD3^+$ cells isolated from the peritoneal cavity or MLN did not produce IFNγ and IL-17 in response to *in vitro* stimulation with HK *S. aureus* (Figure 3.3). In contrast purified CD3 cells from the peritoneal cavity (Figure 3.3 A & B) and MLN (Figure 3.3 C & D) of mice that had been previously exposed to *S. aureus in vivo* for 7 d were capable of responding to *S. aureus* stimulation *in vitro* through production of substantial levels of IFNγ and IL-17. This experiment represents limited replicates, due to the large numbers of animals required to obtain enough purified CD3 cells for *in vitro* antigen stimulation. However this data confirms that the IFNγ and IL-17 secretion observed in total cell stimulation (Figure 3.2) is primarily produced by CD3 T cells.

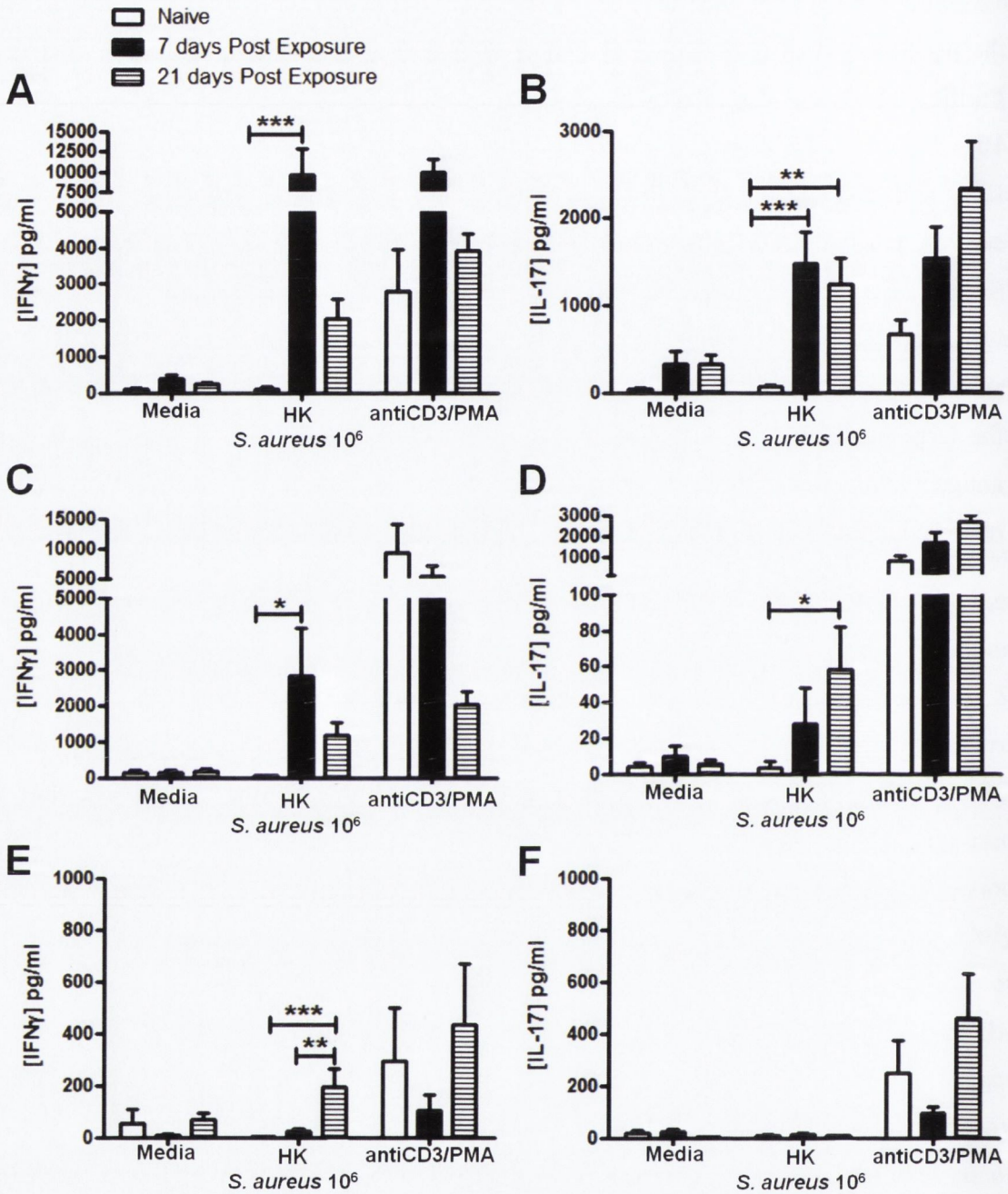


Figure 3.2: Exposure to *S. aureus in vivo* results in the expansion of antigen-specific IFNγ and IL-17 producing cells.

Groups of mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injection. On d 7 and 21 post challenge, total cells from the peritoneal cavity (A, B), MLN (C, D) and spleen (E, F) were isolated. Cells from naive mice were isolated as a control. Isolated cells were re-exposed to heat-killed *S. aureus* (10^6 CFU) *in vitro* for 72 h. IFNγ (A, C, E) and IL-17 (B, D, F) secretion was measured by ELISA. Results expressed as mean±SEM. n=15 for naive, n=12 for 7 d post exposure, n=8 for 21 d post exposure *$p<0.05$, ** $p<0.005$.

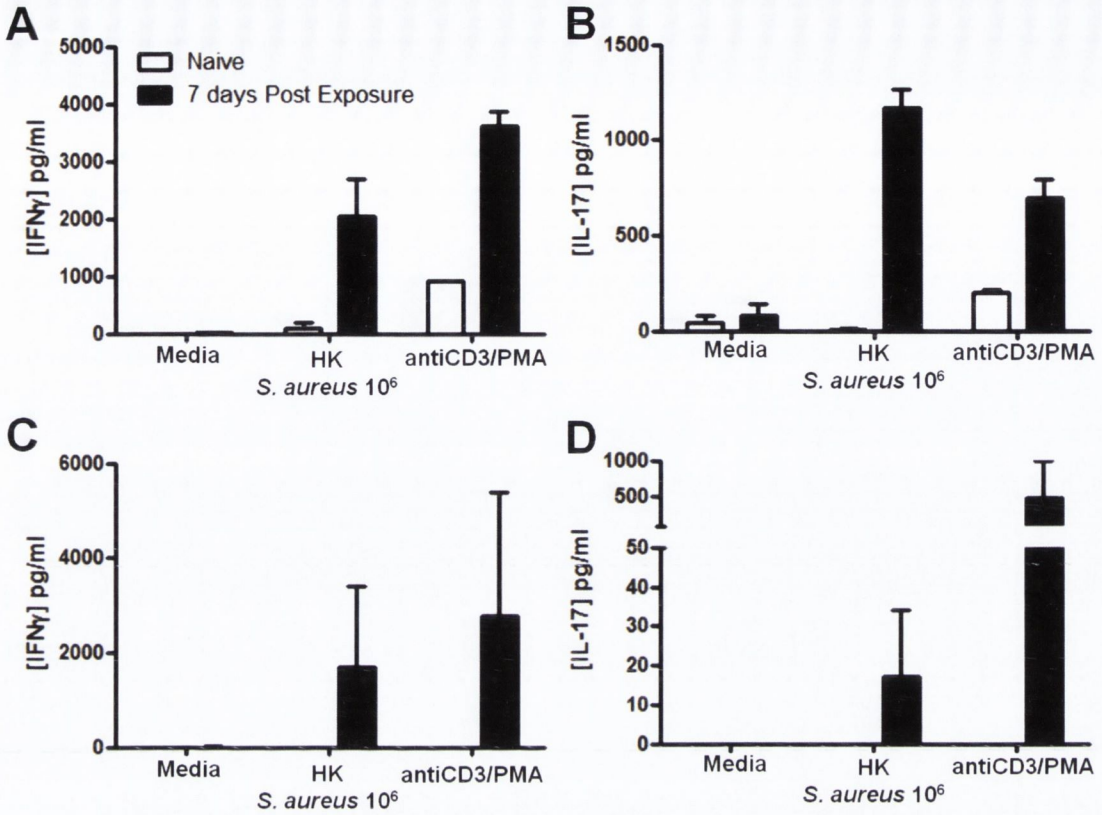


Figure 3.3: Exposure to *S. aureus in vivo* results in the expansion of *S. aureus* antigen-specific $CD3^+$ T cells.

Groups of mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injection. On d 7 post challenge, total cells from the peritoneal cavity (A, B) and MLN (C, D) were isolated. Cells from naive mice were isolated as a control. Total cells were then purified for $CD3^+$ T cells and the remaining APCs irradiated. Purified cells were re-exposed to heat-killed *S. aureus* (10^6 CFU) *in vitro* for 72 h. IFNγ (A, C) and IL-17 (B, D) secretion was measured by ELISA. Results expressed as mean±SEM, n=2 per group.

3.2.3. Prolonged exposure to S. aureus as a consequence of multiple challenges promotes enhanced T cell activation which correlates with protection against subsequent infection

Previous studies established *S. aureus* is cleared very quickly from the peritoneal cavity upon i.p challenge (Figure 3.1). Initial experiments also identified that *in vivo* exposure to *S. aureus* drove increased IL-17 and IFNγ secretion by T cells upon *in vitro* stimulation with staphylococcal antigens (Figure 3.2 & 3.3). Expanding on this, T cell antigen recall responses *in vivo* were assessed. Repeated injections were used to maintain exposure to *S. aureus* thereby increasing the potential to generate or expand *S. aureus* antigen-specific T cells.

Groups of mice were exposed to *S. aureus* ($5x10^8$ CFU) via a single i.p. injection on d 0 or repeated i.p injections on d 0, 7 and 14. The mice were then allowed to recover until d 21 before re-challenge with a subsequent i.p injection of *S. aureus* ($5x10^8$ CFU). In addition a group of naive mice (that had never been exposed to the bacterium) were also challenged as the control group. At 3 and 72 h after the final challenge the peritoneal cavity was lavaged with PBS and the local cytokine secretion and bacterial burden assessed.

IL-17 was detected in the peritoneal fluid of non-exposed mice (~280.89 pg/ml) 3 h post infection (Figure 3.4 A). Of note, no IFNγ secretion was detected in the peritoneal fluid of non-exposed mice (Figure 3.4 B). Increasing the number of exposures to *S. aureus* significantly increased IFNγ (Figure 3.4 B) and IL-17 (Figure 3.4 A) production following the final challenge. To establish if the increased IFNγ and IL-17 responses observed in mice that underwent multiple exposures to the pathogen correlated with enhanced bacterial clearance the bacterial burden at the infection site was established. Mice that had not been previously exposed to *S. aureus* had approximately 5 log bacteria present in the peritoneal cavity 72 h post final challenge (Figure 3.4 C). A single exposure to *S. aureus* 21 d prior to final challenge did not affect bacterial clearance from the peritoneal cavity. However, multiple exposures to *S. aureus* on d 0, 7 and 14 resulted in a significant reduction (~2.1 log reduction) of local bacterial burden, compared to non-exposed mice (Figure 3.4 C).

These data suggest that expansion of T cells following prolonged exposure to *S. aureus* can mediate protection against subsequent *S. aureus* challenge in an IFNγ and/or IL-17 dependent manner. These results validate the model of recurrent peritonitis (Figure 2.1) which is used throughout the remainder of this project.

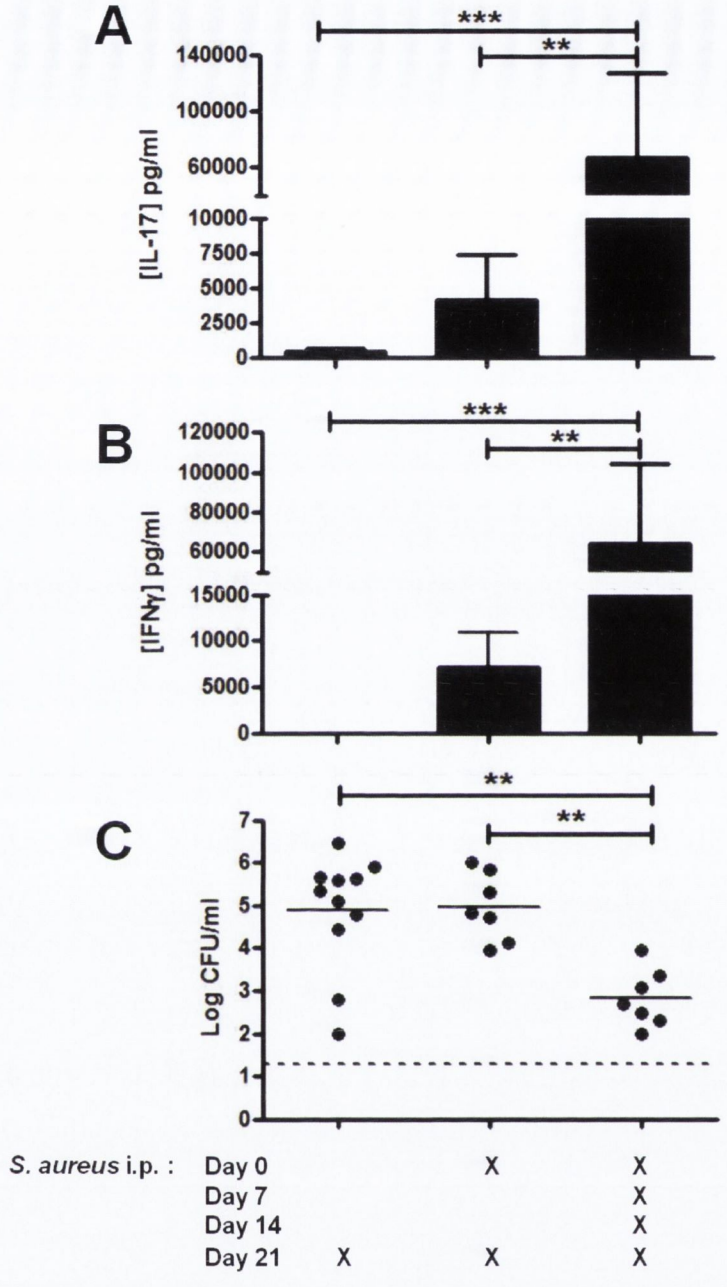


Figure 3.4: Prolonged exposure to *S. aureus* significantly increases lymphocyte derived cytokine production which is associated with increased bacterial clearance upon re-infection

Groups of mice were exposed to *S. aureus* ($5x10^8$ CFU) via a single i.p. injection on d 0 or multiple i.p. injections on d 0, 7 and 14. Prior exposed mice were then re-challenged with an i.p injection of *S. aureus* ($5x10^8$ CFU) on d 21. In addition, a group of naive mice that had not been previously exposed were challenged as a control. At 3 and 72 h post challenge the peritoneal cavity was lavaged with PBS. IL-17 (A) and IFNγ (B) production was detected by ELISA 3 h post infection. Results expressed as mean±SEM. The bacterial burden present in the peritoneal cavity was also assessed 72 h post infection (C). Results expressed as Log CFU/ml with means indicated by line. Dashed lines indicate limit of detection. n=11 for non-exposed group, n=7 for single prior exposure group, n=7 for multiple prior exposure group. Data pooled from 2 independent experiments. **$p<0.005$ ***$p<0.001$

3.2.4. Characterisation of baseline infection and inflammatory levels post prolonged exposure to S. aureus

To confirm that any protection observed against *S. aureus* in prior exposed mice was not due to residual immune responses against persisting staphylococci, experiments were set up to investigate the baseline levels of infection and inflammatory responses after prolonged exposure to *S. aureus*. In order to do this mice received multiple i.p. injections with *S. aureus* ($5x10^8$ CFU) on d 0, 7 and 14 and were then allowed to recover for varying time periods post third exposure in order to assess residual bacterial burden.

On d 21 (7 d post final exposure) or d 35 (21 d post final exposure) the peritoneal cavity was lavaged with PBS and the local bacterial burden assessed. *S. aureus* infection was cleared to the limit of detection (1.3 log) in the peritoneal cavity by d 21, however extending the recovery period to d 35 resulted in even greater clearance of residual staphylococci (Figure 3.5 A). To confirm that the local inflammatory responses in the peritoneal cavity had resolved by d 21 or d 35, total cell counts from the peritoneal cavity were compared to naive mice. There was a significant increase in the total number of cells present in the peritoneal cavity of mice at d 21 (7 d post final exposure), whereas by d 35 (21 d post final exposure) the total number of cells was comparable to naive mice (Figure 3.5 B). This suggests that extending the time period to d 35 (21 d post final exposure) allows for clearance of residual inflammatory cells.

Cytospins were then prepared of PECs to assess differential leukocyte infiltration. In mice that were allowed to recover until d 21, (7 d post final exposure), significantly decreased proportions of macrophages were observed (Figure 3.5 C) alongside a significant increase in the number of residual neutrophils (Figure 3.5 D), compared to naive mice. However by extending the length of recovery to d 35 (21 d post final exposure), the proportions of macrophages (Figure 3.5 C), neutrophils (Figure 3.5 D) and lymphocytes (Figure 3.5 E) in the peritoneal cavity were comparable to naive mice.

Taken together these data confirm that by d 35, (21 d post final exposure to *S. aureus*) infection had been cleared and the inflammatory response at the site of infection completely resolved. Therefore a recurrent i.p. injection strategy consisting of 3 consecutive *S. aureus* i.p. exposures 7 d apart, followed by a 21 d recovery period was utilised for the remainder of this study.

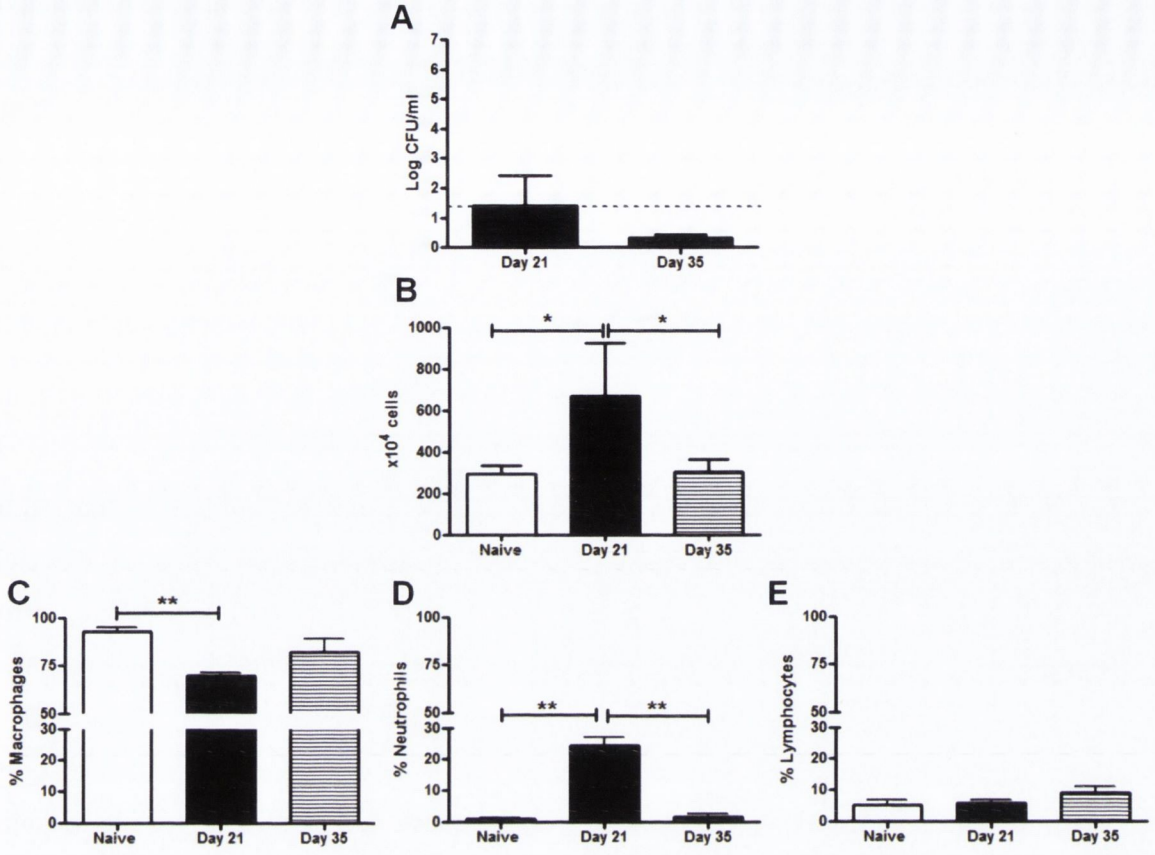


Figure 3.5: Increasing the recovery time post final exposure reduces residual inflammatory responses.

Groups of mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injections on d 0, 7 and 14. Mice were then allowed to recover until d 21 or d 35 after which the peritoneal cavity was lavaged with PBS and local bacterial burden assessed (A). Results expressed as Log CFU/ml of mean±SEM. Dashed lines indicate limit of detection. Total cell numbers were established in the peritoneal cavity (B). Cytospins were prepared using PECs to assess macrophage (C), neutrophil (D) and lymphocyte (E) infiltration. Results expressed as mean±SEM. n=5 for naive, n=5 for d 21, n=8 for d 35. * $p<0.05$, ** $p<0.005$

3.2.5. Prior exposure to S. aureus significantly increases IFNγ and IL-17 producing T cells upon in vitro mitogen stimulation

Previous experiments established that infection had been cleared and immune responses had resolved by d 35 post exposure to multiple i.p. injections of *S. aureus*. The ability of *S. aureus*-primed cells to respond to *in vitro* mitogen stimulation was then investigated. Groups of mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injections on d 0, 7 and 14. On d 35 peritoneal, MLN and spleen cells were isolated for flow cytometry analysis of T cells. Cells isolated from naive mice were used as a control.

A significant increase in the proportions (Figure 3.6 A) and absolute numbers (Figure 3.6 B) of $CD3^+$ cells was detected in the peritoneal cavity of prior exposed mice compared to non-exposed mice. No difference in the frequency or numbers of $CD3^+$ cells was detected in the MLN (Figure 3.6 C & D) or spleen (Figure 3.6 E & F) between naive and prior exposed mice. Further analysis of the cell populations making up the $CD3^+$ cells revealed that $CD4^+$ and $CD8^+$ T cell proportions and numbers in the peritoneal cavity (Figure 3.7 A & B), MLN (Figure 3.7 C & D) and spleen (Figure 3.7 E & F) were unaffected by prior exposure to *S. aureus*. Conversely, γδ T cell proportions and numbers were significantly elevated in the peritoneal cavities (Figure 3.7 A & B) and MLN (Figure 3.7 C & D) of prior exposed mice compared to naive mice, with a similar trend evident in the spleens (Figure 3.7 E & F). This suggests that expansion and maintenance of γδ T cells is occurring at the local site of infection and in the periphery following prior exposure to *S. aureus*.

Following identification of the T cell subsets present in the peritoneal cavity, MLN and spleens of naive and prior exposed mice, the cytokine profile of these cells was investigated using *in vitro* stimulation with the mitogens PMA and ionomycin. Analysis revealed a marked increase in the frequency and absolute numbers of IFNγ producing CD4 and CD8 cells in the peritoneal cavity (Figure 3.8 B & C) and MLN (Figure 3.8 D & E) of prior exposed mice compared to naive mice. Prior exposure did not affect IFNγ production by splenic $CD4^+$ T cells (Figure 3.8 F & G), however significantly increased proportions and numbers of $IFN\gamma^+CD8^+$ T cells were detected in the spleens of prior exposed mice (Figure 3.8 F & G). Prior exposure to *S. aureus* did not affect IFNγ production by $\gamma\delta^+$ T cells at any of the investigated sites (Figure 3.8 B-G), with very low numbers of $IFN\gamma^+\gamma\delta^+$ cells detected compared to CD4 and CD8 T cells (Figure 3.8 C, E & G).

In contrast, a significant increase in IL-17$^+\gamma\delta^+$ T cell frequencies and numbers was detected in the peritoneal cavity (Figure 3.9 B & C) and MLN (Figure 3.9 D & E) of prior exposed mice, with a similar trend evident in the spleen (Figure 3.9 F & G). Overall γδ T cells were the dominant producers of IL-17 with only low frequencies and numbers of IL-17 producing CD4$^+$ or CD8$^+$ T cells detected in the peritoneal cavity (Figure 3.9 B & C) and MLN (Figure 3.9 D & E). However, a significant increase in splenic IL-17$^+$CD4$^+$ T cell numbers was observed in prior exposed mice compared to naïve mice (Figure 3.9 G). Taken together these data point towards a selective expansion of Th1 cells, IFNγ producing CD8 cells and IL-17 producing γδ T cells at the infection site and the periphery as a result of prior exposure to *S. aureus*.

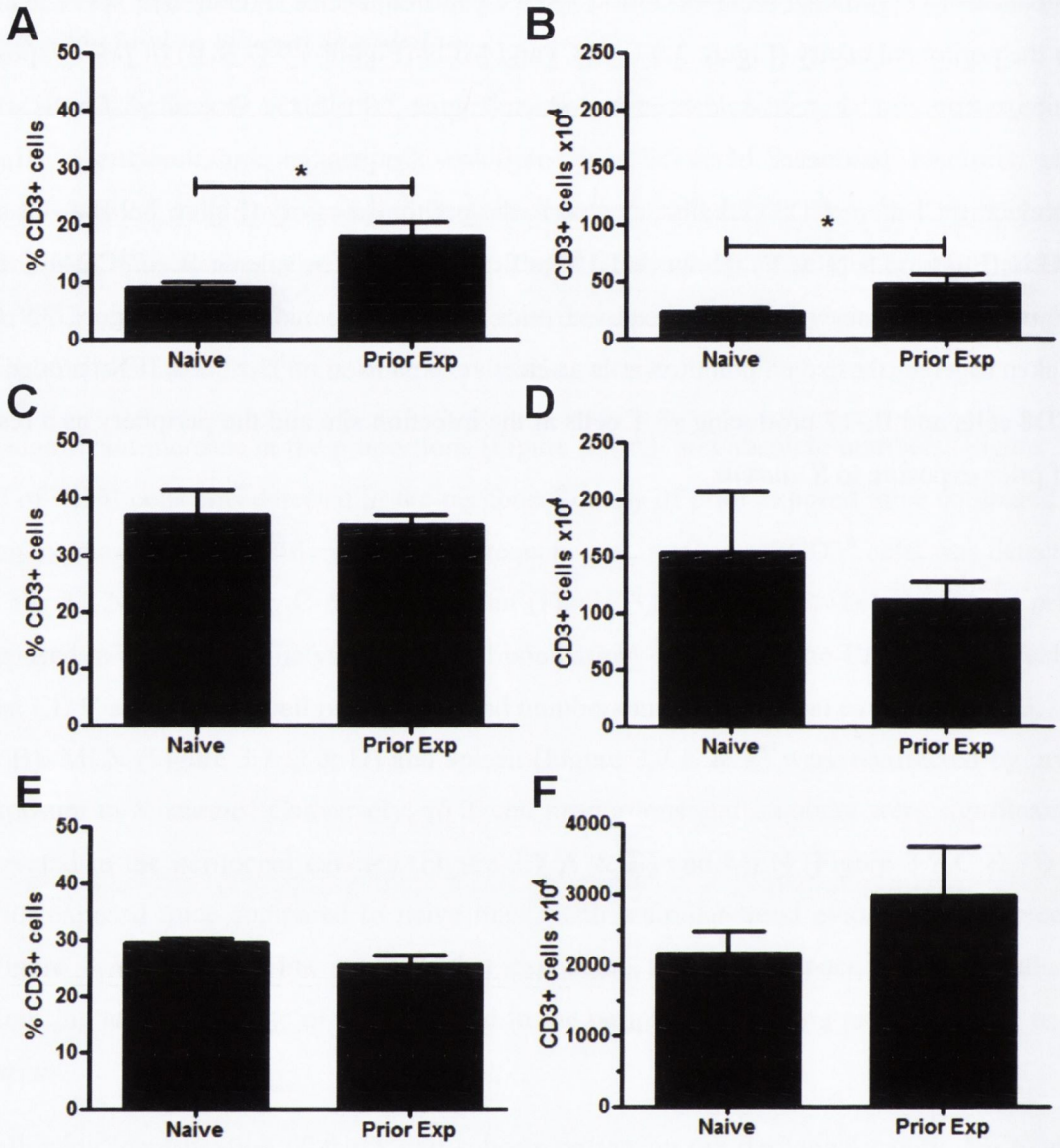


Figure 3.6: Prior exposure to *S. aureus* enhances $CD3^+$ T cells accumulation within the peritoneal cavity

Groups of mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injections on d 0, 7 and 14. Prior exposed mice were allowed to recover until d 35 when peritoneal (A, B), MLN (C, D) and spleen (E, F) cells were isolated to assess the $CD3^+$ cell proportions (A, C, E) and absolute numbers (B, D, F) by flow cytometry. A group of naive mice were used as a control. Results expressed as mean±SEM. n=8 per group. Data pooled from 2 independent experiments. * $p<0.05$

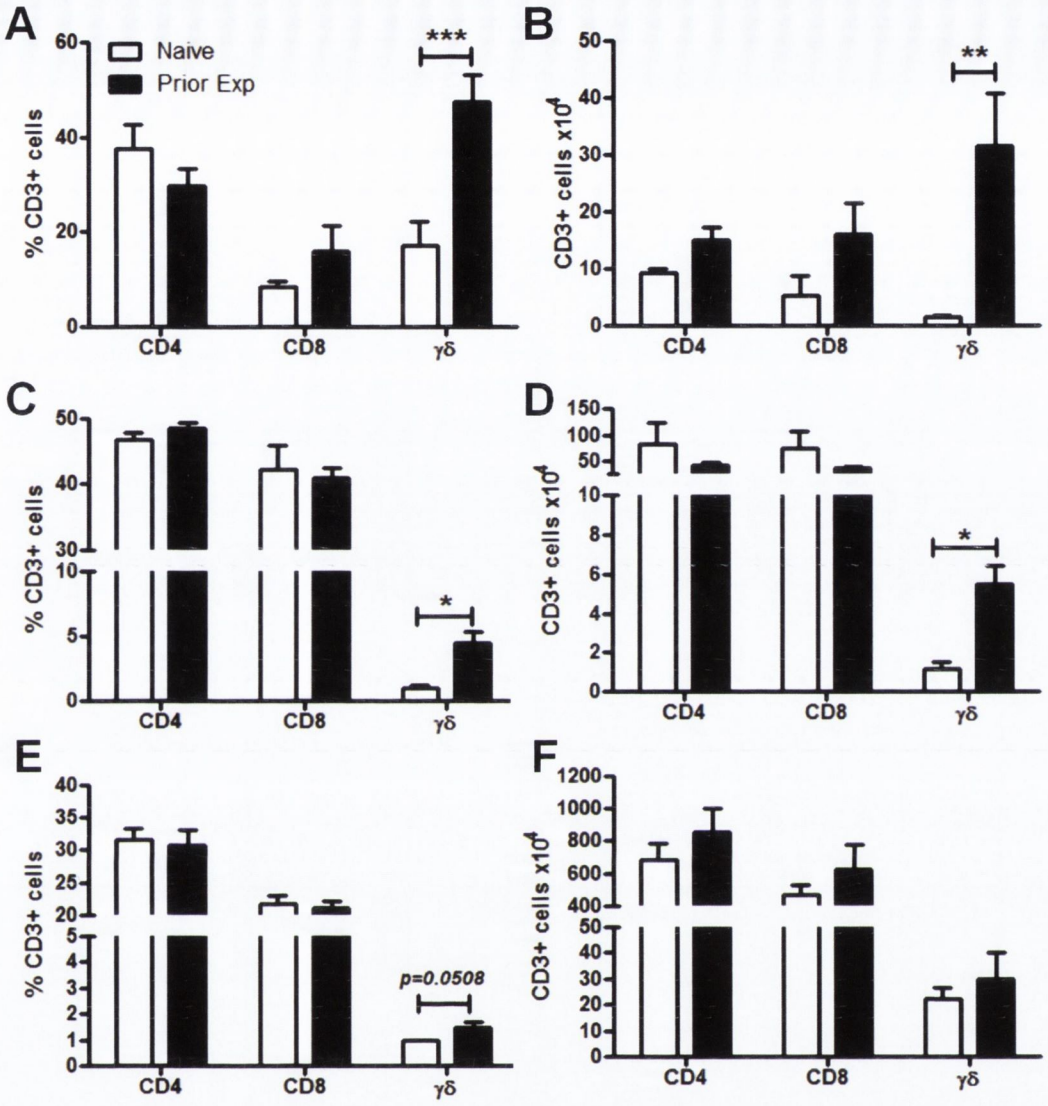


Figure 3.7: Prior exposure to *S. aureus* results in γδ T cell expansion but not CD4 and CD8 T cell expansion

Groups of mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injections on d 0, 7 and 14. Prior exposed mice were allowed to recover until d 35 when peritoneal (A & B), MLN (C & D) and spleen (E & F) cells were isolated to assess the proportions (A, C & E) and absolute numbers (B, D & F) of CD4, CD8 and γδ T cell proportions by flow cytometry. Cells isolated from naive mice were used as a control. Results expressed as mean±SEM. n=8 per group. Data pooled from 2 independent experiments. *$p<0.05$, **$p<0.005$, ***$p<0.001$

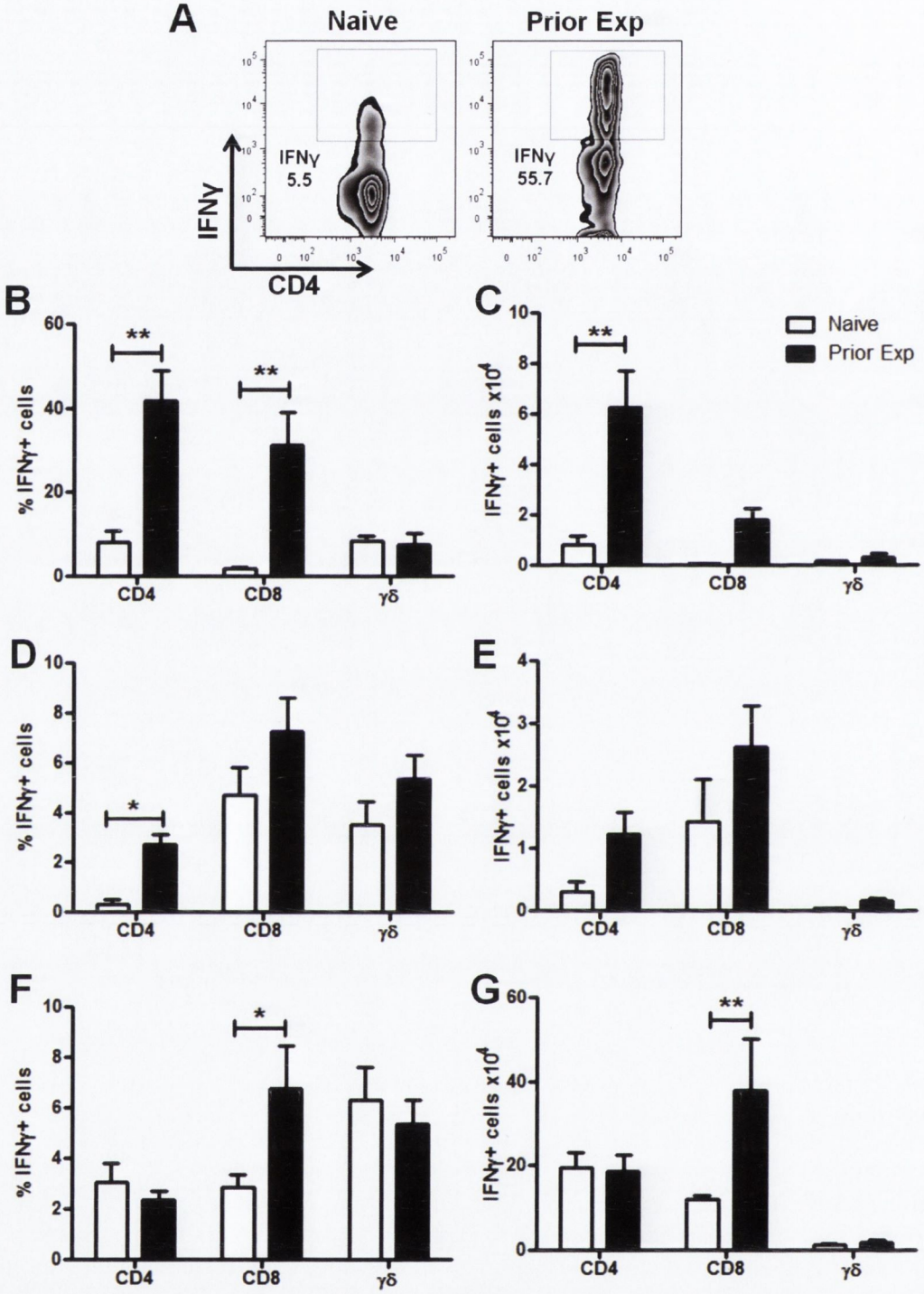


Figure 3.8: Prior exposure to *S. aureus* enhanced IFNγ production by T cells upon *in vitro* mitogen stimulation

Groups of mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injections on d 0, 7 and 14. Prior exposed mice were allowed to recover until d 35 when peritoneal (A-C) MLN (D & E) and spleen (F & G) cells were isolated to assess the proportions (B, D & F) and absolute numbers (C, E & G) of CD4, CD8 and γδ T cells capable of producing IFNγ in response to stimulation with PMA and Ionomycin (37°C for 4 h). Cells isolated from naive mice were used as a control. Results expressed as mean±SEM (B-J) with representative FACS plots from peritoneal CD4 cells shown (A). n=8 per group. Data pooled from 2 independent experiments. * $p<0.05$, ** $p<0.05$

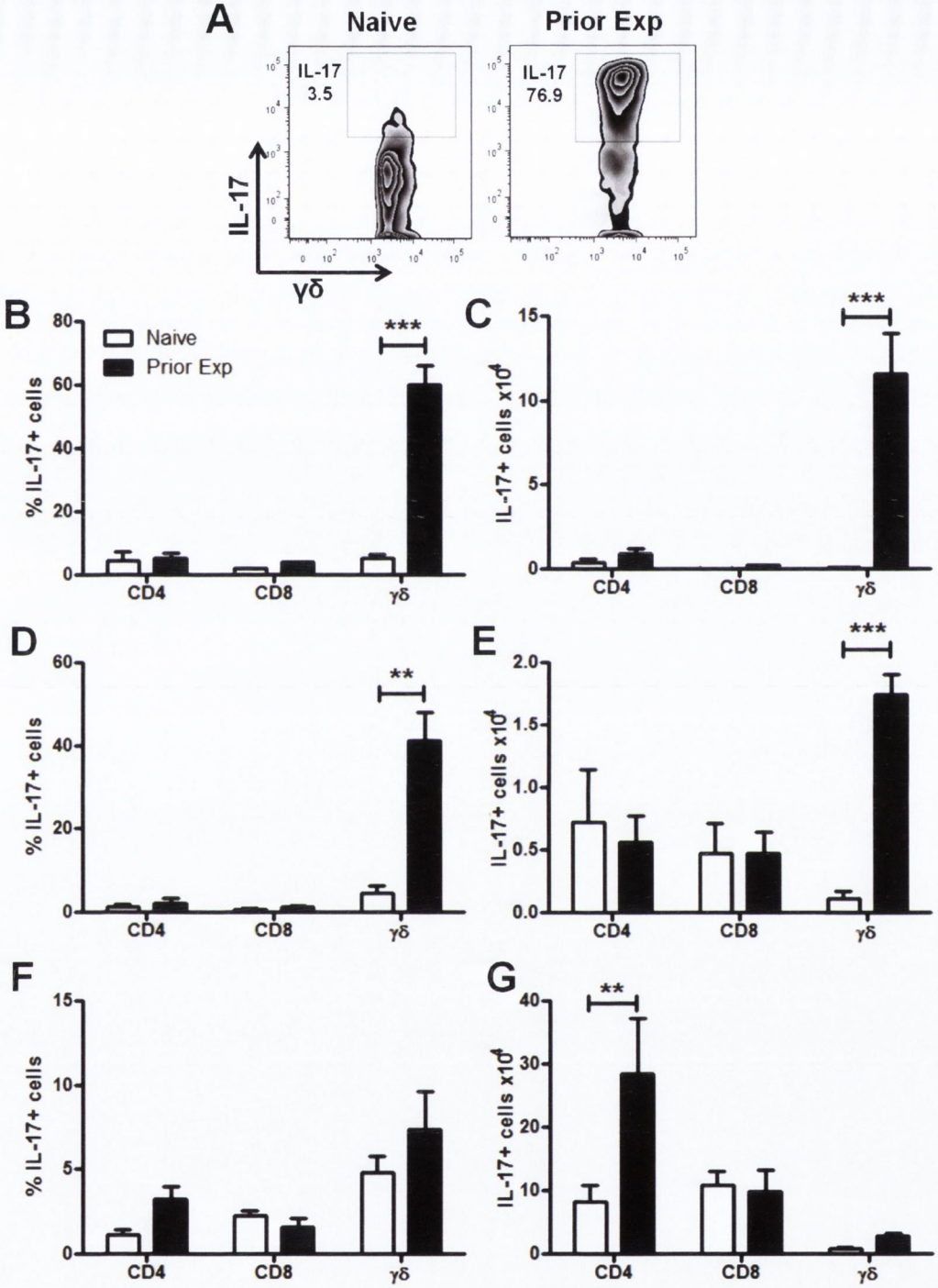


Figure 3.9: Prior exposure to *S. aureus* enhanced IL-17 production by T cells upon *in vitro* mitogen stimulation

Groups of mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injections on d 0, 7 and 14. Prior exposed mice were allowed to recover until d 35 when peritoneal (A-C) MLN (D & E) and spleen (F & G) cells were isolated to assess the proportions (B, D & F) and absolute numbers (C, E & G) of CD4, CD8 and γδ T cells capable of producing IL-17 in response to stimulation with PMA and Ionomycin (37°C for 4 h). Cells isolated from naive mice were used as a control. Results expressed as mean±SEM (B-D) with representative FACS plots from peritoneal γδ T cells shown (A). n=8 per group. Data pooled from 2 independent experiments. * $p<0.05$, ** $p<0.05$, ***$p<0.001$

3.2.6. Prior exposure to S. aureus enhanced anti-staphylococcal antibody responses

The ability of prior exposure to *S. aureus* to drive an antibody response was also investigated. Groups of mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injections on d 0, 7 and 14. On d 35, when mice were infection free, sera were collected to assess anti-*S. aureus* antibody titres and to quantify neutralising antibodies. Prior exposure to *S. aureus* resulted in a significant increase in serum titres of anti-*S. aureus* IgG2a (Figure 3.10 A). The functionality of these antibodies was investigated using an adhesion assay to test the ability of sera to inhibit the binding of *S. aureus* to fibrinogen, an interaction that is dependent upon the surface protein ClfA. Analysis revealed a significant increase in the inhibition of *S. aureus* binding by sera isolated from prior exposed mice compared to sera isolated from naive mice (Figure 3.10 B). This demonstrates that neutralising antibody production increased as a consequence of exposure to *S. aureus*.

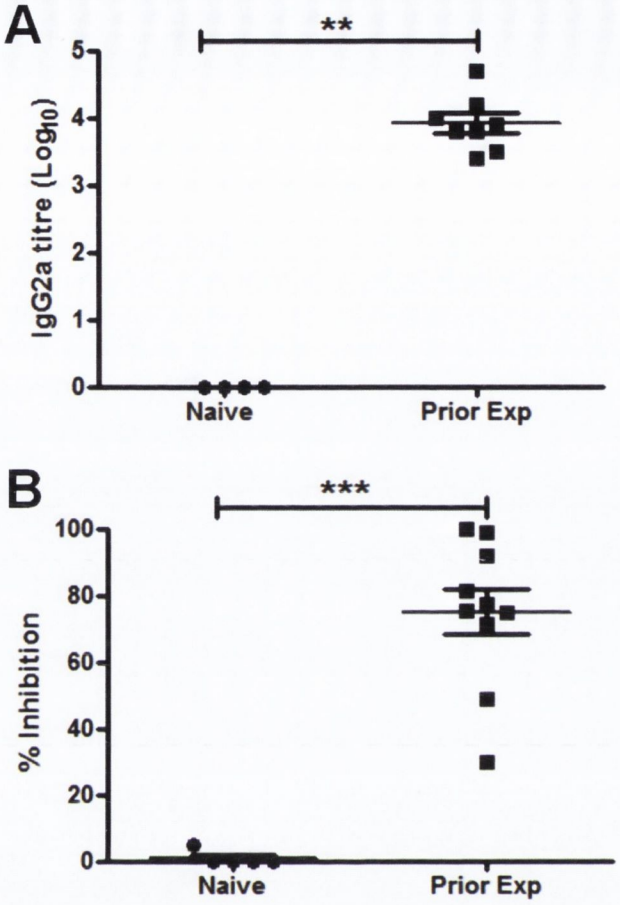


Figure 3.10: Prior exposure to *S. aureus* enhanced anti-staphylococcal antibody responses

Groups of mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injections on d 0, 7 and 14. On d 35 sera from prior exposed mice was isolated. Sera isolated from naive mice were used as a control. Antibody titres reactive against heat killed *S. aureus* were detected by ELISA (A). The presence of neutralising antibodies was determined by measuring the ability of serum to inhibit ClfA mediated *S. aureus* adherence to fibrinogen. *S. aureus* was pre-incubated with serum and adherence to fibrinogen was calculated as a percentage of values measured in control wells lacking serum (B). Results expressed as mean±SEM. n=5 for naive, n=10 for prior exp. Data pooled from 2 independent experiments. ** $p<0.005$, *** $p<0.001$

3.2.7. Prior exposure to S. aureus enhances bacterial clearance during subsequent infection

Having established that prior exposure to *S. aureus* expands populations of IFNγ and IL-17 producing T cells, the ability of these *S. aureus*-primed cells to protect against subsequent infection was investigated. Groups of mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injections on d 0, 7 and 14. Upon recovery on d 35, prior exposed mice were then re-challenged with *S. aureus* ($5x10^8$ CFU) via i.p injection. In addition a group of naive mice that had no prior exposure were challenged as a control. At specific time points post challenge a lavage of the peritoneal cavity was carried out to determine the local bacterial burden. The kidneys, liver and spleen were also isolated to assess the bacterial dissemination.

Prior exposure to *S. aureus* drove enhanced clearance of the bacterium upon re-infection (Figure 3.11). A significant reduction in the bacterial burden was observed in the peritoneal cavities of prior exposed mice 72, 120 and 168 h post challenge (Figure 3.11 A). By 120 h no detectable bacteria were present in the peritoneal lavage of prior exposed mice. However, detectable levels of bacteria (~1.5 log CFU/ml) were still present in the peritoneal cavities of non-exposed mice. Bacterial dissemination was also decreased in prior exposed mice with significantly reduced bacterial burden observed in the kidneys of these mice 72 h post challenge, which was maintained for the remainder of the time course (Figure 3.11 B). Prior exposure to *S. aureus* also had a dramatic effect on clearance of bacteria from the liver, with enhanced clearance in prior exposed mice observed as early as 24 h post challenge which was maintained for the remainder of the time course (Figure 3.11 C). Prior exposure to *S. aureus* did not affect the rate of bacterial clearance from the spleens compared to non-exposed mice (Figure 3.11 D).

Analysis of the time course of *S. aureus* infection revealed that 72 h post infection demonstrated the most significant differences in CFUs, locally and systemically, between prior exposed and non-exposed mice. To confirm 72 h post infection was the optimum time point to investigate bacterial clearance in our model of recurrent peritonitis, prior exposure experiments were repeated to assess the bacterial burden in the various sites at this time point. Analysis of the bacterial burden at 72 h post infection confirmed observations seen in the time course, with significant decreases in the bacterial load detected in the peritoneal cavity (Figure 3.12 A), kidneys (Figure 3.12 B) and liver (Figure

3.12 C). Therefore, this time point is used in the remainder of the report to analyse the bacterial burdens in various experimental settings.

These data demonstrate that prolonged prior exposure to *S. aureus* correlated with increased bacterial clearance upon subsequent re-exposure both at the local site of infection and in the periphery.

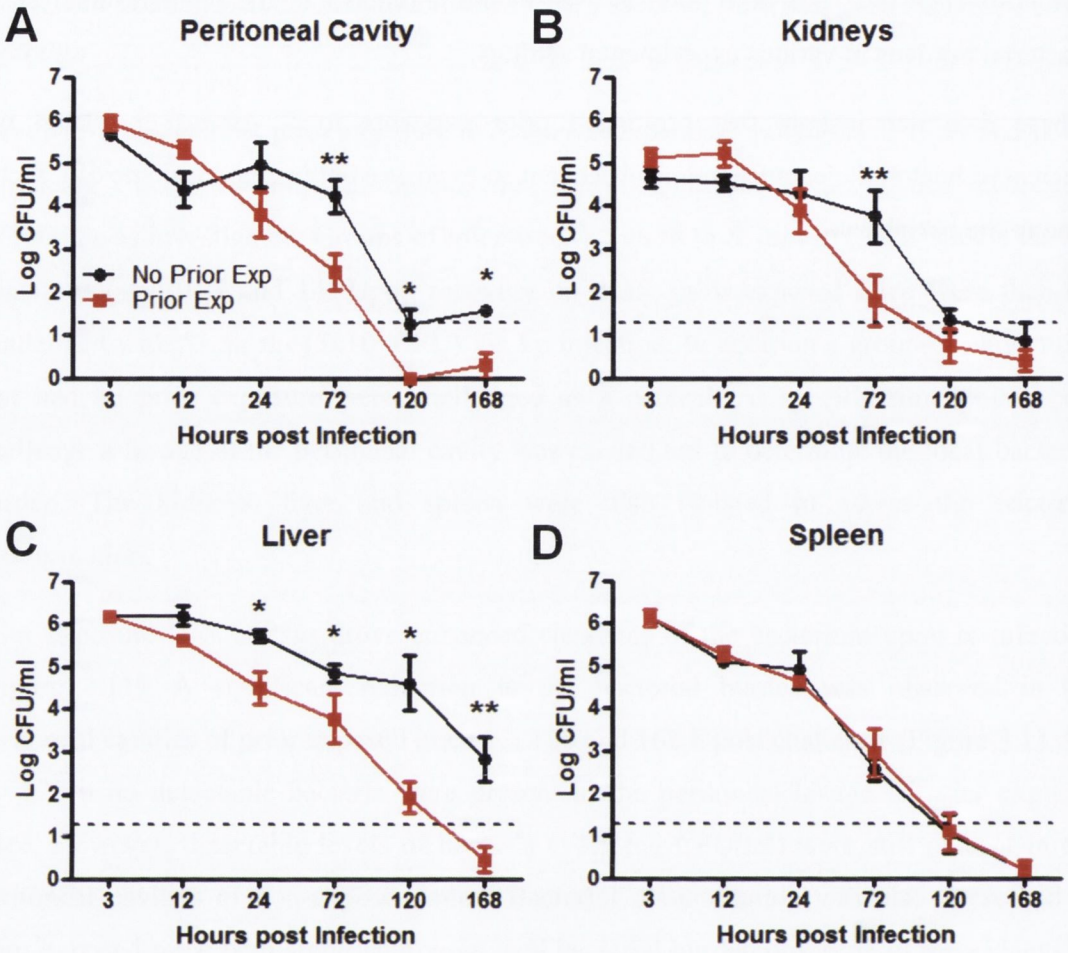


Figure 3.11: Prior exposure to *S. aureus* enhances bacterial clearance upon subsequent challenge.

Groups of mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injections on d 0, 7 and 14. Prior exposed mice were then re-challenged with an i.p injection of *S. aureus* ($5x10^8$ CFU) on d 35 alongside a control group of naive mice. At indicated time points post challenge a lavage of the peritoneal cavity was carried out in order to assess the local bacterial burden (A). The kidneys (B), liver (C) and spleen (D) were also isolated to assess the dissemination of the infection. Results expressed as Log CFU/ml of mean±SEM. Dashed lines indicate limit of detection. n=5 per group. * $p<0.05$, ** $p<0.005$.

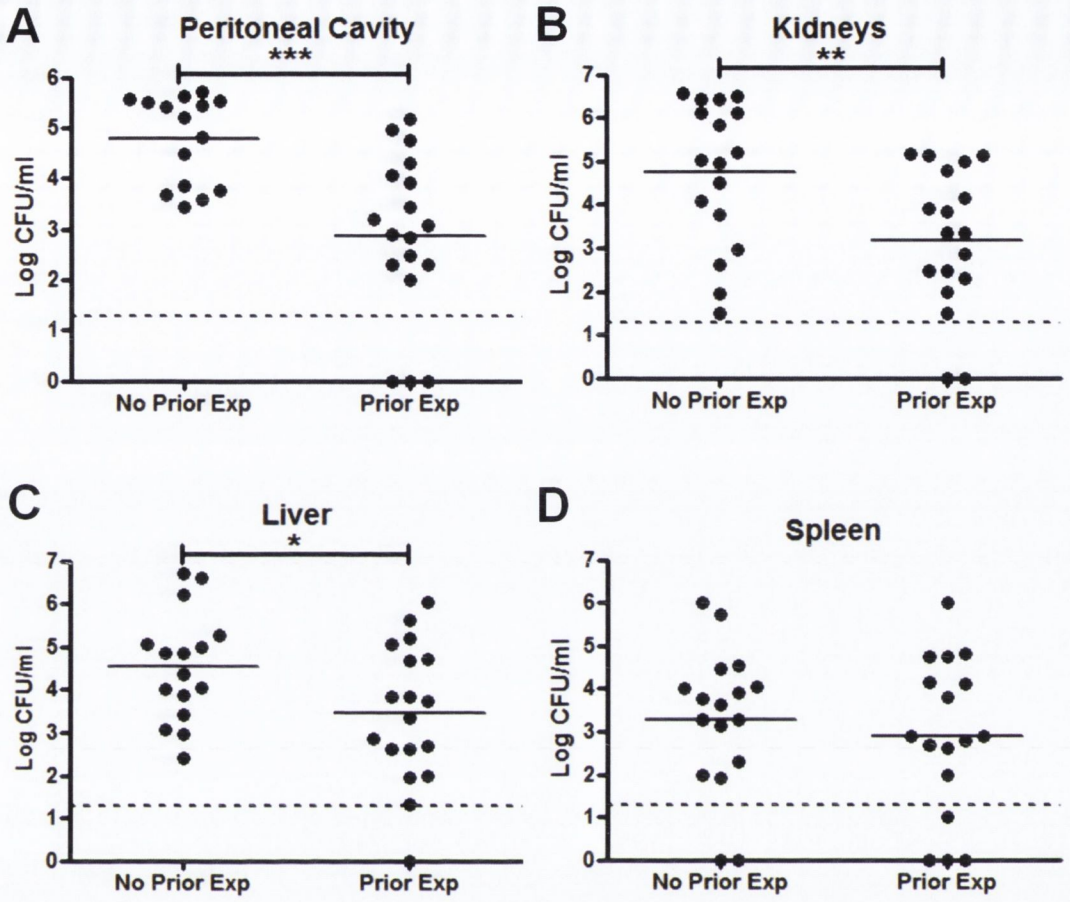


Figure 3.12: Prior exposure to *S. aureus* results in a significant reduction in bacterial burden 72 h post challenge

Groups of mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injections on d 0, 7 and 14. Prior exposed mice were then re-challenged with an i.p injection of *S. aureus* ($5x10^8$ CFU) on d 35 alongside a control group of naive mice. At 72 h post challenge a lavage of the peritoneal cavity was carried out in order to assess the local bacterial burden (A). The kidneys (B), liver (C) and spleen (D) were also isolated to assess the dissemination of the infection. Results expressed as Log CFU/ml with means indicated by line. Dashed lines indicate limit of detection. n=16 for non-exposed, n=18 for prior exposed. Data pooled from 4 independent experiments. *$p<0.05$, ** $p<0.005$

3.2.8. Prior exposure to S. aureus enhances lymphocyte derived cytokine secretion and chemokine production during subsequent infection

To investigate the immune mechanisms mediating enhanced bacterial clearance in prior exposed mice inflammatory cytokine and chemokine production at the local infection site was assessed. Mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injections on d 0, 7 and 14. Upon recovery (d 35), prior exposed mice were re-challenged with *S. aureus* ($5x10^8$ CFU) via i.p. injection, alongside a control group of non-exposed mice. At specific time points post challenge the peritoneal cavity was lavaged with PBS for cytokine analysis by ELISA.

Infection of non-exposed mice with *S. aureus* induced rapid production of innate pro-inflammatory cytokines with IL-1β (Figure 3.13 A), IL-23 (Figure 3.13 B) and TNFα (Figure 3.13 C) detected as early as 3 h post infection. Of note, throughout the time course of infection IL-12 levels were below detection of the lowest standard (39.1 pg/ml) suggesting that this cytokine does not play a substantial role during infection of non-exposed mice (Figure 3.9 D). The levels of IL-1β (Figure 3.13 A), IL-23 (Figure 3.13 B) and IL-12 (Figure 3.13 D) were not significantly affected by prior exposure to *S. aureus*. However TNFα production was significantly increased in prior exposed mice at 3 h post infection compared to non-exposed mice (Figure 3.13 C).

Secretion of the lymphocyte derived cytokines IFNγ, IL-17 and IL-22 were also assessed as well as the anti-inflammatory cytokine IL-10. IFNγ secretion was undetectable in non-exposed mice (Figure 3.14 A). In contrast, IL-17 (Figure 3.14 B), IL-22 (Figure 3.14 C) and IL-10 (Figure 3.14 D) are all produced during *S. aureus* infection in the peritoneal cavity of non-exposed mice as early as 3 h post infection. IFNγ (Figure 3.14 A) and IL-17 (Figure 3.14 B) production were significantly increased in prior exposed mice compared to non-exposed mice at 3 h post infection. No significant differences in IL-22 were detected between the two groups (Figure 3.14 C). Interestingly, significantly elevated levels of anti-inflammatory IL-10 were detected in prior exposed mice 3 h post infection compared to non-exposed mice (Figure 3.14 D).

The levels of the chemokines keratinocyte-derived chemokine; KC (CXCL1), MIP-2 (CXCL2), MCP-1 (CCL2) and regulated on activation, normal T cell expressed and secreted; RANTES (CCL5) were also assessed. In non-exposed mice all the mentioned chemokines were produced in response to *S. aureus* infection (Figure 3.15). Production of KC and MIP2 peaked at 3 h post infection, with MCP-1 and RANTES peaking later at 12 h

post infection. Although prior exposure to *S. aureus* did not significantly affect the levels of KC upon subsequent re-infection (Figure 3.15 A), a marked increase in KC secretion was observed (5608.22 vs. 7368.34 pg/ml in non-exposed vs. prior exposed mice, respectively). Significantly elevated MIP-2 secretion was detected in prior exposed mice 3 h post infection, compared to non-exposed mice (Figure 3.15 B). Prior exposure did not affect MCP-1 secretion upon subsequent re-infection (Figure 3.15 C). RANTES production was significantly increased in prior exposed mice throughout the course of infection, with secretion peaking at 12 h post infection (Figure 3.15 D).

Of note, no detectable levels of IL-1α, IL-4, IL-18 or IL-17F were observed in the peritoneal cavities of either non-exposed or prior exposed mice. Together these data show an increase in lymphocyte derived cytokines and leukocyte recruiting chemokines in prior exposed mice which correlated with enhanced bacterial clearance in these animals, suggesting a role for T cells in mediating this protection. In contrast, no significant changes were detected in innate cytokine levels, apart from TNFα, again suggesting the involvement of the adaptive immune response.

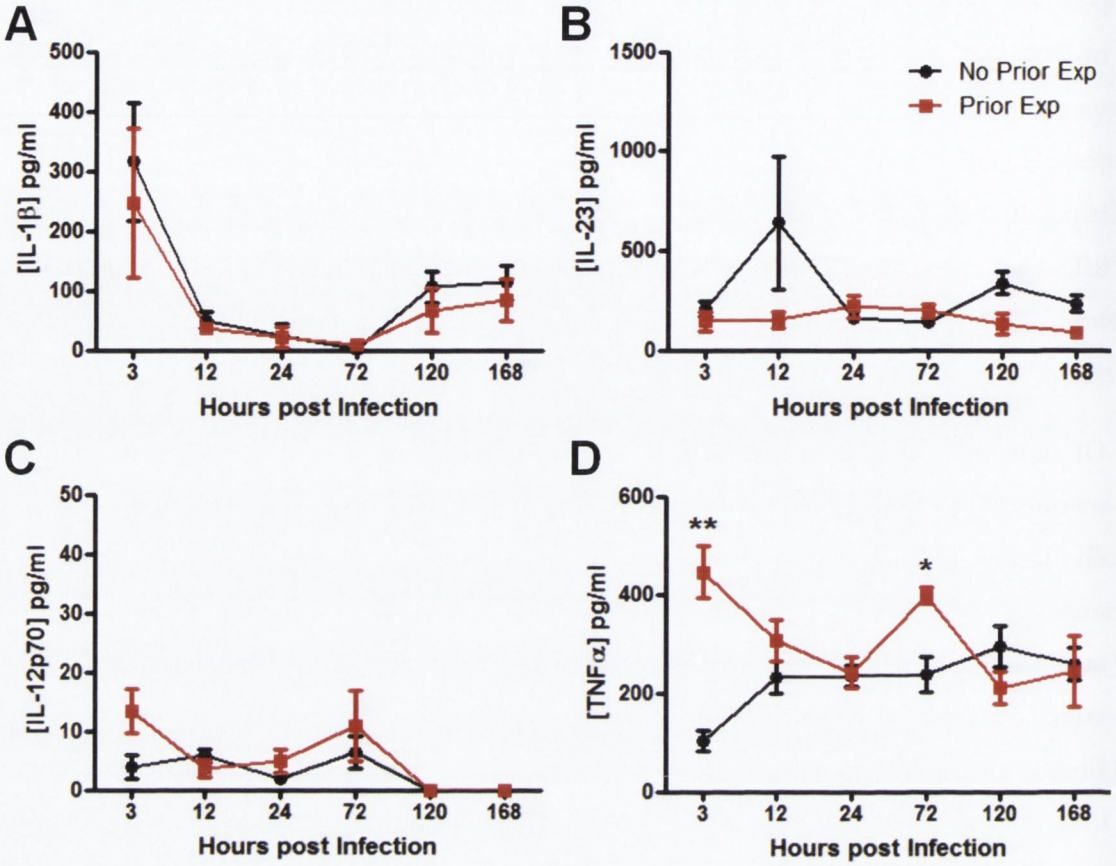


Figure 3.13: Prior exposure to *S. aureus* increased TNFα but not IL-1β, IL-23 and IL-12 secretion during subsequent infection

Groups of mice were exposed to *S. aureus* ($5x10^8$ CFU) via an i.p. injection on d 0, 7 and 14. Prior exposed mice were then re-challenged with an i.p. injection of *S. aureus* ($5x10^8$ CFU) on d 35 alongside a control group of naive mice. At indicated time points post challenge the peritoneal cavity was lavaged with PBS. IL-1β (A), IL-23 (B), IL-12 (C) and TNFα (D) production was detected by ELISA. Results expressed as mean±SEM. n=5 per group.* $p<0.05$, ** $p<0.005$

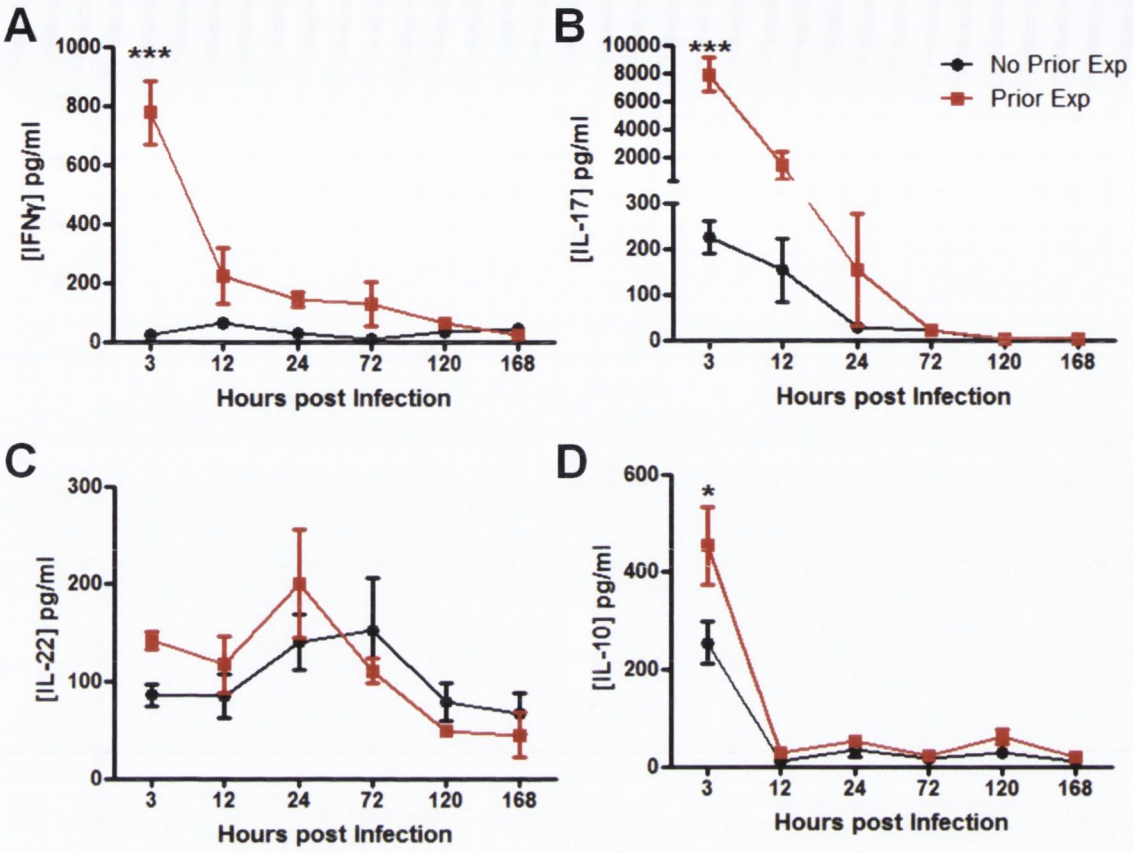


Figure 3.14: Prior exposure to *S. aureus* increased IFNγ, IL-17 and IL-10 but not IL-22 secretion during subsequent infection

Groups of mice were exposed to *S. aureus* ($5x10^8$ CFU) via an i.p. injection on d 0, 7 and 14. Prior exposed mice were then re-challenged with an i.p. injection of *S. aureus* ($5x10^8$ CFU) on d 35 alongside a control group of naive mice. At indicated time points post challenge the peritoneal cavity was lavaged with PBS. IFNγ (A), IL-17 (B), IL-22 (C) and IL-10 (D) production was detected by ELISA. Results expressed as mean±SEM. n=5 per group.* $p<0.05$, ***$p<0.001$

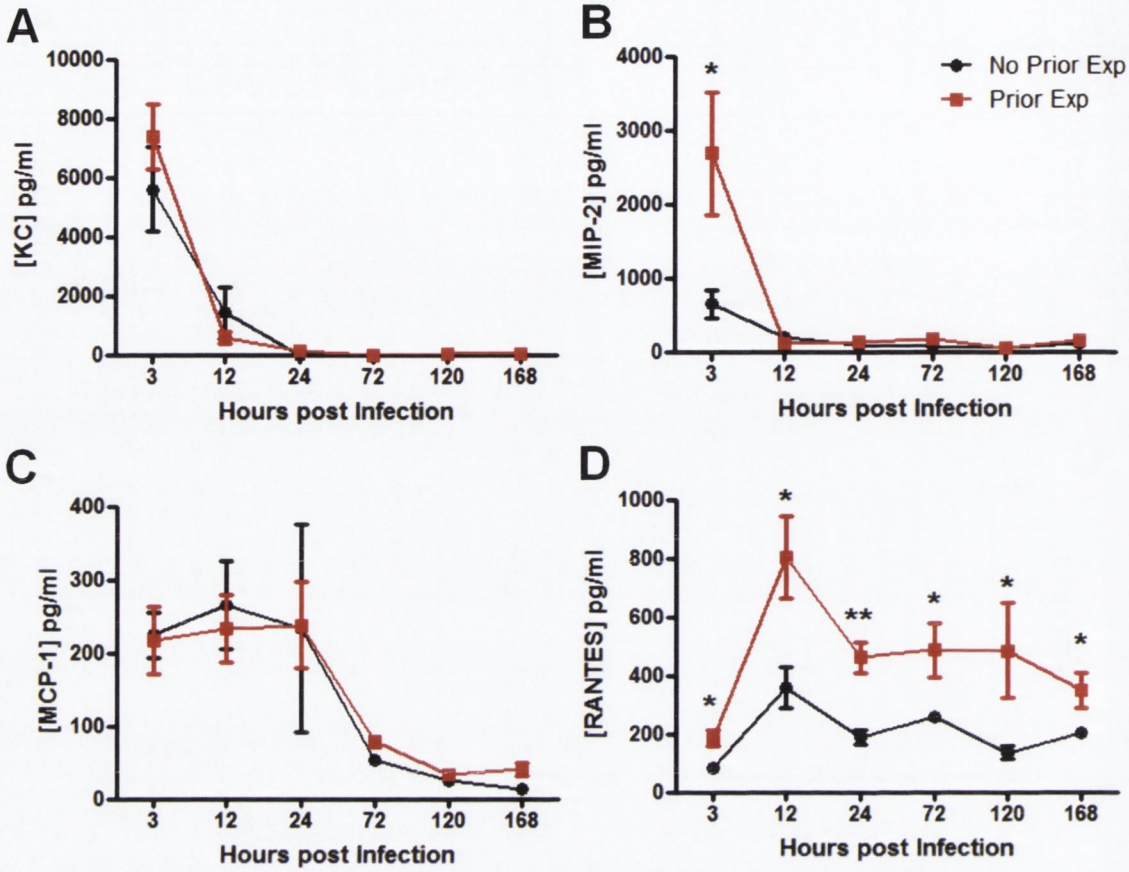


Figure 3.15: Prior exposure to *S. aureus* increased MIP-2 and RANTES but not KC and MCP-1 secretion during subsequent infection

Groups of mice were exposed to *S. aureus* ($5x10^8$ CFU) via an i.p. injection on d 0, 7 and 14. Prior exposed mice were then re-challenged with an i.p. injection of *S. aureus* ($5x10^8$ CFU) on d 35 alongside a control group of naive mice. At indicated time points post challenge the peritoneal cavity was lavaged with PBS. KC (A), MIP-2 (B), MCP-1 (C) and RANTES (D) production was detected by ELISA. Results expressed as mean±SEM. n=5 per group.* $p<0.05$, ** $p<0.005$

3.2.9. Prior exposure to S. aureus significantly increases IFNγ and IL-17 producing T cells during subsequent infection

Having observed a correlation between increased lymphocyte derived cytokines and enhanced bacterial clearance in prior exposed mice compared to non-exposed mice, further characterisation of the specific effector subsets of T cells responsible for cytokine production was carried out. Mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injections on d 0, 7 and 14. Upon recovery (d 35), prior exposed mice were re-challenged with *S. aureus* ($5x10^8$ CFU) via i.p. injection, alongside a control group of non-exposed mice. At specific time points post challenge peritoneal, MLN and splenic cells were isolated in order to assess the proportions of lymphocyte subsets using flow cytometry. Intracellular staining was also carried out to identify the T cells responsible for IFNγ and IL-17 production.

In prior exposed mice there was a significant increase in the numbers of $CD3^+$ cells infiltrating the peritoneal cavity from 72 h post infection onwards (Figure 3.16 A). Overall, prior exposed mice also had significantly elevated numbers of $CD3^+$ cells in the MLN. By 168 h post infection, MLN $CD3^+$ cell numbers matched those observed in non-exposed mice (Figure 3.16 B). Significantly increased numbers of splenic $CD3^+$ cells were detected 3 h post infection in prior exposed mice, with numbers returning to those observed in non-exposed mice by 24 h post infection (Figure 3.16 C).

The specific subsets comprising the $CD3^+$ cells were then assessed. Absolute numbers of $CD4^+$ cells were significantly elevated in the peritoneal cavities of prior exposed mice 120 and 168 h post infection, compared to non-exposed mice (Figure 3.17 A). Analysis of MLN $CD4^+$ cell numbers revealed a significant increase at 3, 12 and 72 h post infection compared to non-exposed mice (Figure 3.17 D). No significant differences in splenic $CD4^+$ numbers were observed between mice that had undergone prior exposure and non-exposed mice (Figure 3.17 G). Prior exposure did not significantly affect peritoneal $CD8^+$ T cell numbers (Figure 3.17 B). Analysis of $CD8^+$ cell numbers in the MLN revealed a significant increase at 3, 12 and 120 h post infection in prior exposed mice compared to non-exposed mice (Figure 3.17 E). Absolute numbers of splenic $CD8^+$ T cells were unaffected by prior exposure (Figure 3.17 H). Peritoneal $\gamma\delta^+$ T cell numbers were significantly elevated 120 and 168 h post infection in prior exposed compared to non-exposed mice (Figure 3.17 C). In the MLN, a significant increase in $\gamma\delta^+$ T cells numbers was detected in prior exposed mice at 3, 12, 72 and 120 h post infection (Figure 3.17 F).

Splenic $\gamma\delta^+$ T cell numbers were significantly elevated in prior exposed mice versus non-exposed mice 120 and 168 h post infection (Figure 3.17 I).

To establish IFNγ production by specific T cell subsets, isolated cells were incubated *in vitro* with PMA, ionomycin and brefeldin A for 4 h at 37°C in order to stimulate the intracellular accumulation of cytokines within the isolated cells. The primary source of IFNγ at the local infection site during *S. aureus* induced peritonitis was $CD4^+$ T cells, with significantly increased proportions of peritoneal $IFN\gamma^+CD4^+$ cells detected in prior exposed versus non-exposed mice 3, 12 and 72 h post challenge (Figure 3.18 A). Absolute numbers of peritoneal $IFN\gamma^+CD4^+$ cells were significantly elevated 72 and 120 h post infection in prior exposed mice compared to non-exposed mice (Figure 3.18 B). No difference in the proportions of IFNγ producing $CD4^+$ cells was detected in the MLN between the two groups (Figure 3.18 C). Absolute numbers of $IFN\gamma^+CD4^+$ cells were significantly enhanced in the MLN of prior exposed mice 12 and 72 h post infection compared to non-exposed mice (Figure 3.18 D). Significantly elevated frequencies (Figure 3.18 E) and numbers (Figure 3.18 F) of IFNγ producing $CD4^+$ cells were also observed in the spleens of prior exposed mice compared to non-exposed mice 12, 24, 72 and 120 h post infection.

Although fewer peritoneal IFNγ producing $CD8^+$ cells were detected than $IFN\gamma^+CD4^+$ cells, significantly increased proportions of IFNγ producing $CD8^+$ cells were detected in the peritoneal cavities of prior exposed versus non-exposed mice 3 h post infection (Figure 3.19 B). Absolute numbers of peritoneal $IFN\gamma^+CD8^+$ cells were significantly increased in prior exposed mice 72 and 120 h post infection (Figure 3.19 C). No significant differences in $IFN\gamma^+CD8^+$ cell frequencies or numbers were observed in the MLN (Figure 3.19 D & E) or spleen (Figure 3.19 F & G) between the two groups.

In this model of *S. aureus* induced peritonitis γδ T cells do not appear to be a major source of IFNγ compared to $CD4^+$ T cells. Interestingly, $IFN\gamma^+\gamma\delta^+$ T cell frequencies were decreased in the peritoneal cavity (Figure 3.20 A), MLN (Figure 3.20 C) and spleens (Figure 3.20 E) of prior exposed versus non-exposed mice. However, analysis of absolute numbers revealed a significant increase in the $IFN\gamma^+\gamma\delta^+$ T cells with the peritoneal cavities (Figure 3.20 B) and MLN (Figure 3.20 D) of prior exposed mice compared to non-exposed mice, due to the greater γδ T cell recruitment observed in prior exposed mice at these time points (Figure 3.17 C & F). No similar trend was observed in the splenic $IFN\gamma^+\gamma\delta^+$ cells of prior exposed mice (Figure 3.20 F).

To assess IL-17 production, isolated cells were incubated with brefeldin A only as previous studies (346), as well work within this thesis (Figure 4.4), have demonstrated that *in vitro* stimulation with PMA and ionomycin can exaggerate IL-17 production, potentially masking subtle *in vivo* effects. Overall very few proportions of IL-17 producing $CD4^+$ (Figure 3.21) and $CD8^+$ cells (Figure 3.22) (<5% of $CD4^+$ or $CD8^+$ cells) were detected in the peritoneal cavities, MLN or spleens of non-exposed mice. Prior exposure did not significantly affect the proportions of IL-17 producing $CD4^+$ (Figure 3.21 A, C & E) and $CD8^+$ (Figure 3.22 A, C & E) cells at any of the investigated sites. Analysis of absolute numbers revealed a significant increase in IL-17^+CD4^+ cell numbers in the peritoneal cavity 72 h post infection (Figure 3.21 B), and the in the MLN 3 and 12 h post infection (Figure 3.21 D), in prior exposed mice. Numbers of IL-17^+CD8^+ cells were significantly increased 120 h post infection in the MLN of prior exposed mice compared to non-exposed mice (Figure 3.22 D), whereas numbers of IL-17 producing CD8 T cells were unaffected by prior exposure in the peritoneal cavity (Figure 3.22 B) and spleen (Figure 3.22 F). The primary source of IL-17 during *S. aureus* induced peritonitis at the local infection site (Figure 3.23 A & B), MLN (Figure 3.23 C & D) and the spleen (Figure 3.23 E & F) were γδ T cells. Proportions and numbers of IL-$17^+\gamma\delta^+$ T cells were significantly elevated in the peritoneal cavities (Figure 3.23 A & B), MLNs (Figure 3.23 C & D) and spleens (Figure 3.23 E & F) of prior exposed mice at 3 h post infection.

Taken together, these data demonstrate an overall increase in the recruitment/expansion of T cells ($CD3^+$) in the peritoneal cavity of prior exposed mice during the course of *S. aureus* infection. This increase in total $CD3^+$ T cells correlated with significantly elevated numbers of peritoneal $CD4^+$ and $\gamma\delta^+$ T cells in prior exposed compared to non-exposed mice. In the MLN, $CD3^+$ T cells were significantly increased from 3-120 h post infection in prior exposed mice. This was associated with significantly elevated numbers of $CD4^+$, $CD8^+$ and $\gamma\delta^+$ T cells in these mice, suggesting all subsets contributed to the increases seen in total CD3 T cell numbers in the MLN. Splenic $CD3^+$ T cells were significantly elevated 3 h post infection in prior exposed mice which correlated with a significant increase in $CD4^+$ and $CD8^+$ T in these mice at this time point. Significantly elevated splenic $\gamma\delta^+$ T cells were detected 120 and 168 h post infection in prior exposed mice, suggesting that these cells are responsible for the non-significant increase in total CD3 T cells observed at these time points. The increase in MLN $CD4^+$ T cell numbers detected early on in infection (3-72 h post challenge) correlated with increased recruitment of $CD4^+$ cells in the peritoneal cavity towards the end of infection (72-168 h post challenge). Prior exposure did

not affect $CD8^+$ cell numbers as dramatically as $CD4^+$ cells at any of the investigated sites. Notably, a striking expansion of $\gamma\delta^+$ T cells was observed at all investigated sites.

Overall, prior exposure to *S. aureus* appeared to drive type 1 immunity upon subsequent re-infection, with increased levels of $IFN\gamma^+CD4^+$ and $IFN\gamma^+CD8^+$ cells detected at the local infection site of prior exposed mice. Additionally, a substantial increase in IL-17 production by γδ T cells was detected in the peritoneal cavities, MLN and spleens of prior exposed mice. This suggests that both Th1 type immunity and IL-17 responses are contributing to the enhanced bacterial clearance observed in prior exposed mice.

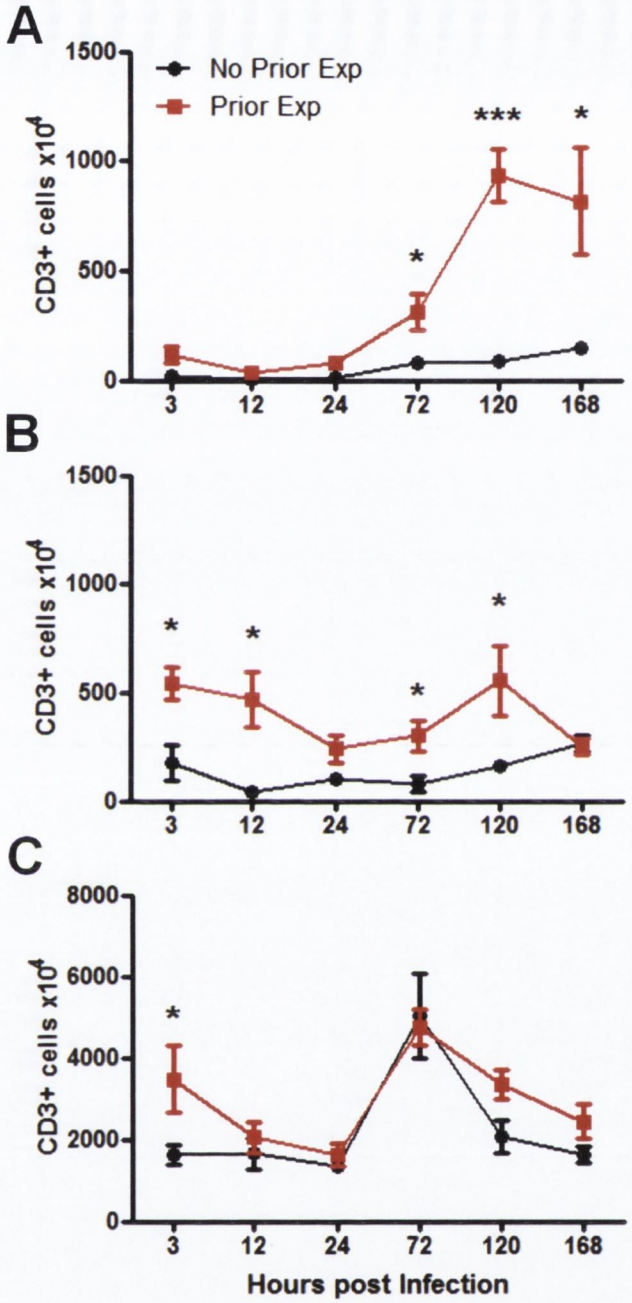


Figure 3.16: Prior exposure to *S. aureus* resulted in a significant influx of $CD3^+$ cells into the peritoneal cavity and MLN but not Spleen during subsequent challenge.

Groups of mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injections on d 0, 7 and 14. Prior exposed mice were then re-challenged with an i.p injection of *S. aureus* ($5x10^8$ CFU) on d 35 alongside a control group of naive mice. At indicated time points post challenge peritoneal (A) MLN (B) and spleen (C) cells were isolated to assess the absolute numbers of $CD3^+$ cell by flow cytometry. Results expressed as mean±SEM. n=5 per group. *$p<0.05$, ***$p<0.001$

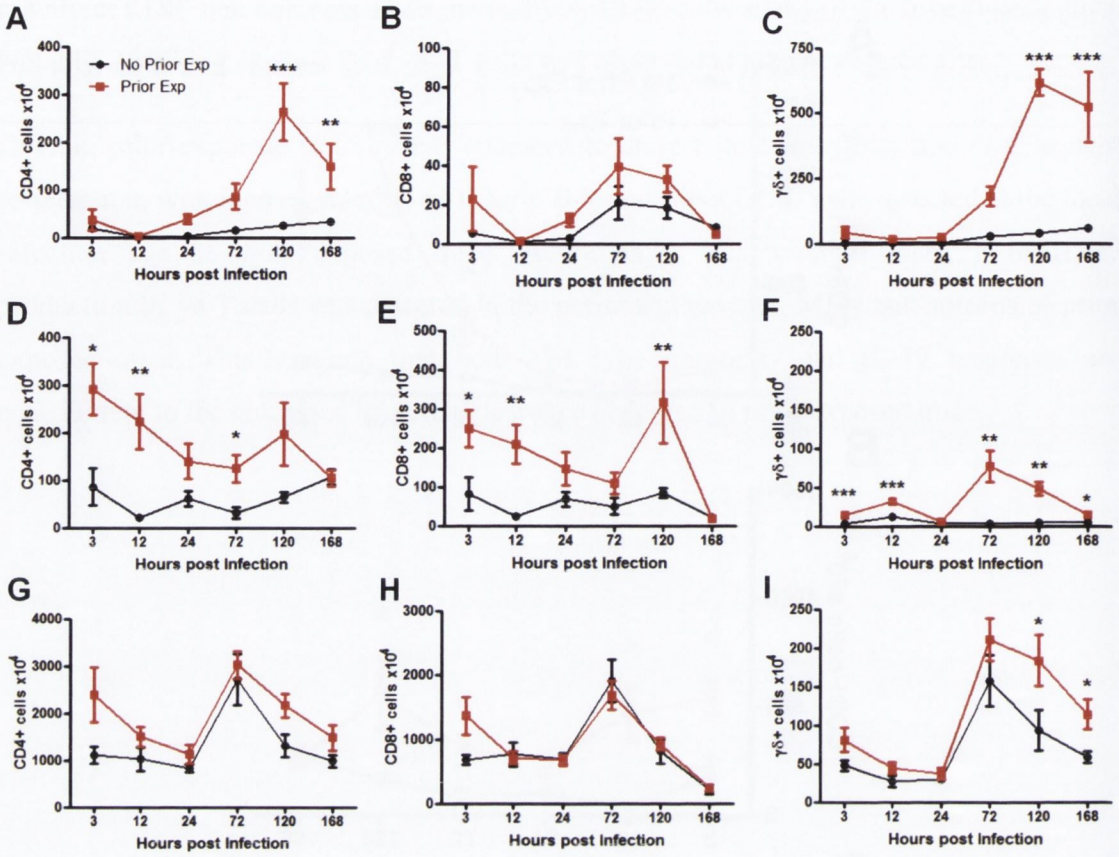


Figure 3.17: Effects of prior exposure to *S. aureus* on T cell subsets in the peritoneal cavity, MLN and spleen during subsequent challenge

Groups of mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injections on d 0, 7 and 14. Prior exposed mice were then re-challenged with an i.p injection of *S. aureus* ($5x10^8$ CFU) on d 35 alongside a control group of naive mice. At indicated time points post challenge peritoneal (A-C) MLN (D-F) and spleen (G-I) cells were isolated to assess absolute numbers of $CD4^+CD3^+$ cells (A, D & G), $CD8^+CD3^+$ cells (B, E & H) and $\gamma\delta^+CD3^+$ cells (C, F & I) by flow cytometry. Results expressed as mean±SEM. n=5 per group. * $p<0.05$, ** $p<0.005$, *** $p<0.001$

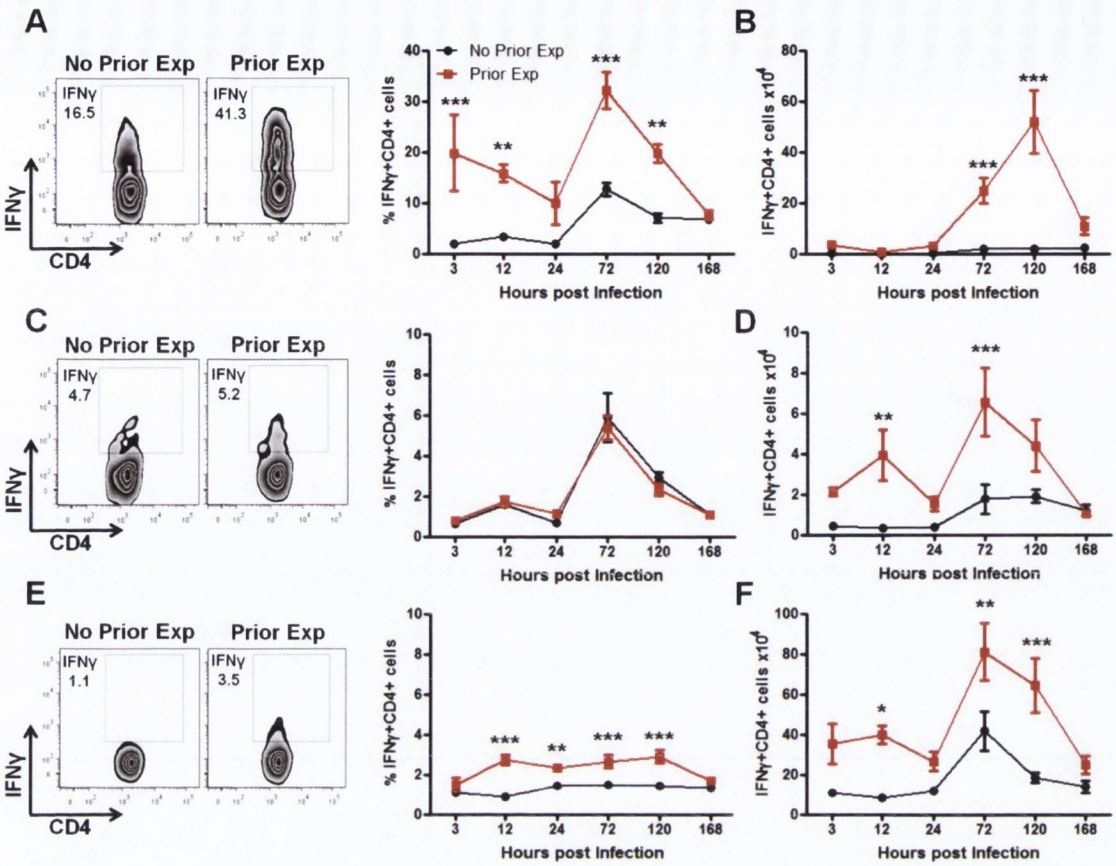


Figure 3.18: Prior exposure to *S. aureus* results in the significant expansion of Th1 cells during subsequent challenge.

Groups of mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injections on d 0, 7 and 14. Prior exposed mice were then re-challenged with an i.p injection of *S. aureus* ($5x10^8$ CFU) on d 35 alongside a control group of naive mice. At indicated time points post challenge peritoneal (A & B) MLN (C & D) and spleen (E & F) cells were isolated to assess the proportions (A, C & E) and absolute numbers (B, D & F) of IFNγ producing CD4 T cells using flow cytometry. Results expressed as mean±SEM, with representative FACS plots from PEC, MLN and spleen CD4 T cells 72 h post infection shown (A, C & E). n=5 per group. *p<0.05, **p<0.005, ***p<0.001

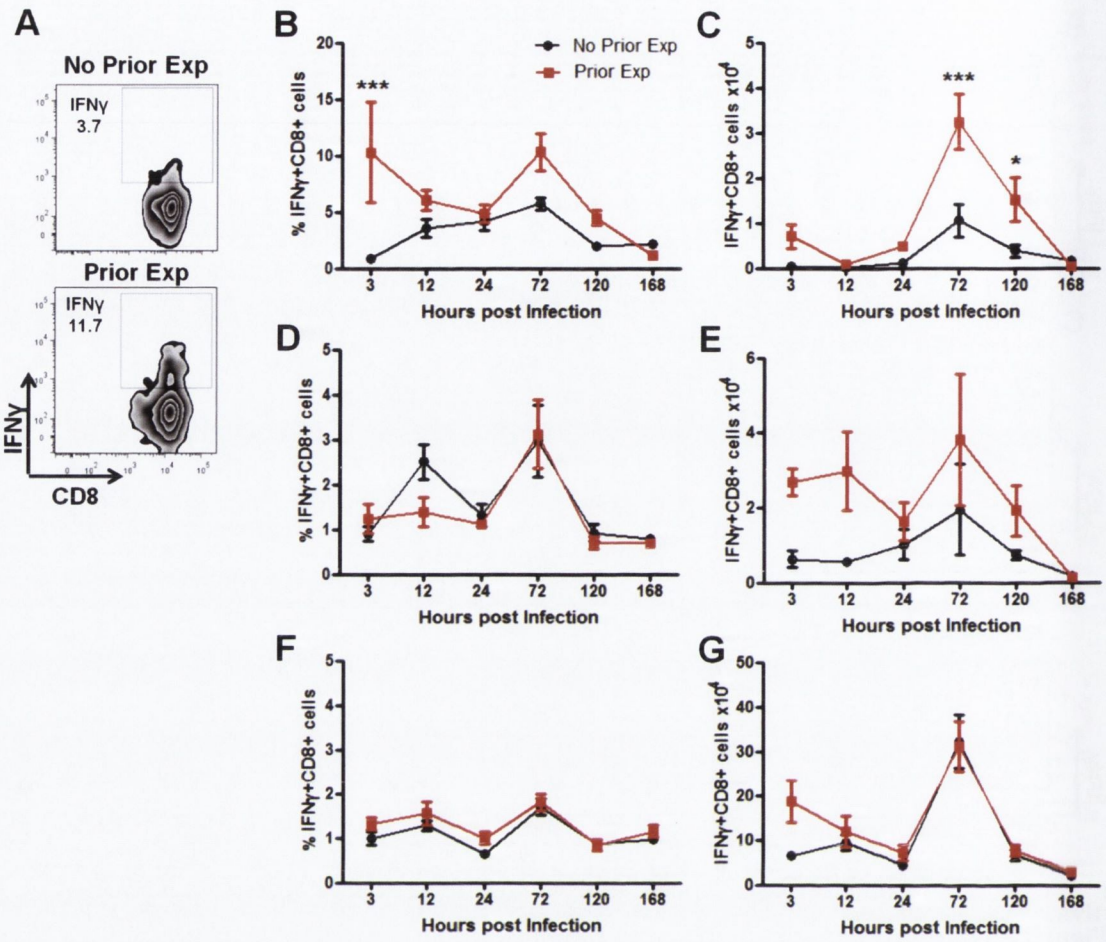


Figure 3.19: Prior exposure to *S. aureus* increases IFNγ production by peritoneal CD8 T cells during subsequent challenge.

Groups of mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injections on d 0, 7 and 14. Prior exposed mice were then re-challenged with an i.p injection of *S. aureus* ($5x10^8$ CFU) on d 35 alongside a control group of naive mice. At indicated time points post challenge peritoneal (A, B & C) MLN (D & E) and spleen (F & G) cells were isolated to assess the proportions (B, D & F) and absolute numbers (C, E & G) of IFNγ producing CD8 T cells using flow cytometry. Results expressed as mean±SEM (B-G) with representative FACS plots from peritoneal CD8 T cells 3 h post infection shown (A). n=5 per group. *$p<0.05$, *** $p<0.001$

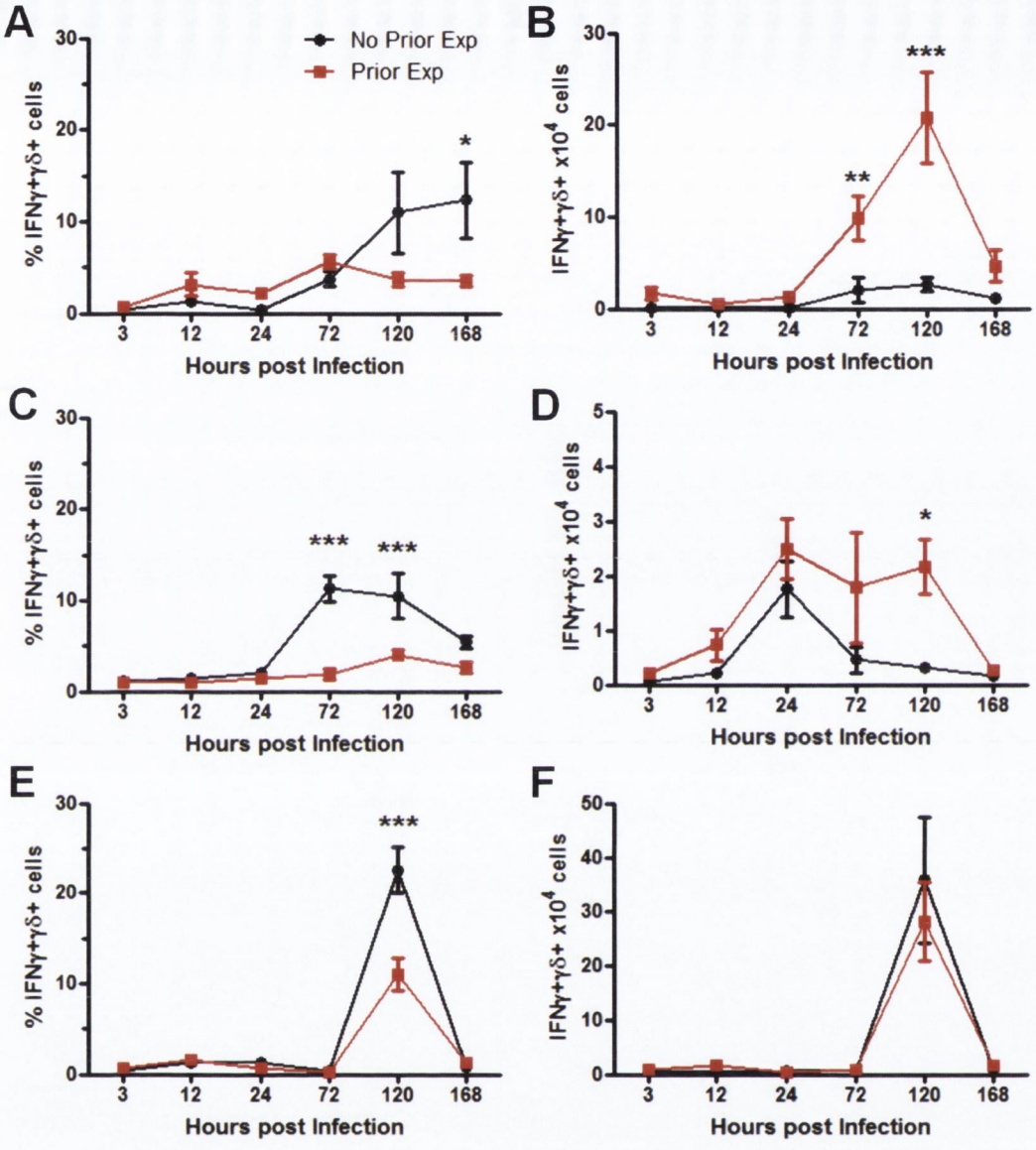


Figure 3.20: Effects of prior exposure to *S. aureus* on IFNγ production by γδ T cells during subsequent challenge.

Groups of mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injections on d 0, 7 and 14. Prior exposed mice were then re-challenged with an i.p injection of *S. aureus* ($5x10^8$ CFU) on d 35 alongside a control group of naive mice. At indicated time points post challenge peritoneal (A & B) MLN (C & D) and spleen (E & F) cells were isolated to assess the proportions (A, C & E) and absolute numbers (B, D & F) of IFNγ producing γδ T cells using flow cytometry. Results expressed as mean±SEM. n=5 per group. *p<0.05, **p<0.005, *** p<0.001

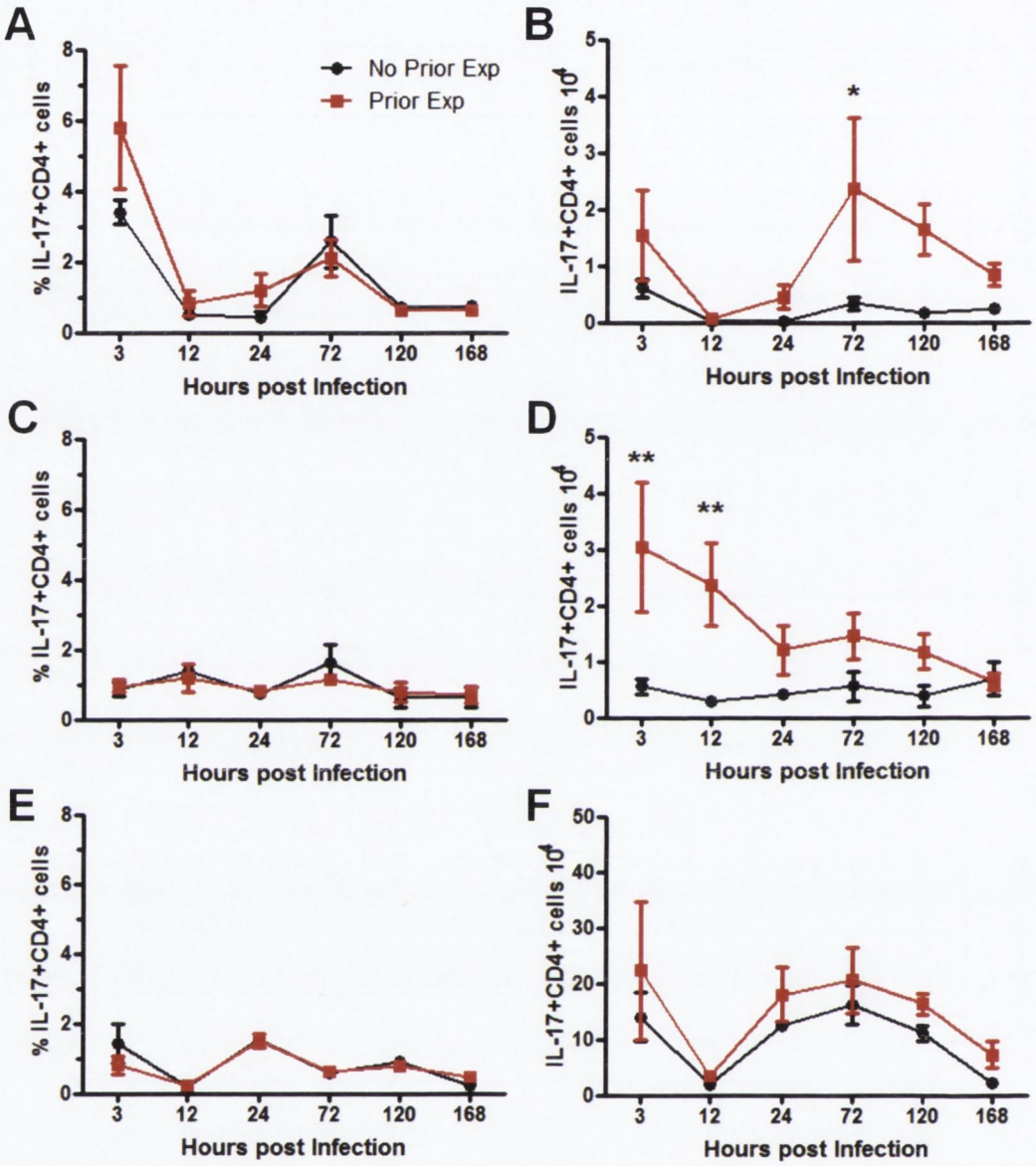


Figure 3.21: Prior exposure to *S. aureus* enhanced IL-17 production by CD4 T cells in the peritoneal cavity and MLN, but not the spleens, during subsequent challenge.

Groups of mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injections on d 0, 7 and 14. Prior exposed mice were then re-challenged with an i.p injection of *S. aureus* ($5x10^8$ CFU) on d 35 alongside a control group of naive mice. At indicated time points post challenge peritoneal (A & B) MLN (C & D) and spleen (E & F) cells were isolated to assess the proportions (A, C & E) and absolute numbers (B, D & F) of IL-17 producing CD4 T cells using flow cytometry. Results expressed as mean±SEM. n=5 per group. *$p<0.05$, **$p<0.005$

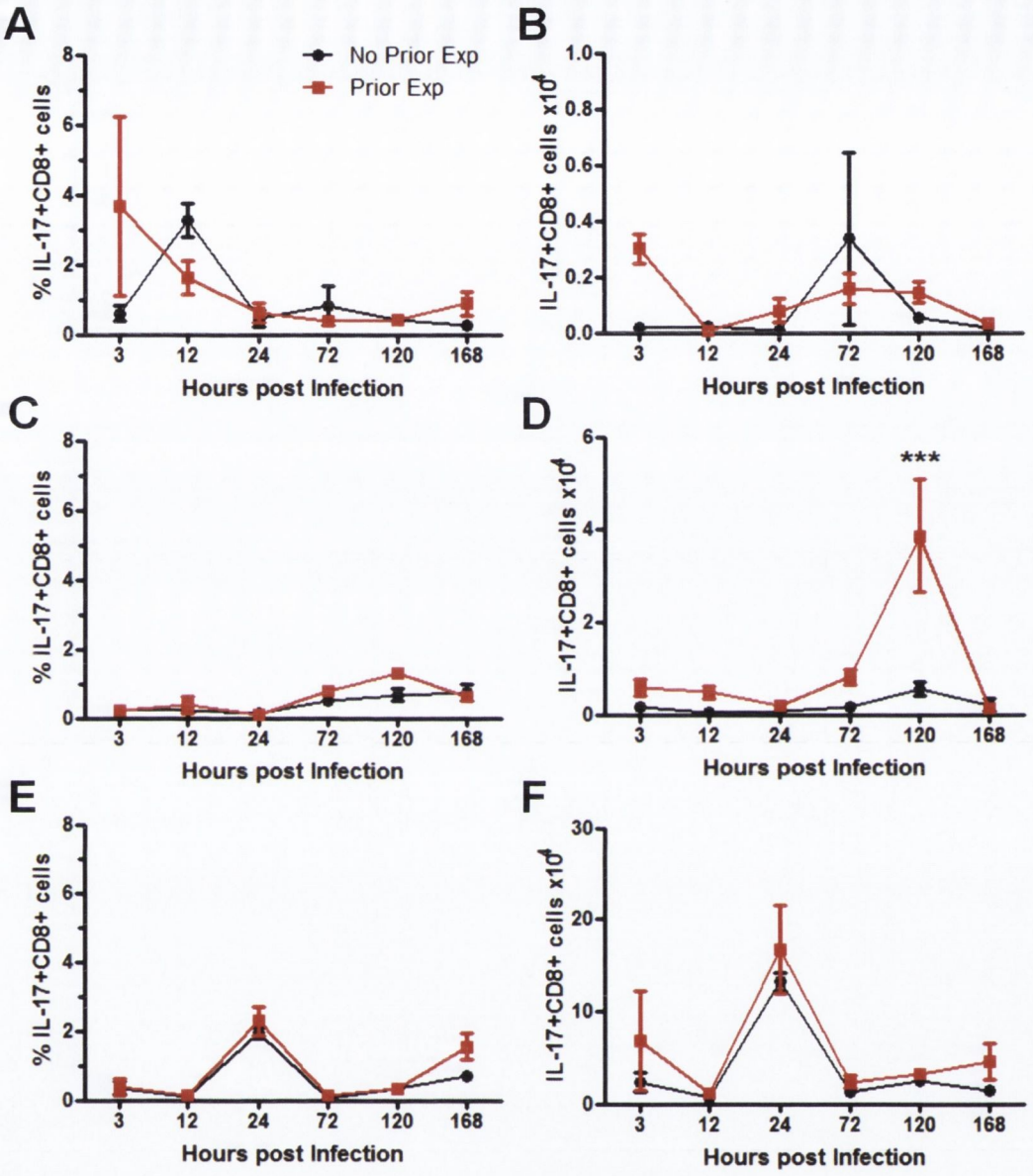


Figure 3.22: Prior exposure to *S. aureus* enhanced IL-17 production by CD8 T cells in the MLN, but not the peritoneal cavity or spleen during subsequent challenge.

Groups of mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injections on d 0, 7 and 14. Prior exposed mice were then re-challenged with an i.p injection of *S. aureus* ($5x10^8$ CFU) on d 35 alongside a control group of naive mice. At indicated time points post challenge peritoneal (A & B) MLN (C & D) and spleen (E & F) cells were isolated to assess the proportions (A, C & E) and absolute numbers (B, D & F) of IL-17 producing CD8 T cells using flow cytometry. Results expressed as mean±SEM. n=5 per group.

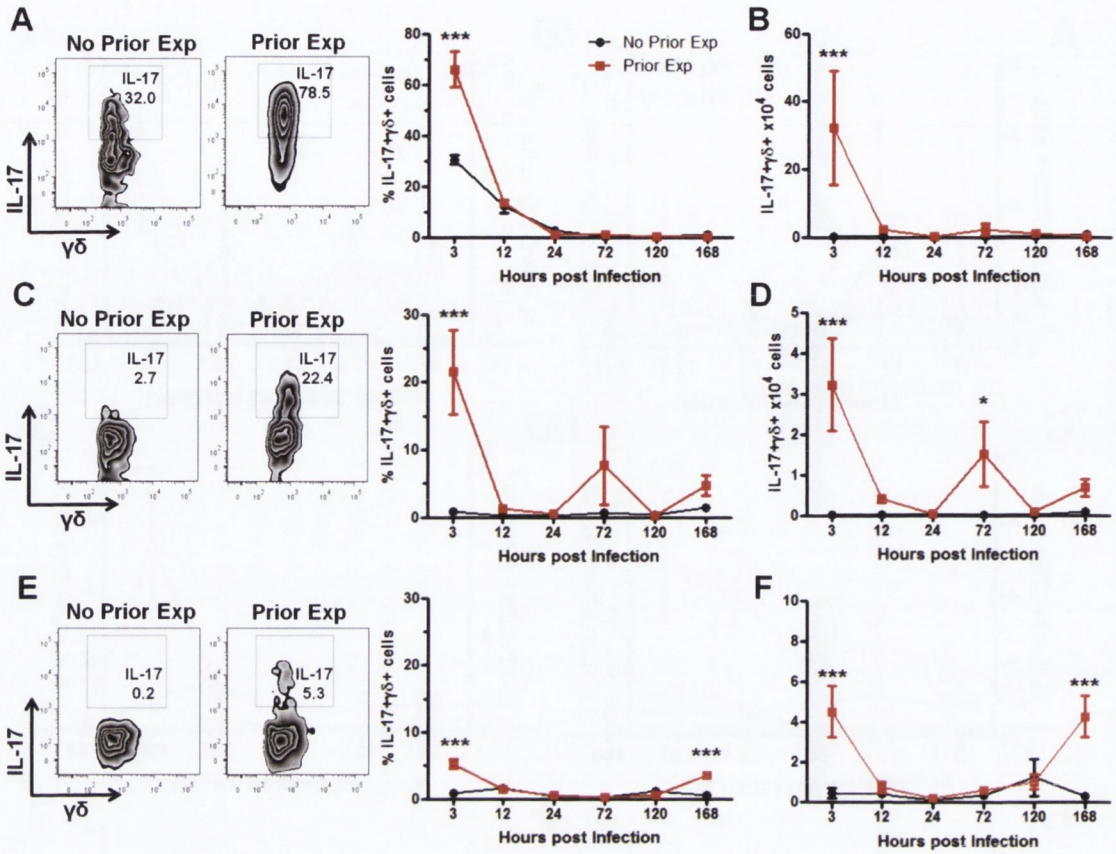


Figure 3.23: Prior exposure to *S. aureus* significantly enhanced IL-17 production by γδ T cells during subsequent challenge.

Groups of mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injections on d 0, 7 and 14. Prior exposed mice were then re-challenged with an i.p injection of *S. aureus* ($5x10^8$ CFU) on d 35 alongside a control group of naive mice. At indicated time points post challenge peritoneal (A & B) MLN (C & D) and spleen (E & F) cells were isolated to assess the proportions (A, C & E) and absolute numbers (B, D & F) of IL-17 producing γδ T cells using flow cytometry. Results expressed as mean±SEM, with representative FACS plots of peritoneal (A), MLN (C) and spleen (E) $\gamma\delta^+$ cells from 3 h post infection shown. n=5 per group. *p<0.05, ***p<0.001

3.2.10. Prior exposure to S. aureus increases phagocyte recruitment and activation during subsequent infection

T cells themselves are not capable of directly killing bacteria, but contribute to anti-bacterial immunity through cytokine induced phagocyte recruitment and activation, which then actively clears the invading pathogen (347). Traditionally, IFNγ is associated with macrophage activation and IL-17 in the recruitment of neutrophils (348, 349). As increased production of IFNγ and IL-17 by T cells was associated with enhanced bacterial clearance in prior exposed mice upon subsequent *S. aureus* challenge, the effects of these cytokines on their downstream effectors i.e. phagocytes, were investigated at the site of infection. Mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injections on d 0, 7 and 14. Upon recovery (d 35), prior exposed mice were re-challenged with *S. aureus* ($5x10^8$ CFU) via i.p. injection, alongside a control group of non-exposed mice. At various time points post challenge PECs were collected, stained for phagocyte markers and analysed by flow cytometry.

A significant increase in the total numbers of $CD11b^+$ cells was detected in the peritoneal cavity of prior exposed mice 3 h post infection compared to non-exposed mice (Figure 3.24 A). By 12 h post infection the numbers of $CD11b^+$ cells had returned to those observed in non-exposed mice. Although no significant difference was observed between the two groups in terms of the proportions of macrophages ($F480^+CD11b^+$) or neutrophils ($Ly6G^+CD11b^+$) (Figure 3.24 B) the absolute numbers of macrophages (Figure 3.24 C) and neutrophils (Figure 3.24 D) were significantly elevated in prior exposed versus non-exposed mice 3 h post infection. By 12 h post infection numbers of macrophages and neutrophils had returned to those observed in non-exposed mice. This data suggests that in prior exposed mice there is a rapid influx of phagocytes comprising of both macrophages and neutrophils, with the early response at 3 h primarily dominated by neutrophils ($143.2x10^4$ macrophages vs. $613.7x10^4$ neutrophils in the peritoneal cavities of prior exposed mice 3 h post infection).

Expression of activation markers by phagocytes was then assessed by quantifying expression of MHC II and CD62L. Activation status was also assessed by measuring ROS activity, using flow cytometry (350). Prior exposure significantly enhanced proportions of MHC II expressing macrophages compared to non-exposed mice at 24 h post infection (Figure 3.25 A). No significant trend was observed upon analysis of absolute numbers of $MHC\ II^+$ macrophages (Figure 3.25 B). L-selectin (CD62L) is constitutively expressed by

circulating neutrophils and upon cell activation is shed from the cell surface (351). Analysis of CD62L expression revealed significantly decreased proportions (Figure 3.25 C) and markedly reduced numbers (Figure 3.25 D) of $CD62L^+$ neutrophils in prior exposed mice 12 and 24 h post infection, suggesting enhanced activation of neutrophils at these time points. Phagocyte activation status was assessed using 123-DHR to measure ROS present within the cell (334). Prior exposure did not significantly affect the proportions of macrophages with ROS activity compared to non-exposed controls (Figure 3.25 E). However, analysis of absolute numbers of macrophages with ROS activity revealed a significant increase in these cells in prior exposed mice compared to non-exposed mice 3 h post infection (Figure 3.25 F). Frequencies of neutrophils with ROS activity were significantly elevated in the peritoneal cavities of prior exposed mice compared to non-exposed mice 12-72 h post infection (Figure 3.25 G). Total numbers of neutrophils with ROS activity revealed a significant increase in these cells in prior exposed mice 3 h post infection (Figure 3.25 H).

Overall this data reveals that prior exposure to *S. aureus* resulted in enhanced recruitment of both macrophages and neutrophils to the infection site at 3 h post infection. Although phagocyte numbers have contracted by 12-24 h post infection, in the prior exposed mice the remaining cells exhibit an increased activation state. Thus prior exposure to *S. aureus* enhanced both initial recruitment of phagocytes to the site of infection and also their activation, during subsequent re-infection.

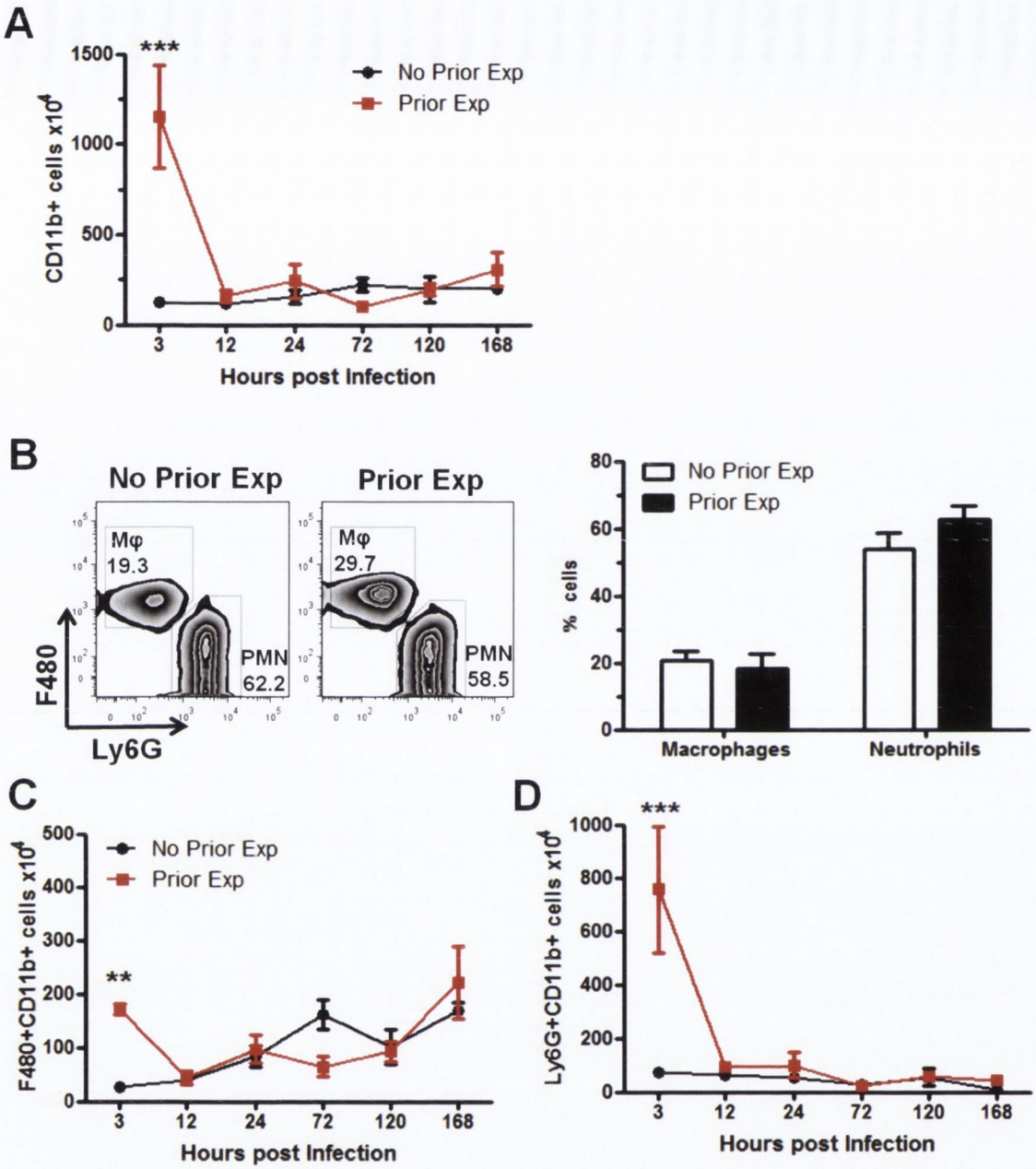


Figure 3.24: Prior exposure to *S. aureus* significantly increases phagocyte influx to the peritoneal cavity during subsequent infection

Groups of mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injections on d 0, 7 and 14. Prior exposed mice were then re-challenged with an i.p injection of *S. aureus* ($5x10^8$ CFU) on d 35 alongside a control group of naive mice. At indicated time points post infection the absolute numbers of $CD11b^+$ cells were assessed by flow cytometry (A). At 3 h post infection the proportions of macrophages ($F480^+Ly6G^-$) and neutrophils ($Ly6G^+F480^-$) were analysed within the $CD11b^+$ population (B). At indicated time points post infection the numbers of macrophages (C) and neutrophils (D) were also determined. Results expressed as mean±SEM (A-D), with representative FACS plots of peritoneal $CD11b^+$ cells 3 h post infection demonstrating the gating strategy used to identify macrophages ($F480^+Ly6G^-$) and neutrophils ($F480^-Ly6G^+$) (B). n=5 per group. ** $p<0.005$, *** $p<0.001$

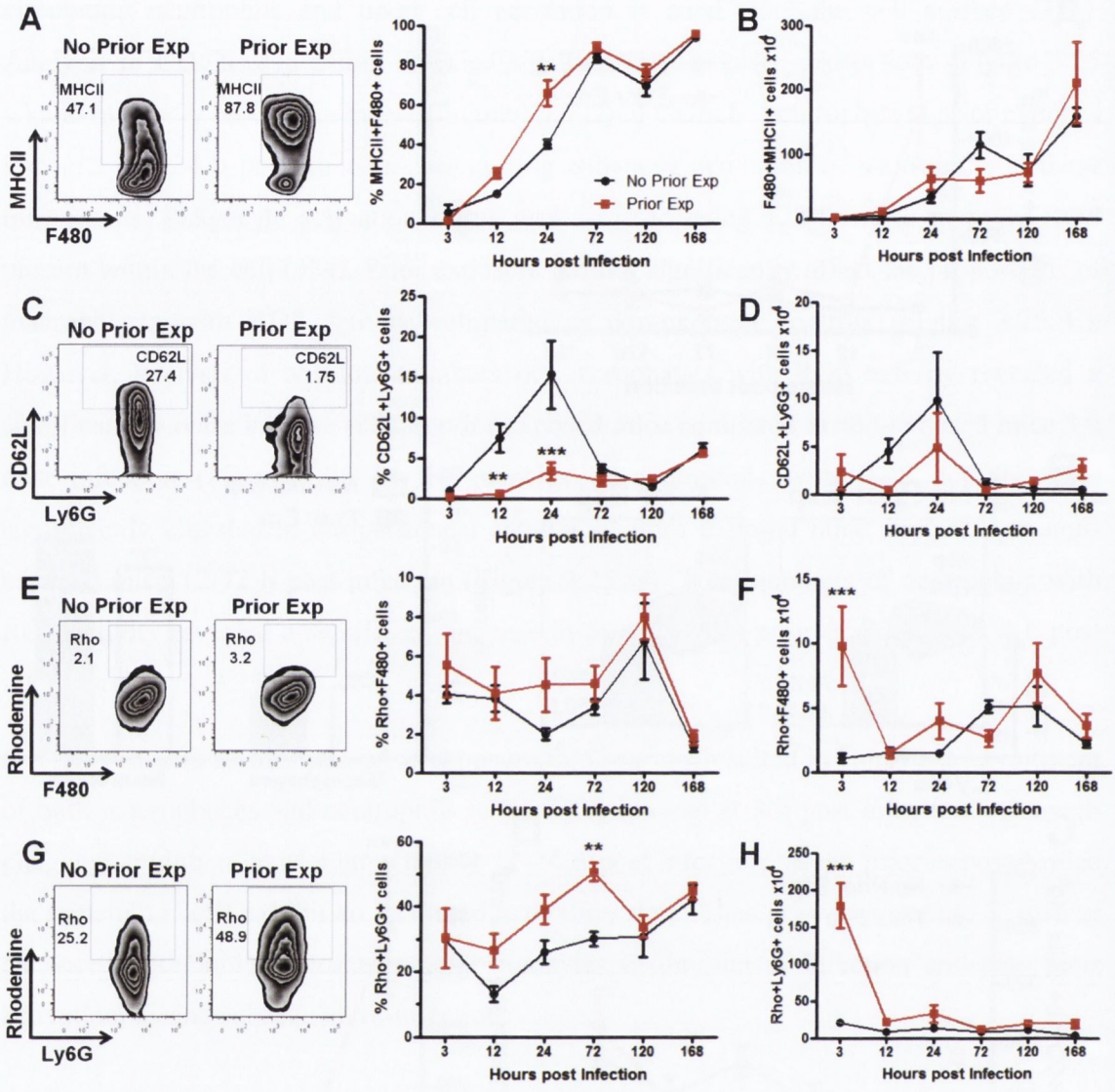


Figure 3.25: Prior exposure to *S. aureus* increases phagocyte activation during subsequent infection

Groups of mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injections d 0, 7 and 14. Prior exposed mice were then re-challenged with an i.p injection of *S. aureus* ($5x10^8$ CFU) on d 35 alongside a control group of naive mice. MHCII expression by macrophages (A & B) and CD62L expression by neutrophils (C & D) was assessed by flow cytometry. ROS activity by macrophages (E & F) and neutrophils (G & H) was detected by rhodemine (Rho) positive cells. Results expressed as mean±SEM of proportions (A, C, E & G) and absolute numbers (B, D, F & H), with representative FACS plots from peritoneal macrophages (A, C) and neutrophils (B, D) 24 h post infection. n=5 per group. **p<0.005, *** p<0.001

3.3. Discussion

Defects in T cell function, both in humans (94, 95, 305) and in mice (165, 169, 320, 322), enhance susceptibility to *S. aureus* infection, leading researchers to question the importance of cellular responses as potential vaccine targets. Indeed, immunisation strategies specifically targeting T cells have been shown to be efficacious in murine models of infection (320, 329, 340). The present study aimed to increase the current understanding of how exposure to *S. aureus* modulates T cell responses and identify if these responses are beneficial to the host upon subsequent infection.

Initial studies using *in vitro* antigen recall assays established that a single *in vivo* exposure to *S. aureus* is capable of expanding populations of IFNγ and IL-17 producing T cells. Similar observations were made by Gomez *et al*, where intra-mammary immunisation with live attenuated *S. aureus* drove IFNγ secretion by CD4 and CD8 T cells upon antigen stimulation *in vitro* (330), and by Joshi *et al* where vaccination with IsdB drove enhanced IL-17 production by CD4 T cells upon re-stimulation with IsdB *ex vivo* (352). Based on these results, the ability of these *S. aureus* expanded T cells to respond to *in vivo* stimulation was established. Previously, a clinical study by Wertheim *et al* (254), demonstrated that sustained exposure to *S. aureus* through colonisation was protective upon invasive infection, suggesting that prolonged exposure can impact the immune system, though no mechanism behind this protection was elucidated. Thus, how the immune system reacts to long-term exposure to *S. aureus* was investigated using a novel murine model of recurrent peritonitis. In this model mice underwent prior exposure to *S. aureus* via multiple i.p. injections on d 0, 7 and 14, followed by a recovery period until d 35 when both infection and inflammation had completely resolved. These prior exposed mice were then re-challenged with *S. aureus* alongside a control group of non-exposed mice, enabling investigation into whether prolonged exposure to *S. aureus* could impact subsequent T cell responses. A limiting factor of this work is that only a single interval period of 21 d between final exposure and re-challenge was investigated and therefore further studies establishing the longevity of the memory T cell responses are needed. However, despite this limitation this study is one of the first to address how long-term exposure to *S. aureus* impacts upon the immune response.

Critically, prolonged prior exposure to *S. aureus* enhanced bacterial clearance during subsequent infection and was associated with a significant increase in IFNγ secretion by CD4 and CD8 T cells, as well as elevated IL-17 production by γδ T cells. IFNγ production

by CD4 T cells (Th1 cells) has previously been shown to be essential for vaccine induced immunity (329, 330). However Lin *et al* documented that IL-17 producing $CD4^+$ cells (Th17 cells) in conjunction with Th1 cells were necessary to mediate full prophylactic protection (329). In contrast, in this model of prior exposure to *S. aureus*, only minimal IL-17 production by CD4 or CD8 T cells was observed in either non-exposed or prior exposed mice, with the primary producers of IL-17 at both the infection site (peritoneal cavity), draining MLN and spleen being γδ T cells. Numerous studies to date have established an important protective role for γδ T cells in murine models of acute *S. aureus* infection (165, 169, 322), through the ability of this cytokine to regulate CXC chemokine driven neutrophil recruitment (165, 169).

In this model, enhanced bacterial clearance induced by prior exposure was associated with both type I immunity and IL-17 responses suggesting that both are required to mediate effective clearance of *S. aureus*. Traditionally, type I immunity is associated with protection against intracellular bacteria, such as *L. monocytogenes* (57, 130, 131) and *Mycobacterium spp.* (58, 59, 126, 127). Emerging evidence suggests that *S. aureus* can survive intracellularly in numerous cell types (263, 296-300, 353) as a means of immune evasion and dissemination (315). Thus, induction of type I immunity may aid in the clearance of these intracellular organisms. Additionally, type I immunity enhances anti-bacterial macrophage effector functions (61, 62, 64, 65) as well as inducing expression of co-stimulatory molecules and MHC enabling more efficient antigen presentation (59), further contributing to protective immunity by facilitating antigen presentation to additional T cells. Alternatively, IL-17 responses are known to be important for protection against extracellular bacteria and have been documented to be important in anti-staphylococcal immunity through downstream effects on neutrophil recruitment (165, 169). Thus in this model of prior exposure type I immunity may be responsible for eradication of intracellular staphylococci and driving enhanced macrophage responses, whereas IL-17 secretion drives increased neutrophil recruitment involved in elimination of extracellular staphylococci. Therefore the IFNγ and IL-17 responses work in concert to effectively clear *S. aureus* infection.

Emerging evidence reflects this paradigm with efficient protection against invading bacteria at mucosal sites benefiting from synergy of both Th1 and IL-17 mediated responses. Adoptive transfer of Th1 and Th17 cells purified from mice previously infected with *B. pertussis*, into naive mice, enhanced protection against subsequent challenge

compared to transfer of either cell type alone (345). Defects in Th1 and Th17 cell lineages are observed in IL-12 and IL-23 deficient mice respectively, however both experience exacerbated *K. pneumoniae* infection, suggesting that both Th1 and Th17 cells are contributing to immunity in this model (74). Additionally, vaccination against *M. tuberculosis* expanded a population of IL-23 dependent IL-17 producing T cells, which preceded the Th1 response. Ablation of IL-23 signalling significantly impaired IFNγ recall responses, leading the authors to postulate that Th17 cells were indirectly required for anti-mycobacterial Th1 responses (354). Furthermore, IL-17 signalling was required for Th1 cell responses and host resistance against the intracellular bacterium *Francis tularensis* (355). These reports suggest that IL-17 responses in the periphery could provide surveillance which, when triggered by bacterial threat, would initially induce a rapid IL-17 response followed by a Th1 response, ultimately leading to protective immunity against bacteria such as staphylococci, that have the ability to survive in both an intracellular and extracellular environment. Prior exposure to *S. aureus* reflects this concept, as IL-17 secretion commenced before IFNγ in prior exposed mice, with substantial levels of IL-17 observed as early 1 h post infection (Figure 4.10), whereas IFNγ was undetectable in prior exposed mice at this time point (data not shown).

The pro-inflammatory cytokines IL-1β and IL-23 are known to drive innate IL-17 production by γδ T cells (149), however levels of these cytokines were unaffected by prior exposure. IL-12 is essential in driving the differentiation of Th1 cells (57, 60) and again, prior exposure to *S. aureus* did not significantly affect IL-12 secretion. This suggests that the enhanced production of IFNγ and IL-17 by T cells observed in prior exposed mice is not occurring non-specifically as a result of increased innate cytokine signalling, but rather from increased recognition of staphylococcal antigens. Elevated IL-10 levels were also observed in prior exposed mice, though no cellular source of IL-10 was identified. IL-10 signalling has been shown to be critical in controlling inflammatory responses induced by infection (106, 107) with ablation of IL-10 leading to increased mortality through uncontrolled immune mediated damage. Therefore the increase of IL-10 during acute infection may help control the effects of increased IL-17 and IFNγ production, preventing chronic inflammation from taking place.

Given that T cells are incapable of directly killing bacteria, enhanced bacterial clearance induced by prior exposure is likely mediated through the downstream effects of T cell derived cytokines. Prior exposure to *S. aureus* resulted in increased levels of RANTES, a

chemokine involved in T cell recruitment (356) and proliferation (357), and was associated with increased CD3 T cell accumulation in the peritoneal cavities of prior exposed mice throughout the course of infection. Specifically, RANTES strongly attracts memory T cells (356) indicating that the enhanced T cell expansion detected in prior exposed mice were acquired memory T cell responses. RANTES is also associated with a Th1 type immune response (358, 359). Thus, elevated RANTES levels may be contributing to the enhanced Th1 responses detected in prior exposed mice. Additionally, RANTES is involved in macrophage recruitment (360, 361), and enhanced RANTES secretion could account for the elevated numbers of macrophages observed 3 h post infection in prior exposed mice.

Prior exposure also drove enhanced secretion of KC and MIP-2 early on in infection, which correlated with increased neutrophil recruitment to the peritoneal cavity. KC and MIP-2 bind to the same receptor, CXCR2, on the surface of neutrophils with blockade of this receptor resulting in decreased neutrophil recruitment (362). It is therefore likely that both these chemokines drove the enhanced neutrophil recruitment observed in prior exposed mice. Although prior exposure did not significantly increase the production of KC during subsequent *S. aureus* infection, a substantial increase in KC was still observed. Given that KC has been reported to peak prior to MIP-2 secretion (312, 363), it is possible that KC secretion peaked prior to 3 h post infection, therefore further studies may be required to examine KC secretion as early as 30 mins post *S. aureus* infection.

The elevated chemokine secretion observed in prior exposed mice was associated with significantly enhanced macrophage and neutrophil recruitment in the early stages of infection. This increase in recruitment was closely followed by enhanced activation of phagocytes in prior exposed mice 12-24 h post infection. As reduced bacterial load is first observed in prior exposed mice 24 h post infection, it suggests that enhanced phagocyte activation is responsible for the active clearance of the bacteria. IFNγ signalling is known to promote MHC complex expression enabling more efficient antigen presentation (59). In this model enhanced IFNγ secretion correlated with increased MHC II expression by macrophages in prior exposed versus non-exposed mice. Additionally RANTES, along with MIP-1α, MIP-1β and lymphotactin, have been shown to cooperate with IFNγ to co-activate macrophages leading to upregulation of CD40, NO, IL-12 and TNFα by macrophages during *L. monocytogenes* infection (364). Thus increased IFNγ and RANTES secretion together could be driving increased macrophage activation that in turn contributes to enhanced clearance of infection in prior exposed mice. IFNγ signalling also

enhances respiratory burst and the formation of ROS by upregulating gp91phox, a subunit of NADPH oxidase, in both macrophages and neutrophils (64), suggesting that elevated IFNγ levels observed in prior exposed mice may also contribute to the increase in ROS activity detected by neutrophils. In addition, IL-17 signalling has been demonstrated to upregulate ROS in neutrophils (365), possibly synergising with IFNγ signalling to drive enhanced ROS activity within prior exposed neutrophils. Given that IL-17 signalling drives CXC chemokine production (88, 96), it is likely that enhanced IL-17 responses in prior exposed mice are responsible for increased MIP-2 and KC secretion leading to the initial rapid influx of neutrophils. Taken together, it is tempting to speculate that elevated IL-17 responses observed in prior exposed mice initially drive enhanced recruitment of phagocytes to the infection site, where increased IFNγ signalling is then responsible for activating and controlling the phagocyte response.

Overall, this study demonstrates that prolonged exposure to *S. aureus* can prime specific T cell subsets which mediate enhanced bacterial clearance against subsequent infection through their downstream effects on phagocyte recruitment and activation. Though further work is required to ascertain the longevity of this protective memory T cell response, it identifies the importance of type I immunity, i.e. IFNγ producing CD4 and CD8 cells, and IL-17 producing γδ T cells in protective memory responses against *S. aureus* infection. The remainder of this thesis will further characterise the relative importance of memory γδ T cell (Chapter 4) and Th1 cells (Chapter 5) in enhancing clearance of *S. aureus* infection. Of note, prior exposure also induced anti-staphylococcal antibody responses so protection induced by prior exposure may not be exclusively T cell mediated. This concept will be further investigated in Chapter 5 where novel adjuvant technology was used to drive cellular immunity in the absence of an antibody response.

Chapter 4

S. aureus exposure expands a population of memory γδ T cells which enhance bacterial clearance during subsequent infection

4.1. Introduction

A growing literature supports the important role played by the cytokine IL-17 in anti-bacterial immunity. It is generally accepted that defective IL-17 signalling results in diminished neutrophil recruitment and impaired bacterial clearance (86, 96, 151, 155, 366). In the context of *S. aureus*, sub-epithelial infections of IL-17A$^{-/-}$IL-17F$^{-/-}$ double knock-out mice (321) and subcutaneous (169) or surgical site (322) infections of IL-17R-deficient mice resulted in increased bacterial burden and disease pathology. Inhibition of IL-17 responses through administration of the anti-inflammatory cytokines IL-19 and IL-20 also led to increased bacterial burden in a model of staphylococcal cutaneous infection (328). Lin *et al.* further demonstrated that vaccination with a candidal surface protein (rAlsp3-N), which is structurally similar to the staphylococcal surface protein ClfA, protected against *S. aureus* systemic infection in WT mice but was ineffective in IL-17A deficient mice (329). Similarly, vaccination with ClfA protected WT mice against systemic *S. aureus* infection, but was ineffective in IL-17A deficient mice (367). Thus, in murine models at least, IL-17 appears to play a critical role in both natural and vaccine induced immunity against *S. aureus* infections.

Even more compelling evidence exists in humans, as patients with HIES, who have mutations in the gene encoding STAT3 leading to impaired Th17 cell function, suffer from recurrent and often severe *S. aureus* infections (94, 95). Furthermore, patients with atopic dermatitis are more susceptible to colonisation by *S. aureus* (343), due in part to decreased IL-17 responses (344). These findings have thrust IL-17 to the forefront of research into anti-staphylococcal immunity highlighting this cytokine as one of the most important potential targets for anti-*S. aureus* immunotherapies (336, 368).

Given the importance of IL-17 in anti-staphylococcal immunity, identifying the cellular sources of this cytokine will be vital for the design of novel anti-*S. aureus* vaccines that promote this arm of cellular immunity. γδ T cells have recently been identified as a potent source of innate IL-17 and implicated in host protection in murine models of *S. aureus* infection. In a cutaneous infection model, γδ T cell deficient mice were found to have reduced neutrophil recruitment to the infection site and impaired bacterial clearance, compared to WT controls (169). Recruitment of neutrophils was dependent upon IL-17 production by a specific subset of Vγ3 T cells, the predominant γδ subset in the epidermis. γδ T cells, specifically Vγ1.1 and Vγ2, were also shown to have a protective role in *S. aureus* induced pneumonia through their capacity to produce IL-17, with increased bacterial loads detected in δTCR$^{-/-}$ mice compared to WT controls (165). Recently, γδ T

cells were reported to be the dominant source of IL-17 in a surgical site infection model, where IL-17R$^{-/-}$ and δTCR$^{-/-}$ mice displayed increased susceptibility to *S. aureus* infection (322).

In humans, circulating γδ T cell numbers increase dramatically upon microbial infection, in some cases reaching more than 50% of peripheral T cells within days (135). Human γδ T cells, like αβ T cells, are capable of antigen recall and can recognise a bacterial antigen upon re-exposure to the organism, allowing for a more rapid and efficient immune response (181, 192, 204). However, unlike classical T cells, γδ T cells are also capable of recognising conserved self and foreign non-peptide antigens, such as phosphoantigens (135). Phosphoantigens can directly activate γδ T cells but activation is enhanced in the presence of monocytes which most likely help present the phosphoantigen to the γδ TCR (369, 370). Using a humanised chimeric severe combined immunodeficiency disease (SCID) model, it was demonstrated that phosphoantigen activated human Vγ9Vδ2 cells can mediate resistance to murine *S. aureus* infection (371), suggesting that human γδ T cells may play a role in anti-staphylococcal immunity.

Traditionally, studies investigating the induction of cellular memory by vaccines have primarily focused on αβ T cells. γδ T cells were not considered vaccine targets due to their lack of adaptive features such as antigen-specificity and immunological memory. Murine γδ T cells resembling pathogen-specific human γδ T cells and bearing the characteristics of adaptive αβ T cells have recently been identified in a model of enteric *L. monocytogenes* infection (173). These *Listeria*-specific memory γδ T cells were of the Vγ4Vδ1 subset, exhibited a CD27^{-}CD44^{hi} profile and produced increased amounts of IFNγ and IL-17 upon re-exposure to *L. monocytogenes*. Of note, these memory γδ T cells displayed contextual specificity and were only induced via the mucosal route not the systemic, suggesting mucosal priming plays a key role in memory γδ T cells responses. Although pathogen-specific γδ T cells had been identified in humans, their murine equivalent was lacking, hindering investigation into the potential of targeting these cells through vaccination. The identification of murine memory γδ T cells will now facilitate investigations into the targeting of these cells by vaccines.

This current study aims to extend initial findings where γδ T cells were established as the primary source of IL-17 during *S. aureus* induced peritonitis, by identifying the distinct Vγ subsets responsible for IL-17 production in both prior exposed and non-exposed mice. Importantly, the mechanisms responsible for inducing IL-17 production by γδ T cells will

be investigated in both naive and prior exposed mice, in order to verify if enhanced IL-17 secretion by prior exposed γδ T cells is indicative of a memory response. Finally, adoptive transfer studies will attempt to ascertain if *S. aureus* primed/memory γδ T cells can mediate enhanced clearance of *S. aureus* infection, thus identifying γδ T cells as potential anti-*S. aureus* vaccine targets.

4.2. Results

4.2.1. γδ T cells are the major source of IL-17 during S. aureus induced peritonitis.

Previous publications have demonstrated that IL-17 plays a protective role during *S. aureus* infection (94, 95, 169, 321, 322). Hence, IL-17 secretion, as well as IL-1β and IL-23 secretion (known to drive IL-17 production (149)), were investigated in this systemic infection model of *S. aureus* induced peritonitis, where mice received a single i.p. injection of *S. aureus* ($5x10^8$ CFU) or sterile PBS as a control. IL-1β (Figure 4.1 A) and IL-17A (Figure 4.1 C) were rapidly produced in the peritoneal cavity upon infection. Both IL-17A and IL-1β were maximal at 3 h and had decreased to the levels observed in control mice by 24 h post infection. IL-23 was also produced upon *S. aureus* infection, with IL-23 secretion peaking by 12 h post infection (Figure 4.1 B). IL-1α and IL-18 are also known to drive IL-17 production by γδ T cells (372), and IL-17F can compensate for IL-17A during *S. aureus* infection (321). However, secretion of IL-1α, IL-18 or IL-17F was not detected in the peritoneal fluid of infected mice (data not shown). This lack of IL-17F induction by *S. aureus* is consistent with previous work by the Host Pathogen Interactions Group demonstrating no role for IL-17F during *S. aureus* surgical site infection (322).

IL-17 was primarily produced by $CD3^+$ T cells upon infection with *S. aureus* (0.5% $CD3^+IL\text{-}17^+$ vs. 0.05% $CD3^-IL\text{-}17^+$ of total PECs). Within the $CD3^+$ population, $\gamma\delta^+$ T cells were the primary source of IL-17, whereas few $CD4^+$ and $CD8^+$ T cells expressed IL-17 at this stage (Figure 4.1 D). IL-17 producing T cells were also detected in the MLN during *S. aureus* infection. Again, γδ T cells comprised the major source of IL-17, although significantly fewer cells were cytokine-positive in the lymphoid tissues (Figure 4.1 E). Of note, we did not detect any IFNγ production by γδ T cells isolated from *S. aureus* infected mice (Figure 4.1 F).

IL-17 producing $\gamma\delta^+$ T cell proportions (Figure 4.2 A) and absolute numbers (Figure 4.2 B) were significantly elevated in the peritoneal cavity as early as 1 h post infection, and continued to increase up to 3 h post infection. By 24 h post infection peritoneal $\gamma\delta^+$ T cells had ceased to produce IL-17, but remained in the peritoneal cavity (Figure 3.25). Interestingly, a second wave of IL-17 producing γδ T cells appeared in the peritoneal cavity with increased $IL\text{-}17^+\gamma\delta^+$ T cells detected by 72 h post infection. This second phase of $IL\text{-}17^+\gamma\delta$ T cell accumulation was not detected in the MLN where the frequency (Figure 4.2 C) and absolute numbers (Figure 4.2 D) of these cells was comparable to the PBS treated group by 12 h post infection.

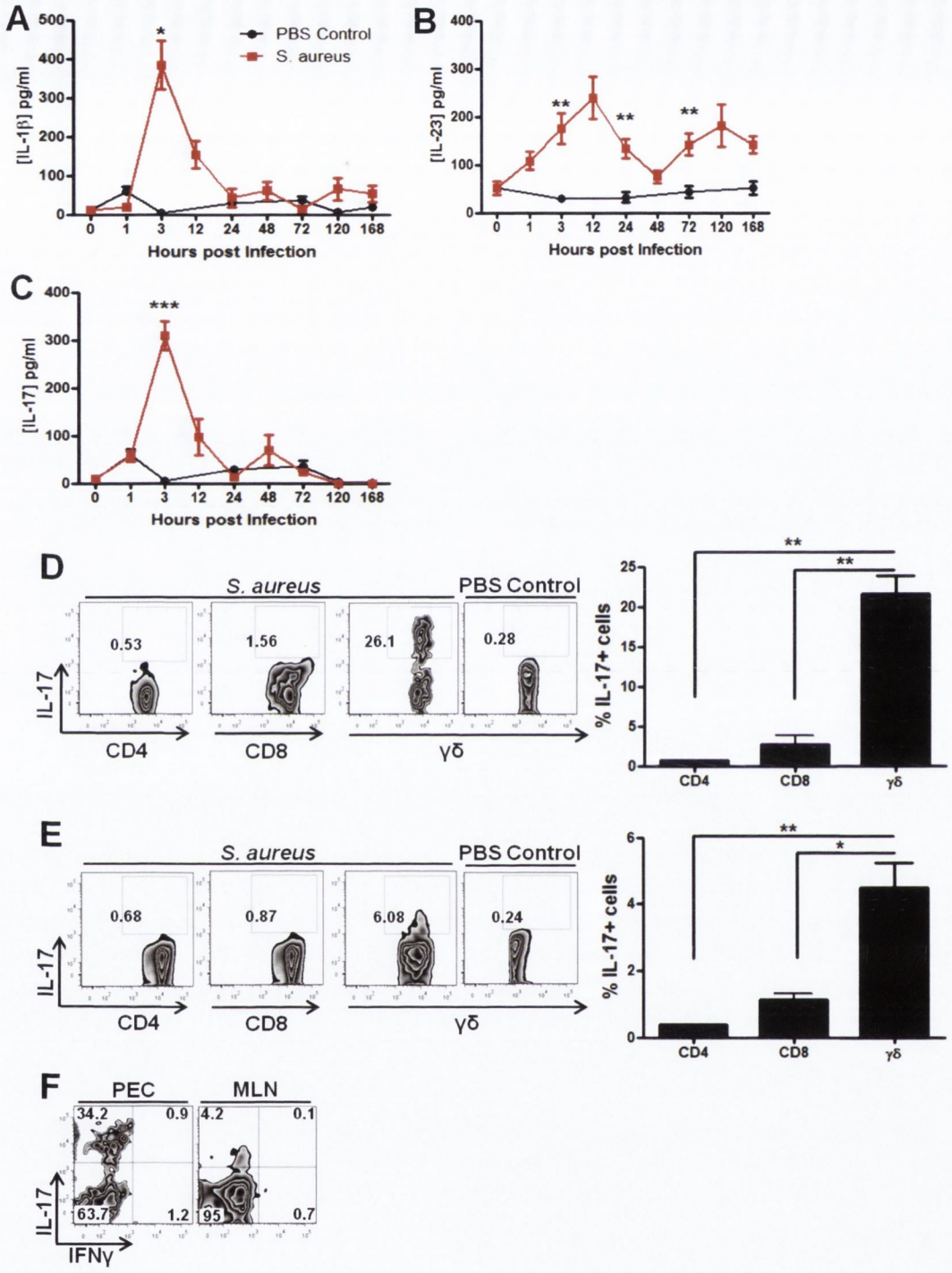


Figure 4.1: γδ T cells are the primary source of IL-17 during *S. aureus* induced peritonitis

Mice were infected with *S. aureus* ($5x10^8$ CFU) via i.p. injection. Secreted IL-1β (A), IL-23 (B) and IL-17A (C) in the peritoneal fluid was measured by ELISA at indicated time points post infection. At 3 h post infection PECs (D) and MLN cells (E) were isolated and IL-17 production by CD4, CD8 and γδ T cells assessed by flow cytometry. Results expressed as mean±SEM of n=5-10 per group, with representative FACS plots shown (C, D). Data pooled from 2 independent experiments. In addition, IFNγ production by γδ T cells was also assessed (F). Results expressed as representative FACs plots of n=5. *$p<0.05$, **$p<0.005$, ***$p<0.001$.

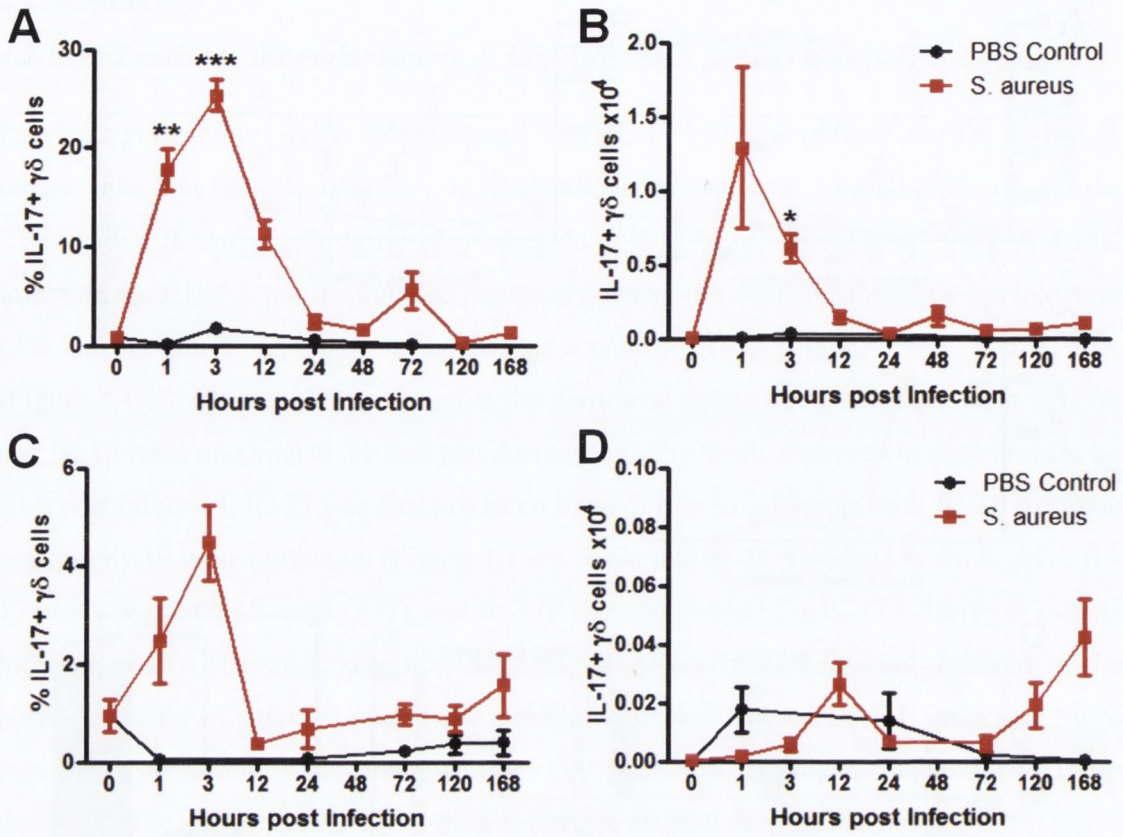


Figure 4.2: IL-17 production by γδ T cells during the course of *S. aureus* infection

Mice were infected with *S. aureus* ($5x10^8$ CFU) via i.p. injection. At the indicated times post infection, the peritoneal cavity was lavaged and the MLN collected. The proportions (A) and absolute numbers (B) of peritoneal IL-$17^+\gamma\delta^+$ cells, and the proportions (C) and absolute numbers (D) of IL-$17^+\gamma\delta^+$ cells within the MLN, were assessed by flow cytometry. Results expressed as mean±SEM. n=5-10 per group. Data pooled from 2 independent experiments. *p<0.05, **p<0.005, ***p<0.001.

4.2.2. IL-1RI signalling is required for production of IL-17 by γδ T cells during S. aureus infection

Previous studies have reported that IL-17 production by γδ T cells during *S. aureus* infection is IL-1β dependent (9, 322). To confirm a role for IL-1RI signalling in regulating IL-17 production by γδ T cells during *S. aureus* induced peritonitis, WT and IL-1R$^{-/-}$ mice were infected with *S. aureus* (5x10^{8}CFU) via i.p. injection. At 3 h post infection, PECs were isolated and IL-17 production by γδ T cells assessed. IL-17 secretion in the peritoneal fluid of infected IL-1RI$^{-/-}$ mice was significantly reduced in comparison to infected WT mice with no significant difference in IL-17 levels detected between PBS treated and *S. aureus* infected IL-1RI$^{-/-}$ mice (Figure 4.3 A). Of note the levels of secreted IL-23, another cytokine reported to drive innate production of IL-17 by γδ T cells (149), did not significantly differ between WT and IL-1RI$^{-/-}$ mice (data not shown). Ablation of IL-1RI signalling did not affect recruitment of γδ T cells to the peritoneal cavity, as the total number of peritoneal γδ$^+$ T cells was comparable in both *S. aureus* infected WT and IL-1RI$^{-/-}$ mice (Figure 4.3 B). However, IL-17 production by these γδ T cells was abrogated in the IL-1RI$^{-/-}$ mice, with significantly reduced proportions (Figure 4.3 C) and numbers (Figure 4.3 D) of IL-17$^+$γδ$^+$ T cells detected in the peritoneal cavity of IL-1R$^{-/-}$ mice compared to WT mice. These data demonstrate a critical role for IL-1R signalling in the early induction of IL-17 by γδ T cells during *S. aureus* induced peritonitis.

Cells isolated from infected WT or IL-1RI$^{-/-}$ mice were also stimulated with PMA and ionomycin prior to staining for flow cytometry. Analysis revealed that *in vitro* mitogen stimulation significantly increased the proportions of peritoneal IL-17$^+$γδ$^+$ cells detected in *S. aureus* infected IL-1RI$^{-/-}$ mice to levels comparable to WT mice (Figure 4.4). This was unusual as ELISA data revealed that IL-17 secretion in IL-1RI$^{-/-}$ mice was significantly impaired compared to WT mice (Figure 4.3 A). This suggests that PMA and ionomycin treatment over-stimulates production of IL-17 by γδ T cells masking true *in vivo* trends, and as such, all intracellular FACS analysis of γδ T cells throughout this thesis was carried out with brefeldin A stimulation alone.

As defective IL-17 signalling results in diminished neutrophil recruitment and impaired bacterial clearance (86, 96, 151, 155, 366), chemokine production and neutrophil influx were compared between *S. aureus* infected WT and IL-1RI deficient mice. WT and IL-1RI$^{-/-}$ mice were infected with *S. aureus* (5x10^{8}CFU) via i.p. injection. At 3 h post infection the peritoneal cavity was lavaged and the local chemokine production assessed by

ELISA. At 72 h post infection the influx of neutrophils into the peritoneal cavity was established using flow cytometry. Ablation of IL-1RI signalling led to reduced chemokine secretion upon infection with *S. aureus*, with significantly decreased levels of KC (Figure 4.5 A) and MIP-2 (Figure 4.5 B) detected in the peritoneal fluid of IL-1RI$^{-/-}$ mice compared to WT mice, 3 h post infection. The downstream effects of this impaired chemokine production resulted in significantly reduced influx of neutrophil proportions (Figure 4.5 C) and absolute numbers (Figure 4.5 D) to the peritoneal cavity 72 h post infection.

Having established that γδ T cells deficient in IL-1RI produced significantly less IL-17 than γδ cells from WT mice and as defects in IL-17 increase host susceptibility to *S. aureus* infection (169, 321, 322), the capacity of WT and IL-1RI$^{-/-}$ to clear *S. aureus* peritonitis was assessed. WT and IL-1RI$^{-/-}$ mice were infected with *S. aureus* ($5x10^8$ CFU) via i.p. injection. At 72 h post infection the peritoneal cavity was lavaged with PBS and the kidneys, liver and spleen isolated in order to assess bacterial burden. Analysis of the bacterial burden revealed that deficiency in IL-1RI signalling (and thus IL-17), did not significantly affect the clearance of *S. aureus* from the local infection site at this time point (Figure 4.6 A). However, significantly greater levels of bacteria were detected in the kidneys (Figure 4.6 B), liver (Figure 4.6 C) and spleen (Figure 4.6 D) of IL-1RI$^{-/-}$ mice compared to WT mice, suggesting that together impaired IL-1RI and IL-17 responses led to increased staphylococcal dissemination to peripheral tissues.

Taken together these data demonstrate the importance of IL-1β signalling in driving IL-17 production by naive γδ T cells in response to *S. aureus* induced peritonitis. It also highlights the importance of the downstream effects of IL-1β and subsequent IL-17 responses in regulating neutrophil infiltration and aiding in bacterial clearance.

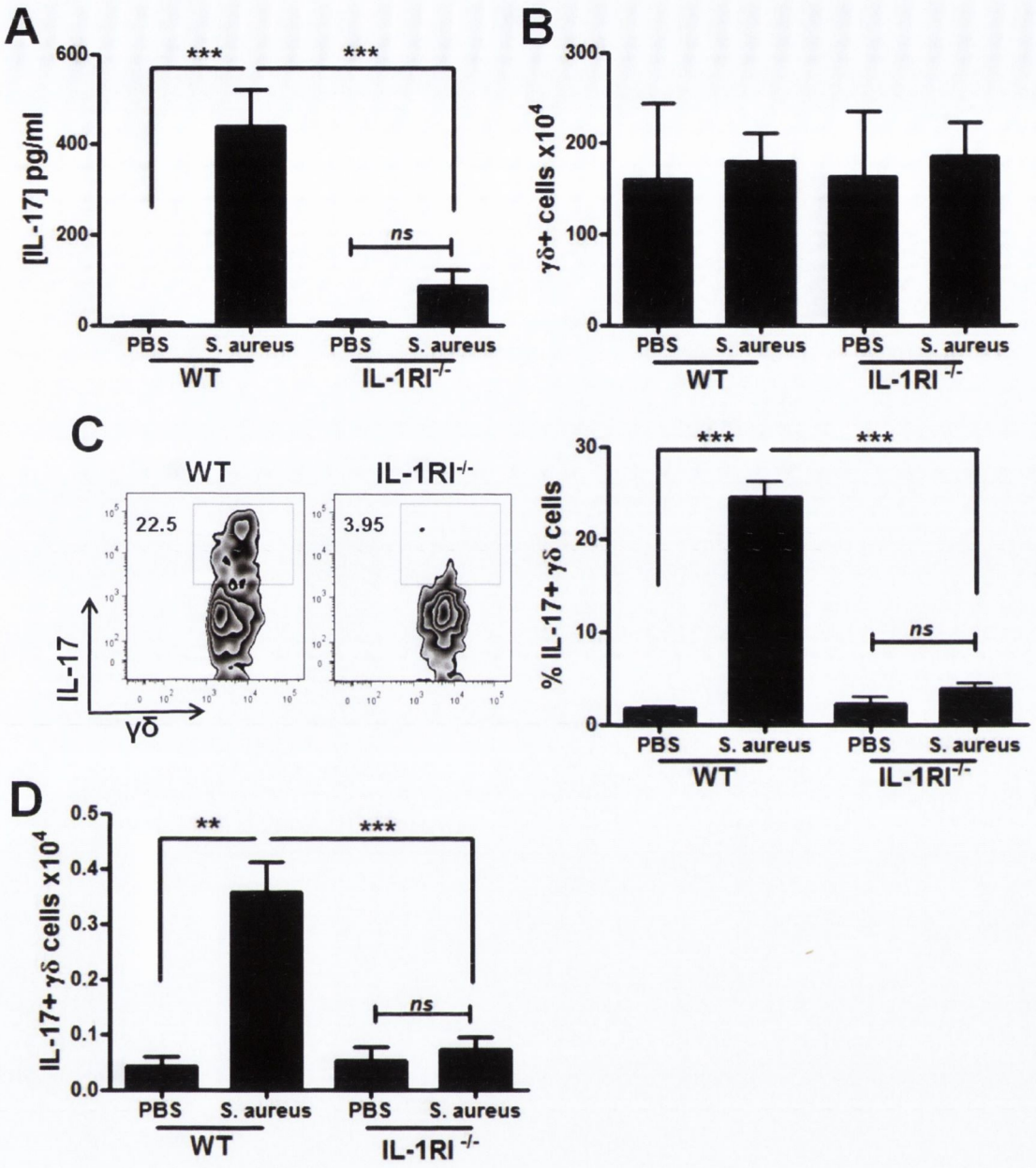


Figure 4.3: Ablation of IL-1RI signalling impairs IL-17 production by γδ T cells during *S. aureus* infection

IL-1RI$^{-/-}$ and WT mice were infected with *S. aureus* (5x10^8 CFU) via i.p. injection and at 3 h post infection the peritoneal cavity was lavaged with PBS. Secreted IL-17A in the peritoneal fluid was measured by ELISA (A). γδ T cell influx to the peritoneal cavity was assessed by flow cytometry (B). The proportions (C) and absolute numbers (D) of IL-17 producing γδ T cells were assessed by flow cytometry following *ex vivo* stimulation with brefeldin A. Results expressed as mean±SEM with representative FACS plots shown (C). n=5 for PBS treated groups, n=10 for *S. aureus* infected groups. Data pooled from 2 independent experiments. **p<0.005, ***p<0.001.

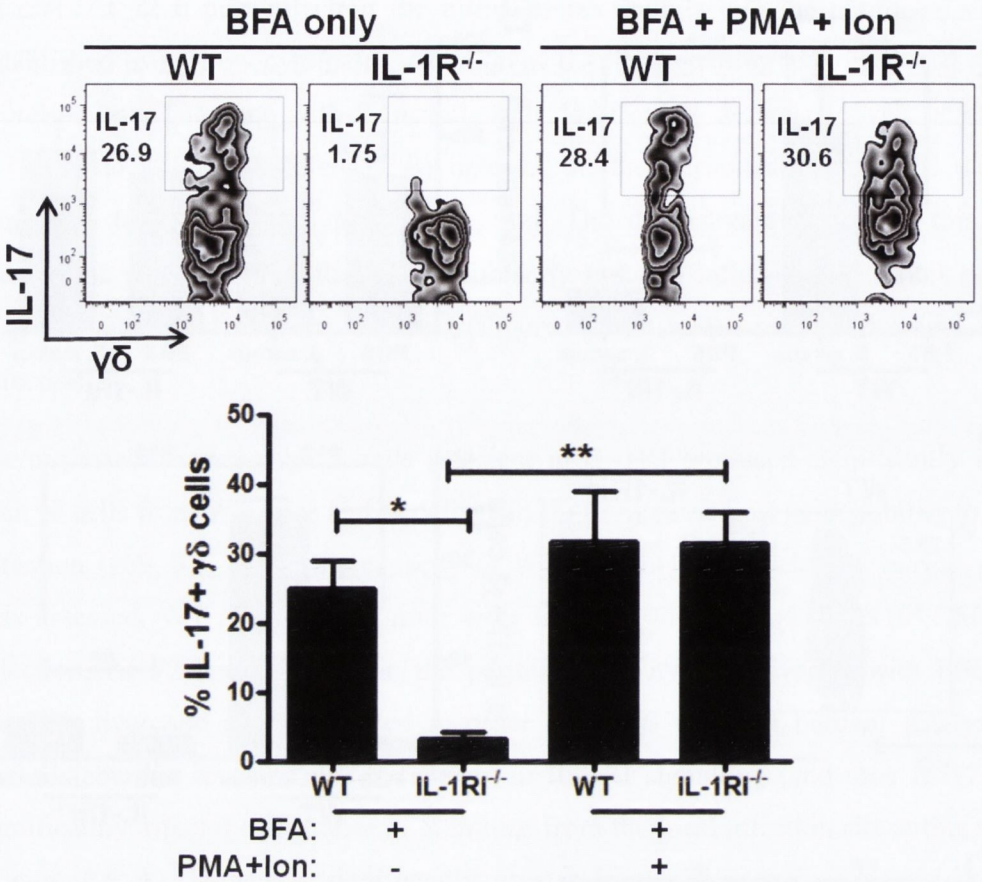


Figure 4.4: Simulation with PMA and Ionomycin exaggerates IL-17 production by γδ T cells isolated from IL-1RI$^{-/-}$ mice

IL-1RI$^{-/-}$ and WT mice were infected with *S. aureus* (5x10^8 CFU) via i.p. injection and at 3 h post infection the peritoneal cavity was lavaged with PBS. PECs were stimulated *ex vivo* with brefeldin A (BFA) in the presence or absence of PMA and Ionomycin (Ion) prior to analysis of IL-17$^+$γδ T cells by flow cytometry. Results expressed as mean±SEM with representative flow cytometry plots shown. n=5 per group. *p<0.05, **p<0.005

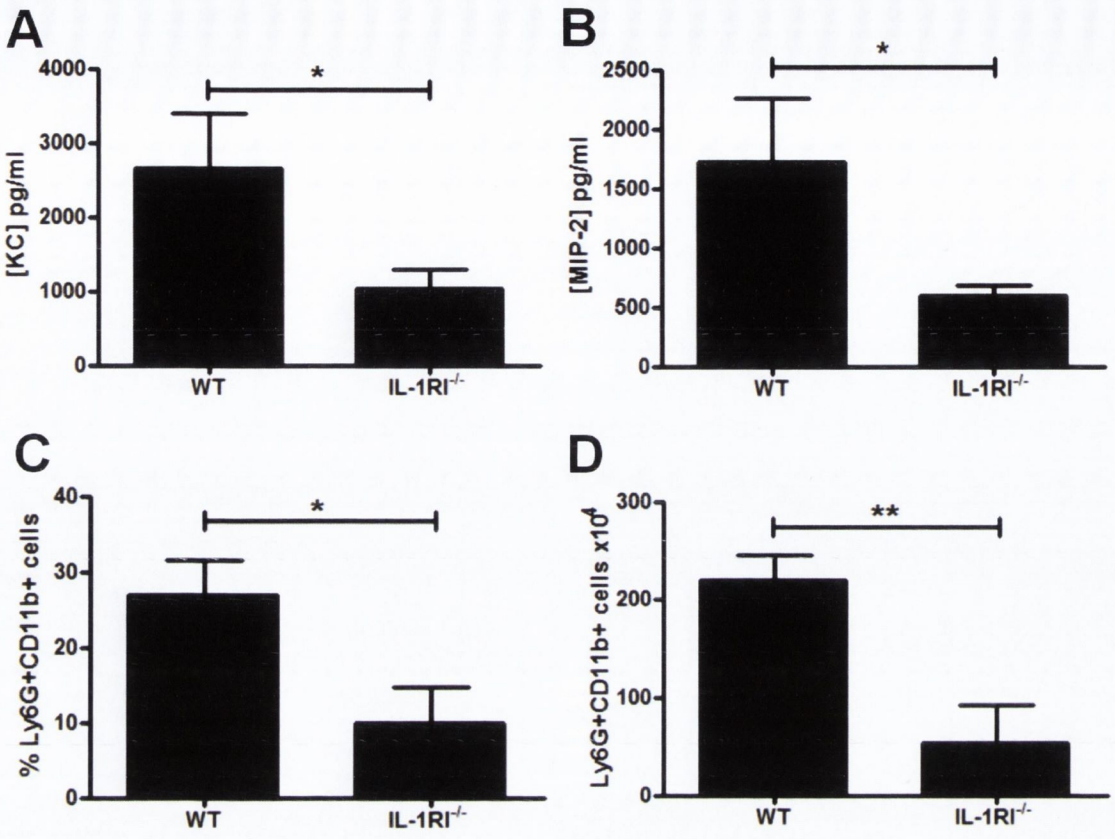


Figure 4.5: Ablation of IL-1RI signalling impairs chemokine production and neutrophil influx during *S. aureus* infection

IL-1RI$^{-/-}$ and WT mice were infected with *S. aureus* ($5x10^8$ CFU) via i.p. injection. At 3 h post-infection the peritoneal cavity was lavaged with PBS and secreted KC (A) and MIP-2 (B) measured in the peritoneal fluid by ELISA. At 72 h post infection PECs from WT and IL-1RI$^{-/-}$ were isolated and neutrophil ($CD11b^+Ly6G^+F480^-$) proportions (C) and absolute numbers (D) assessed by flow cytometry. Results expressed as mean±SEM. n=5 per group. *$<p<0.05$, **$p<0.005$.

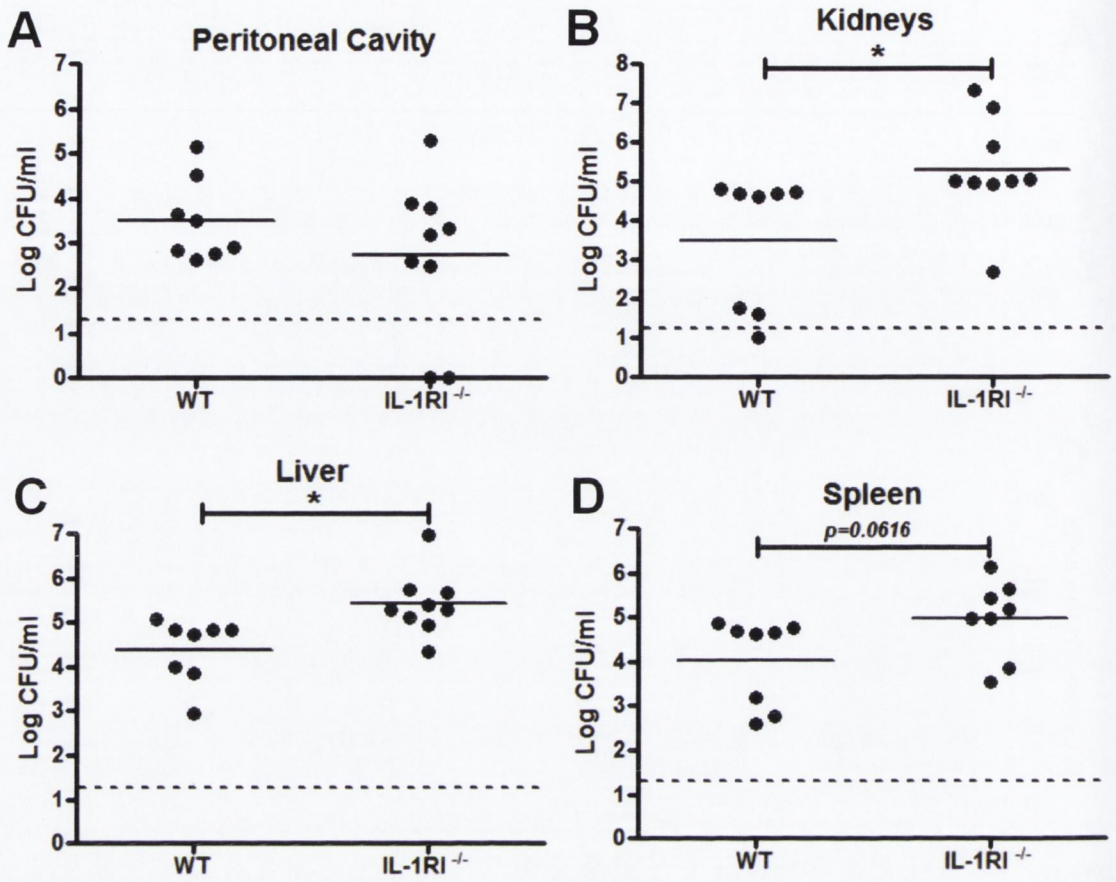


Figure 4.6: Ablation of IL-1RI signalling impairs clearance of *S. aureus* infection

IL-1RI$^{-/-}$ and WT mice were infected with *S. aureus* ($5x10^8$ CFU) via i.p. injection. At 72 h post infection the peritoneal cavity was lavaged with PBS to assess the local bacterial burden (A). The kidneys (B), liver (C) and spleen (D) were also collected to assess the spread of infection. Results expressed as Log CFU/ml with means indicated by bar. Dashed lines indicate limit of detection. n=8-9 per group. Data pooled from 2 independent experiments.*<$p<0.05$.

4.2.3. Differential recruitment of γδ T cell subsets occurs over the course of S. aureus infection

Individual γδ T cell subsets are often associated with tissue-specific homing and functions (139). In order to characterise the γδ T cell subsets responsible for IL-17 production throughout the time course of *S. aureus* induced peritonitis, mice were infected with *S. aureus* ($5x10^8$ CFU) via i.p. injection and PECs and MLN cells isolated at various time points post-infection and stained with antibodies against CD3, γδTCR, Vγ1.1, Vγ2 and Vγ3 surface markers. By 3 h post infection a distinct population of $V\gamma1.1^+$, $V\gamma2^+$ and a population of double negative cells could be observed within the peritoneal cavity (Figure 4.7 A). Of note, no $V\gamma3^+$ cells were detectable by flow cytometry in the PEC (Figure 4.7 B) or MLN (data not shown). Based on previous studies (154), it was hypothesised that peritoneal $V\gamma1.1^-V\gamma2^-$ cells were in fact $V\gamma4^+$ cells, for which no fluorochrome-conjugated antibody is commercially available.

To confirm this, $V\gamma1.1^-V\gamma2^-$ cells were purified from PECs and MLNs of mice 3 h post *S. aureus* infection using cell sorting. RNA was extracted from the purified $V\gamma1.1^-V\gamma2^-$ cells, reverse transcribed into cDNA and each Vγ gene amplified by PCR. Gel electrophoresis of the amplimers revealed that the $V\gamma1.1^-V\gamma2^-$ cells were indeed $V\gamma4^+$ cells (Figure 4.7 C). Furthermore, the sequence of the amplified DNA, excised from the gel, was translated and aligned with the Vγ4 protein sequence confirming that the $V\gamma1.1^-V\gamma2^-$ cells were $V\gamma4^+$ cells (Figure 4.7 D). These $V\gamma1.1^-V\gamma2^-$ cells are therefore referred to as $V\gamma4^+$ cells in the figure and throughout the remainder of this report.

Flow cytometry analysis of PECs 1 h post *S. aureus* infection, revealed that the γδ T cell population consisted primarily of $V\gamma1.1^+$ T cells and $V\gamma2^+$ T cells (Figure 4.8 A). By 3 h post infection γδ cells were composed of approximately equal frequencies of $V\gamma1.1^+$, $V\gamma2^+$ and $V\gamma4^+$ cells. At 72 h post infection, the frequency of $V\gamma4^+$ cells was significantly increased while the frequency of $V\gamma2^+$ cells had significantly decreased in the peritoneal cavity (Figure 4.8 A). The frequency of $V\gamma1.1^+$ T cells as a proportion of total γδ T cells did not change over the course of acute infection; however there was a significant increase in the absolute numbers of $V\gamma1.1^+$ and $V\gamma4^+$ T cells at 72 h post infection (Figure 4.7 B). These data demonstrate that during the course of *S. aureus* infection in the peritoneal cavity there is a shift in the dominant γδ subsets present from $V\gamma1.1^+$ and $V\gamma2^+$ early in infection to $V\gamma4^+$ in the later phases. Expansion of $V\gamma1.1^+$ and $V\gamma4^+$ cell frequencies was also observed in the MLN at 72 h post infection (Figure 4.8 C). Analysis of absolute

numbers revealed a similar trend with increased numbers of $V\gamma1.1^+$ and $V\gamma4^+$ cells observed 72 h post infection (Figure 4.8 D).

Vγ subset proportions were analysed 168 h or 7 d post infection to profile the γδ T cells subset composition present upon re-exposure to *S. aureus* in the prior exposure model. Resident peritoneal γδ T cells comprised primarily of $V\gamma4^+$ cells (~75%) by 168 h post infection, compared to naive mice (~30%) (Figure 4.8 E). $V\gamma4^+$ T cells also comprised the majority of MLN γδ T cells (~53%) compared to naive mice (~33%), although they were not as dominant as was observed in the PEC (Figure 4.8 E). These results suggest that during the course of infection $V\gamma4^+$ T cells have been selectively expanded at both the infection site and in the draining MLN. This profile is maintained until the resolution of *S. aureus* infection from the peritoneal cavity by 168 h post injection. It is therefore likely, that these $V\gamma4^+$ cells are responding to staphylococcal antigens upon re-exposure to *S. aureus*.

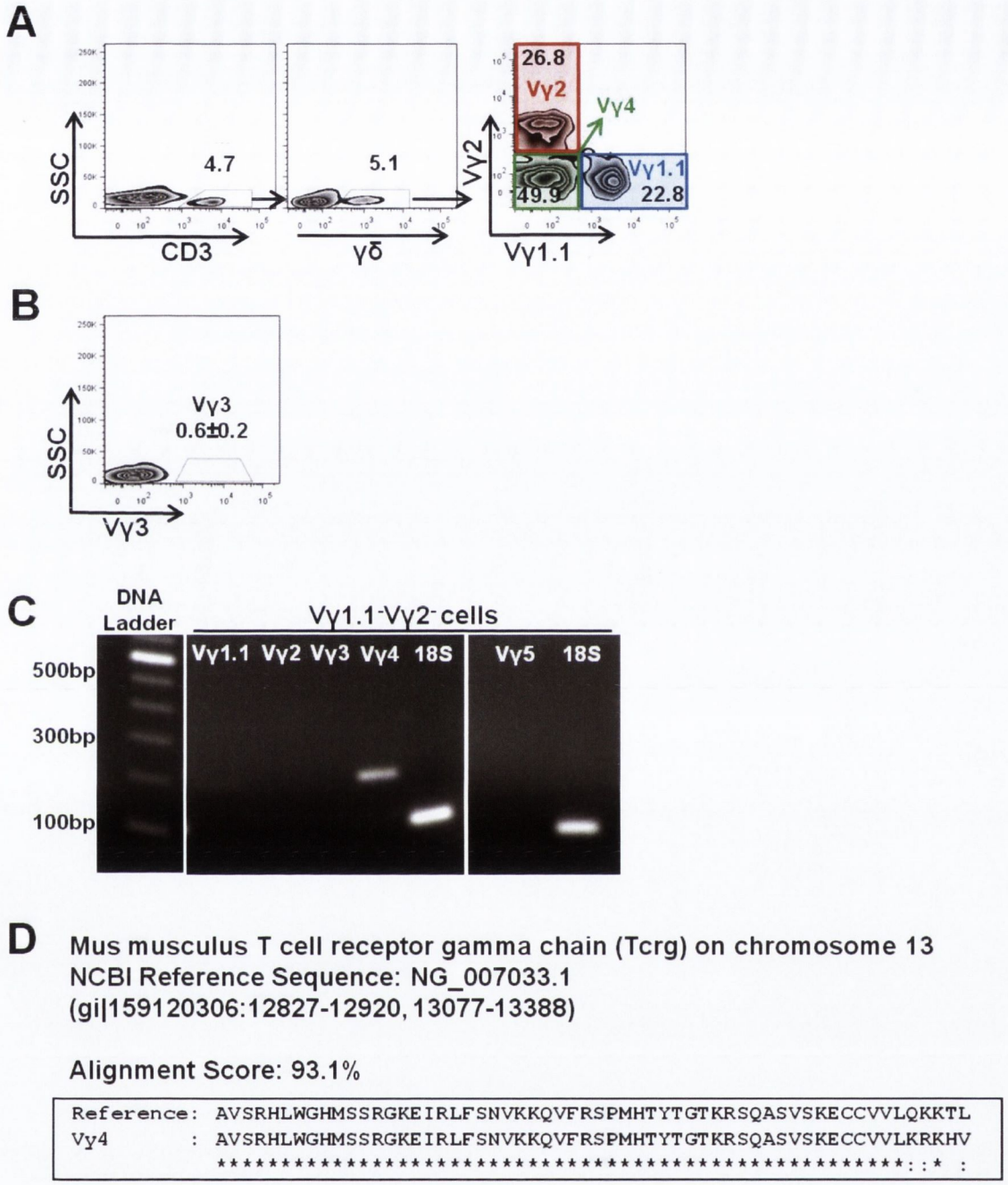


Figure 4.7: Identification of Vγ1.1⁻Vγ2⁻ cells recruited during *S. aureus* infection as Vγ4 T cells

Mice were infected with *S. aureus* ($5x10^8$ CFU) via i.p. injection. PECs (A & B) were harvested from mice at 3 h post infection, surface stained with fluorochrome-conjugated antibodies against CD3, γδTCR, Vγ1.1, Vγ2 and Vγ3 and analysed by flow cytometry (A & B). In addition, PECs and MLN cells were harvested and pooled from mice at 3 h post infection and Vγ1.1⁻Vγ2⁻ cells purified by cell sorting. RNA was extracted from the purified Vγ1.1⁻Vγ2⁻ cells, reverse transcribed into cDNA and each Vγ gene amplified by PCR. Amplimers were then run on a 1% agarose gel and imaged under UV light (C). Amplified DNA was excised from the gel, translated and aligned with the Vγ4 sequence (NCBI reference gene (NG_007033.1)) (D).

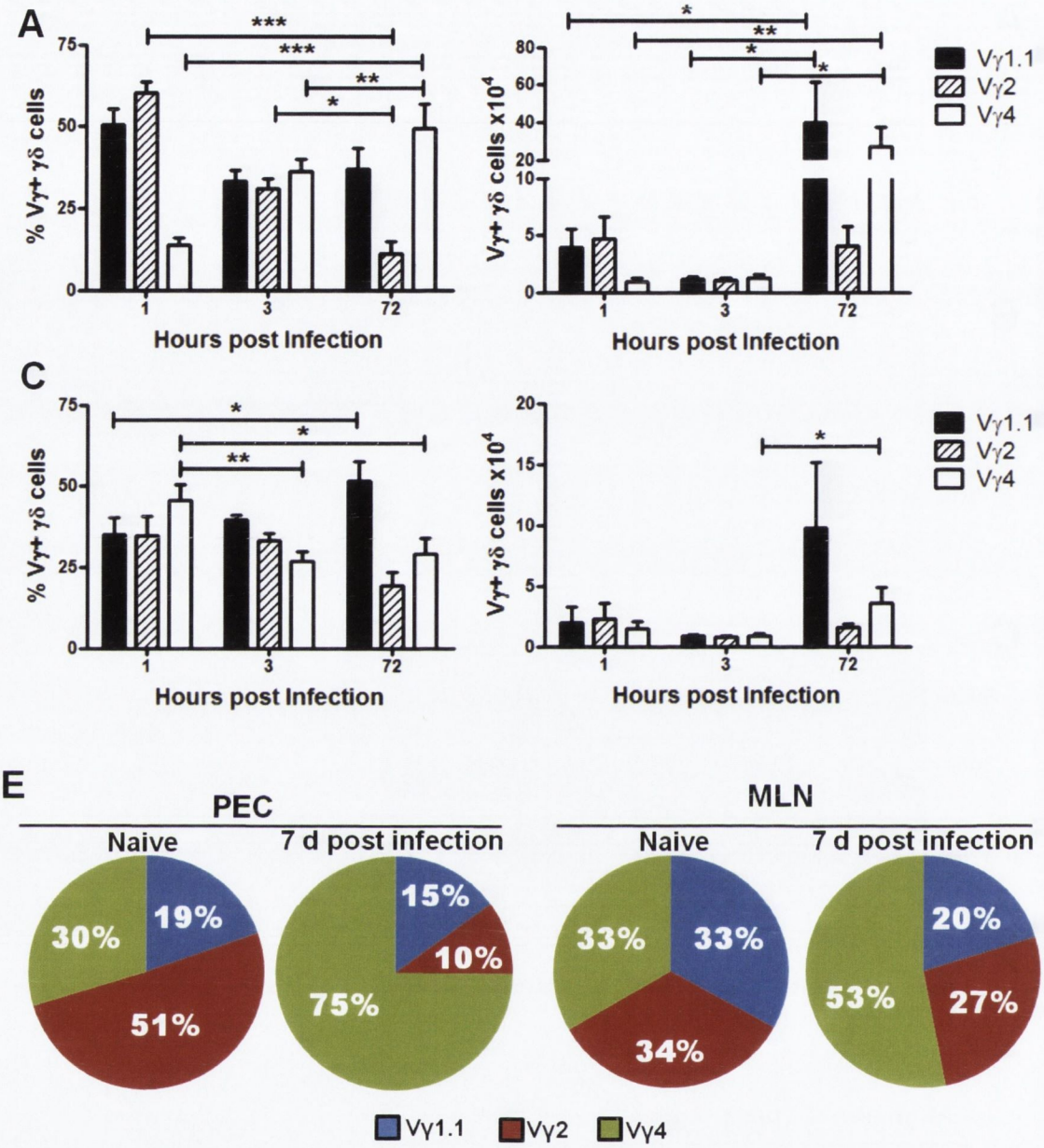


Figure 4.8: Differential recruitment of γδ T cell subsets during the course of *S. aureus* peritoneal infection

Mice were infected with *S. aureus* ($5x10^8$ CFU) via i.p. injection. At indicated time points post infection, the peritoneal proportions (A) and absolute numbers (B), and the MLN proportions (C) and absolute numbers (D) of $V\gamma1.1^+$, $V\gamma2^+$ and $V\gamma4^+$ cells was assessed by flow cytometry. Results expressed as mean±SEM of each subset within the $\gamma\delta^+$ gate, n=5-12 per group. Data pooled from 2-3 independent experiments. In addition,168 h post infection, PEC and MLN cells were isolated to assess the proportions of $V\gamma1.1^+$, $V\gamma2^+$ and $V\gamma4^+$ cells (E). Results expressed as pie charts of average percentage of $V\gamma^+$ cells. n=5 per group. *p<0.05, **p<0.005, ***p<0.001.

4.2.4. Prior exposure to S. aureus preferentially expands a population of Vγ4$^+$ T cells with an activated memory phenotype capable of enhanced IL-17 production during subsequent infection

During the course of *S. aureus* infection there is a shift in the composition of γδ T cells from primarily Vγ1.1$^+$ and Vγ2$^+$ cells in the acute phase of infection to Vγ4$^+$ cells upon resolution of infection (Figure 4.8 E). These Vγ4$^+$ cells may represent a primed γδ subset capable of responding to subsequent *S. aureus* infection. To test this hypothesis, groups of mice were repeatedly exposed to *S. aureus* (5x10^8 CFU i.p. on d 0, 7 and 14) and allowed to clear the infection (21 d post the final exposure, Section 3.2.4). At this stage (d 35) Vγ4$^+$ T cells were the prominent subset in PEC of prior exposed, but currently uninfected mice (Figure 4.9 A). This was consistent with previous results comparing the composition of γδ T cells between naive mice and mice 7 d post infection, where expansion of Vγ4 was maintained until bacterial clearance (Figure 4.8 E). Using the prior exposure model, this shift in subset composition to a Vγ4 dominant profile was maintained for up to 3 weeks post final exposure. Prior exposure to *S. aureus* did not significantly affect the frequency of peritoneal Vγ1.1$^+$ cells but did result in a significant decrease in the proportions of Vγ2$^+$ cells. Interestingly, a predominance of Vγ4$^+$ T cells was also observed in the MLN at this stage (Figure 4.9 B). Crucially, these γδ T cells from prior exposed mice did not produce IL-17 (Figure 4.9 C) and consistent with this secreted levels of IL-17 were undetectable in the peritoneal lavage fluid (data not shown).

On d 35 (21 d post the final exposure) mice were then re-challenged by i.p. injection of *S. aureus* (5x10^8 CFU), as were a group of naive mice as a control. At specific time points post-challenge, local IL-17 secretion was assessed and PECs and MLN cells isolated to characterise the subsets of γδ T cells responsible for IL-17 production. IL-17 secretion was significantly increased in the peritoneal cavity of prior exposed mice compared to non-exposed mice 1 and 3 h post challenge (Figure 4.10 A). Elevated IL-17 production was associated with a significant increase in the both the frequency (Figure 4.10 B) and absolute number (Figure 4.10 C) of IL-17 producing γδ T cells in the peritoneal cavities of prior exposed mice, compared with non-exposed mice as early as 1 h post challenge. Notably, prior exposure to *S. aureus* did not induce the expansion of CD4$^+$IL-17$^+$ (Th17) or CD8$^+$IL-17$^+$ cells in either the peritoneum or the MLN (previously shown in Section 3.2.9). To confirm that this effect was not strain-specific mice underwent prior exposure with an alternative *S. aureus* strain, SH1000 (331, 332). Prior exposure using a different *S. aureus* strain also drove increased IL-17 production by γδ T cells upon challenge with

SH1000 (11.1±1.1 vs. 31.7±4.7 IL-17$^+\gamma\delta^+$ cells in prior exposed vs. non-exposed mice respectively).

Analysis of the individual γδ T cell subsets revealed an increase in IL-17 expression by all subsets present in the peritoneal cavity at both 1 h (Figure 4.11 A) and 3 h (Figure 4.11 B) post infection in prior exposed versus non-exposed mice. In particular IL-17 production by the V$\gamma4^+$ population was significantly enhanced, with a similar albeit lesser trend evident in the V$\gamma1.1^+$ subset in prior exposed mice.

Significantly increased frequencies (Figure 4.12 B) and absolute numbers (Figure 4.12 C) of IL-17$^+\gamma\delta^+$ T cells were observed in the MLN of prior exposed mice both 1 and 3 h post infection. Analysis of the individual γδ T cell subsets revealed a marked increase in IL-17 expression by V$\gamma2^+$ and V$\gamma4^+$ subsets present in the MLN at 1 h post infection in prior exposed versus non-exposed mice (Figure 4.13 A). By 3 h post infection, IL-17 production by all subsets was significantly increased in prior exposed mice compared to non-exposed mice (Figure 4.13 B). In particular, IL-17 production by the V$\gamma4^+$ population was dramatically enhanced in the MLN of prior exposed mice.

Surface expression of CD44 and CD27 by γδ T cells was investigated to identify whether prior exposed γδ T cells exhibited an effector memory cell phenotype akin to the memory γδ T cells identified by Sheridan *et al* (173). A significant increase in CD44 expressing IL-17$^+\gamma\delta^+$ T cells was detected in both the peritoneum (Figure 4.14 A & B) and MLN (Figure 4.14 C & D) of prior exposed mice compared to non-exposed mice 3 h post infection. In addition, IL-17 producing γδ T cells in the peritoneum and MLN of both non-exposed and prior exposed mice were CD27$^-$ (Figure 4.14 E).

Taken together, these results suggest that exposure to *S. aureus* induces a population of "primed" γδ T cells, predominantly of the Vγ4 subset, which exhibit an activated memory phenotype (CD27$^-$CD44^{hi}), and are capable of elevated IL-17 production upon subsequent infection with the organism which possibly contributes to the enhanced bacterial clearance observed in this model (as shown in Section 3.2.7).

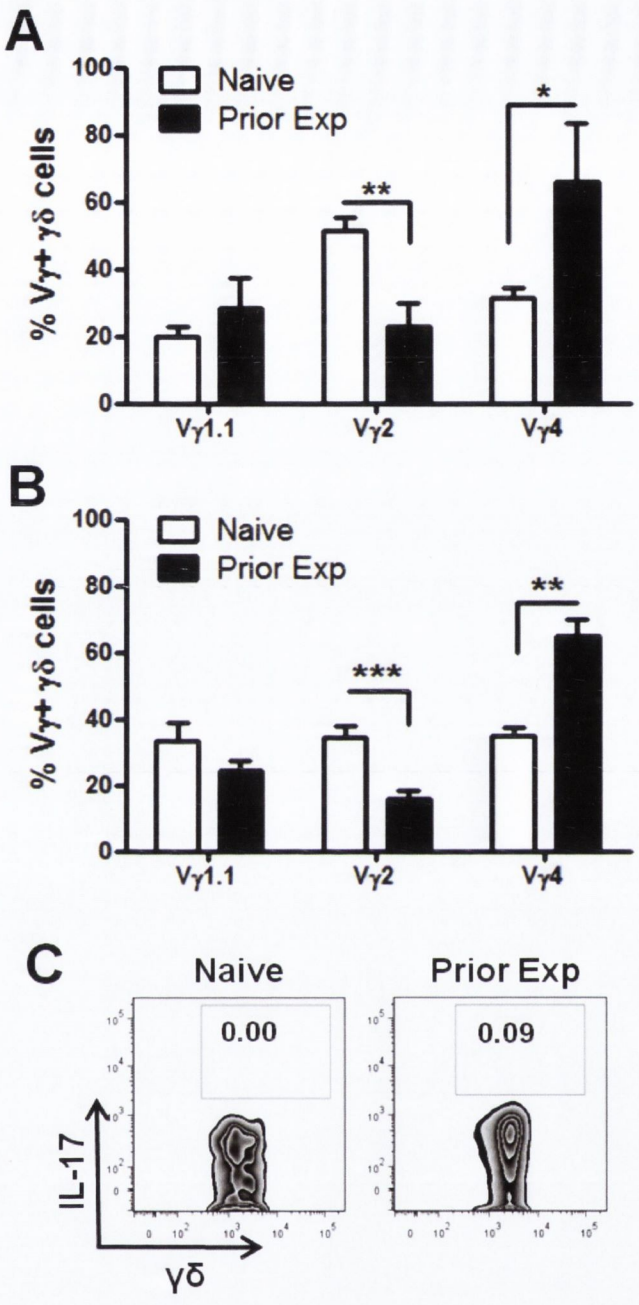


Figure 4.9: Elevated frequency of Vγ4$^+$ T cells in prior exposed mice

Groups of mice were exposed to *S. aureus* (5x10^8 CFU) via i.p. injections on d 0, 7 and 14. Following recovery on d 35 (when mice were infection free), PECs and MLN cells were isolated, cultured with Brefeldin A, and stained for surface CD3, γδTCR, Vγ1.1, Vγ2 and Vγ3, and intracellular IL-17, and analysed by flow cytometry. The Vγ subsets comprising the γδ T cell population in the peritoneal cavity (A) and the MLN (B) were assessed and compared to naive cells. IL-17-expression by peritoneal γδ T cells from naive or prior exposed mice was compared (C). Results expressed as mean±SEM (A, B), with representative FACS plot shown (C). n=8 per group. Data pooled from 2 independent experiments. *p<0.05, **p<0.005, ***p<0.001.

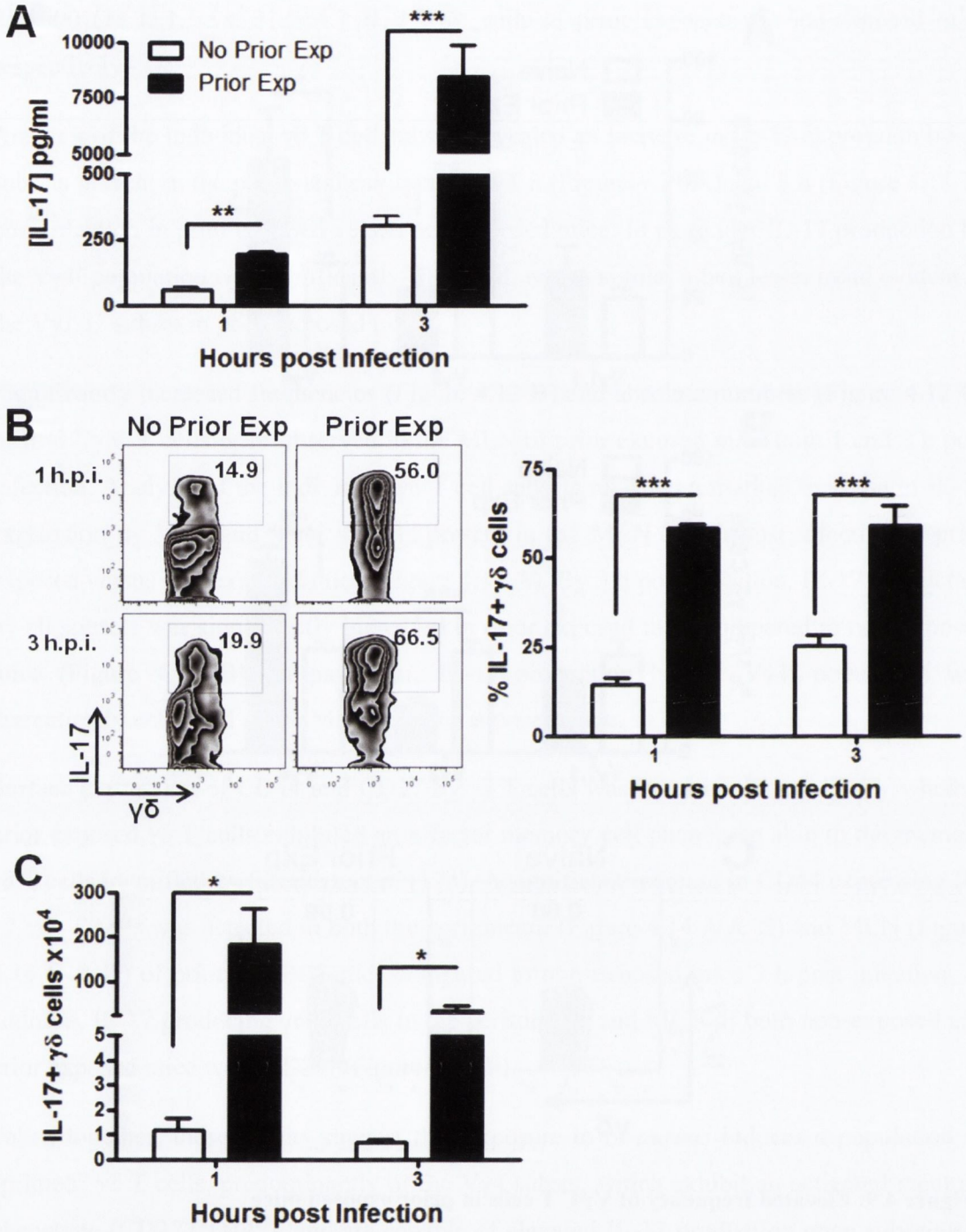


Figure 4.10: Prior exposure to *S. aureus* enhances IL-17 production by γδ T cells as early as 1 h post infection

Groups of mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injections on d 0, 7 and 14. Mice were then re-challenged with *S. aureus* ($5x10^8$ CFU) on d 35, as were a group of naive controls. At 1 and 3 h post challenge, secreted IL-17 in the peritoneal fluid was measured by ELISA (A). At 1 and 3 h post challenge, PECs were isolated and cultured with Brefeldin A to analyse proportions (B) and absolute numbers (C) of IL-17 producing γδ T cells by flow cytometry. Results expressed as mean±SEM, with representative FACS plot shown (B). n=12 per group. Data pooled 3 independent experiments. *$p<0.05$, **$p<0.005$, ***$p<0.001$.

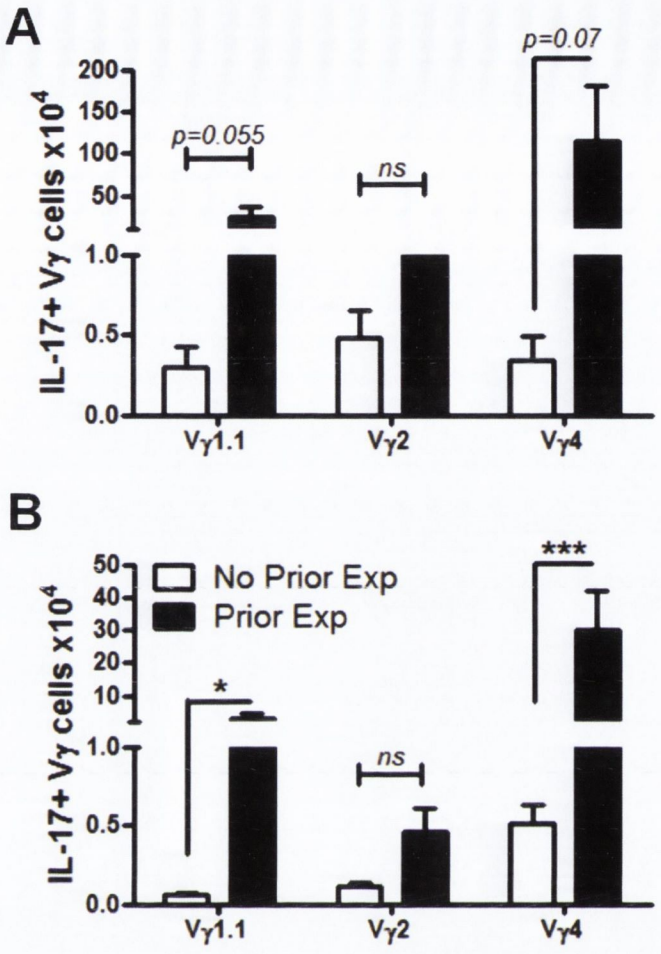


Figure 4.11: Prior exposure to *S. aureus* enhances IL-17 production by peritoneal Vγ4 T cells

Groups of mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injections on d 0, 7 and 14. Mice were then re-challenged with *S. aureus* ($5x10^8$ CFU) on d 35, as were a group of naive controls. At 1 h (A) and 3 h (B) post challenge, isolated PECs were cultured with Brefeldin A, then stained for surface CD3, γδTCR, Vγ1.1 and Vγ2 and intracellular IL-17, and analysed by flow cytometry. Results expressed as mean±SEM of n=12 per group. Data pooled from 3 independent experiments. *p<0.05, **p<0.005, ***p<0.001.

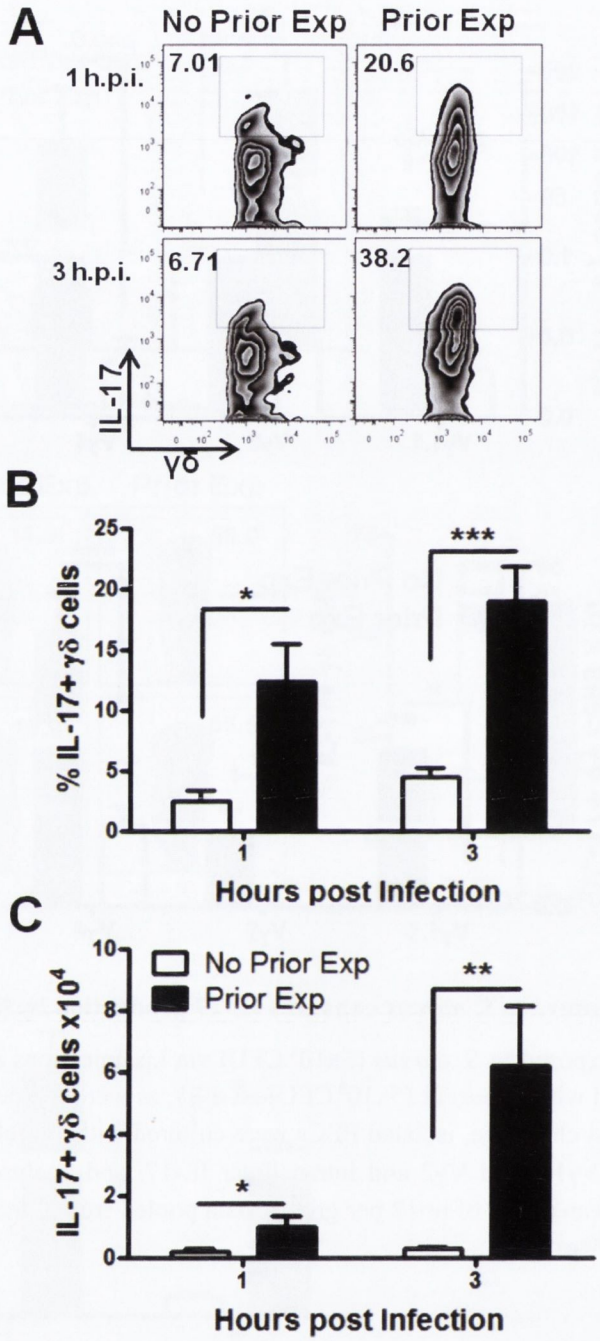


Figure 4.12: Prior exposure to *S. aureus* enhances IL-17 production by MLN γδ T cells as early as 1 h post infection

Groups of mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injections on d 0, 7 and 14. Mice were then re-challenged with *S. aureus* ($5x10^8$ CFU) on d 35, as were a group of naive controls. At 1 and 3 h post challenge, MLN cells were isolated and cultured with Brefeldin A to analyse proportions (B) and absolute numbers (C) of IL-17 producing γδ T cells by flow cytometry. Results expressed as mean±SEM (B, C) with representative FACS plots shown (A). n=12 per group. Data pooled from 3 independent experiments.*$p<0.05$, **$p<0.005$, ***$p<0.001$.

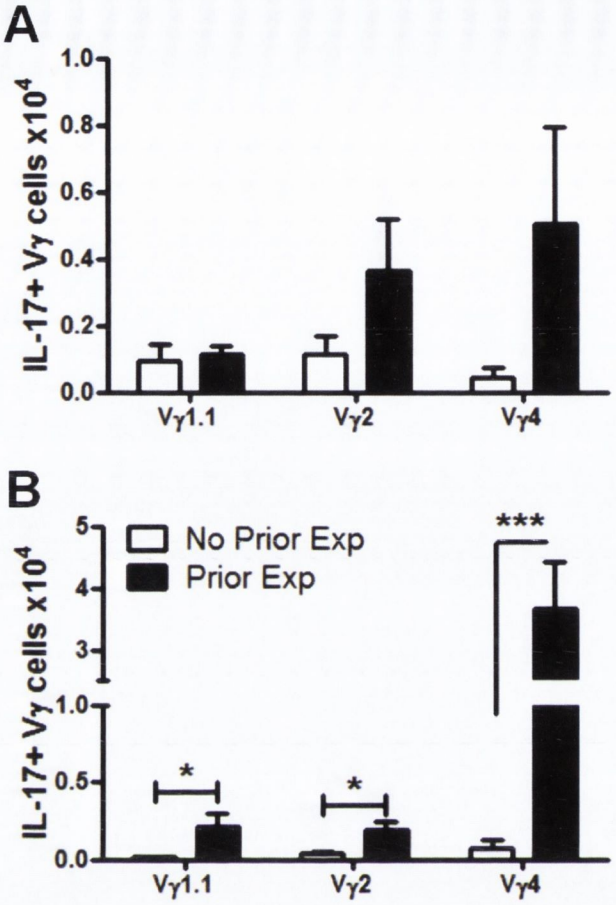


Figure 4.13: Prior exposure to *S. aureus* enhances IL-17 production by MLN Vγ4 T cells

Groups of mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injections on d 0, 7 and 14. Mice were then re-challenged with *S. aureus* ($5x10^8$ CFU) on d 35, as were a group of naive controls. At 1 h (A) and 3 h (B) post challenge, isolated MLN cells were cultured with Brefeldin A, then stained for surface CD3, γδTCR, Vγ1.1 and Vγ2 and intracellular IL-17, and analysed by flow cytometry. Results expressed as mean±SEM of n=12 per group. Data pooled from 3 independent experiments. *$p<0.05$, ***$p<0.001$.

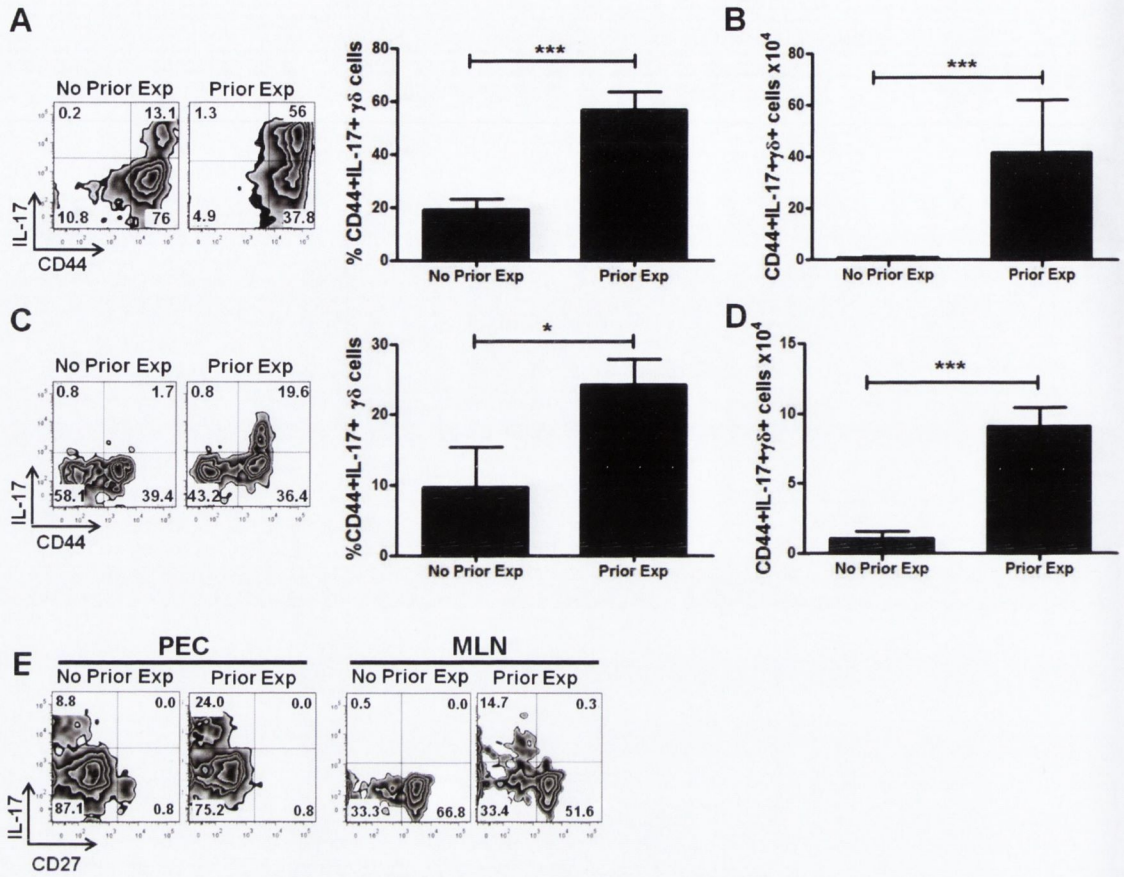


Figure 4.14: Prior exposure to *S. aureus* enhances proportions of CD27$^-$CD44^{hi} γδ T cells during subsequent infection

Groups of mice were exposed to *S. aureus* (5x10^8 CFU) via i.p. injections on d 0, 7 and 14. Mice were then re-challenged with *S. aureus* (5x10^8 CFU) on d 35, as were a group of naive controls. At 3 h post challenge, the proportions (A &C) and absolute numbers (B & D) of CD44$^+$IL-17$^+$ γδ T cells present in peritoneal cavity (A & B) and MLN (C & D) was analysed by flow cytometry. CD27 expression by IL-17$^+$ γδ T cells was also assessed (E). Results expressed as mean±SEM with representative FACS plots shown (A, C). A representative FACS plot only is shown for (E). n=8 per group. Data pooled from 2 independent experiments.*p<0.05, **p<0.005, ***p<0.001.

4.2.5. Enhanced IL-17 production by γδ T cells during re-exposure to S. aureus is not a result of increased innate cytokine signalling

During *S. aureus* infection in naive mice, IL-17 production by peritoneal γδ T cells was critically dependent upon IL-1RI signalling (Figure 4.3). Hence, the effects of prior exposure to *S. aureus* on the IL-1 and IL-23 pathway were investigated. Despite observing significantly enhanced IL-17 secretion in prior exposed mice compared to non-exposed mice upon infection (Figure 4.10 A), no difference in IL-1β (Figure 4.15 A) or IL-23 (Figure 4.15 B) secretion was detected between the two groups. IL-1α and IL-18, which can also stimulate IL-17 production by γδ T cells (55), were undetectable in the peritoneal cavities of either non-exposed or prior exposed mice during *S. aureus* infection (data not shown).

Microbial colonisation has been shown to increase the expression of IL-1R on the surface of IL-17-producing γδ T cells, leading to increased IL-17 production in response to IL-1β signalling (373). However, no difference was detected in the mean fluorescence intensity (MFI) of IL-1R expression on peritoneal IL-17^{+}γδ^{+} T cells isolated from prior exposed and non-exposed mice at 1 and 3 h post challenge (Figure 4.15 C). A similar pattern was observed in the MLN (Figure 4.15 D). These findings suggest an alternative, non IL-1-dependant mechanism for the enhanced IL-17 expression by *S. aureus*-primed γδ T cells.

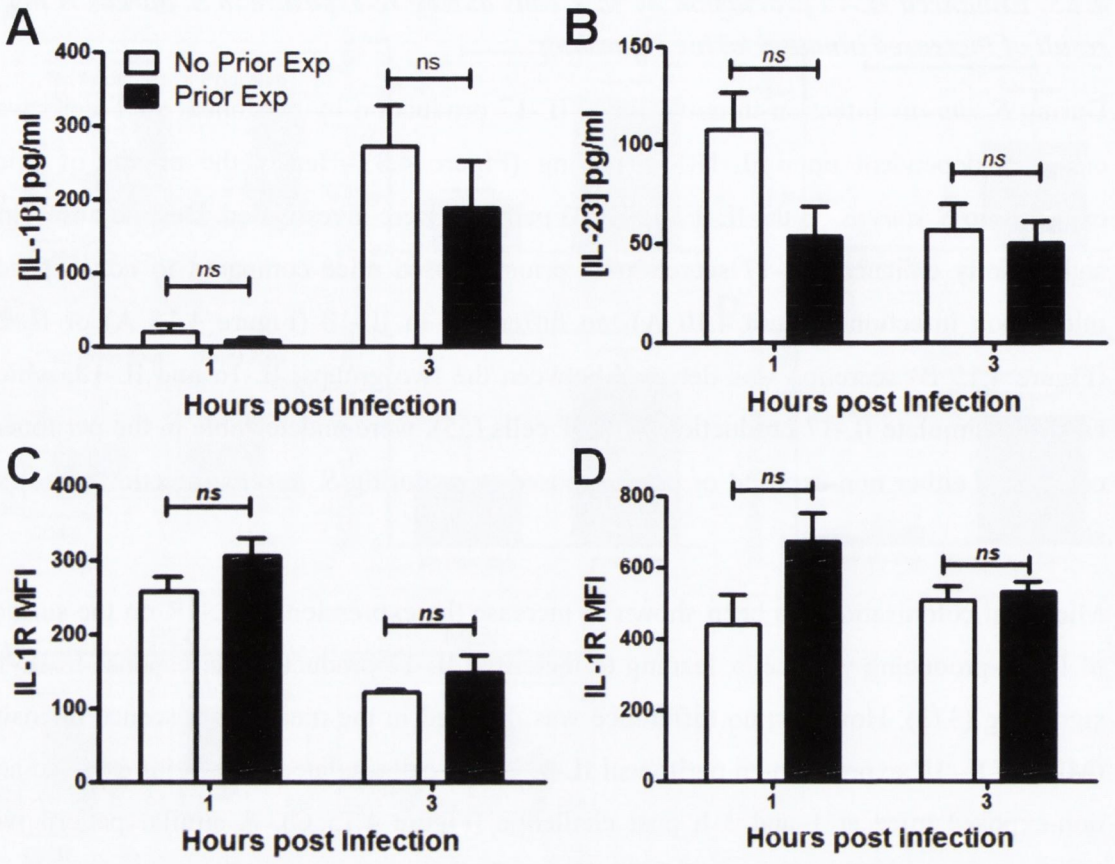


Figure 4.15: Activation of the IL-1 or IL-23 pathways in the peritoneal cavity are unaffected by prior exposure to *S. aureus*

Groups of mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injections on d 0, 7 and 14. Mice were allowed to recover for 21 d before being re-challenged with *S. aureus* ($5x10^8$ CFU) on d 35, as were a control group of naïve mice. At 1 h and 3 h post challenge IL-1β (A) and IL-23 (B) levels were measured in the peritoneal fluid by ELISA. PEC (C) and MLN cells (D) were isolated, cultured with Brefeldin A, to assess IL-1R expression by MFI on the surface of IL-$17^+\gamma\delta^+$ T cells. Results expressed as mean±SEM. Data pooled from 2 independent experiments. n=9 per group.

4.2.6. Inhibition of IL-1RI or IL-23R signalling does not significantly impair IL-17 production by S. aureus-primed γδ T cells

To further investigate the mechanisms responsible for enhanced IL-17 production by γδ T cells following prior exposure to *S. aureus* an *in vitro* co-culture system was set up in which γδ T cells isolated from naive or prior exposed mice that were infection free (d 35; 21 d post final exposure to *S. aureus*) were cultured with peritoneal macrophages infected *in vitro* with *S. aureus* (MOI 1:10). Following 18 h of culture, IL-17 expression by γδ T cells was assessed by flow cytometry and IL-17 secretion was measured by ELISA.

Secreted IL-17 was significantly increased in the supernatants of *S. aureus*-primed γδ T cells compared to naive γδ T cells upon *in vitro* co-culture with *S. aureus* infected macrophages (Figure 4.16 A). Flow cytometry analysis confirmed that γδ T cells were responsible for the enhanced IL-17 production (Figure 4.16 B). *S. aureus*-primed γδ T cells appeared to be exclusively IL-17 producing as no IFNγ production was detected in the culture supernatants (data not shown). Moreover, macrophages stimulated with *S. aureus* alone, in the absence of γδ T cells, did not produce IL-17 (Figure 4.18 A).

To determine the role played by innate cytokines in the memory response of *S. aureus*-primed γδ T cells, blocking antibodies to both the IL-1RI and the IL-23R were added to the co-culture system. Blockade of the IL-1R dose dependently inhibited IL-17 production by γδ T cells from naive mice and at the higher concentration of antibody (4μg/ml) resulted in the complete abrogation of IL-17 secretion (Figure 4.16 A). This was consistent with our *in vivo* observations that demonstrated significantly reduced IL-17 production by IL-1RI deficient γδ T cells (Figure 4.3). Conversely, blockade of the IL-1R, using either concentration of antibody, only partially reduced IL-17 secretion by prior exposed γδ T cell, and this reduction was not significant (Figure 4.16 A). Again flow cytometry analysis confirmed that IL-1R blockade substantially reduced IL-17 production by naive γδ T cells, but not by prior exposed γδ T cells (Figure 4.16 B). Blockade of the IL-23R had no effect on IL-17 production by either naive or prior exposed γδ T cells (Figure 4.16 A).

These results complement the *in vivo* data, demonstrating that *in vitro,* there is a differential requirement for innate IL-1 signalling in the IL-17-mediated response to *S. aureus* infection by primed γδ T cells and those from naive mice. This suggests that *S. aureus*-primed γδ T cells produce increased IL-17 upon recognition of staphylococcal antigens, indicative of an adaptive immune response.

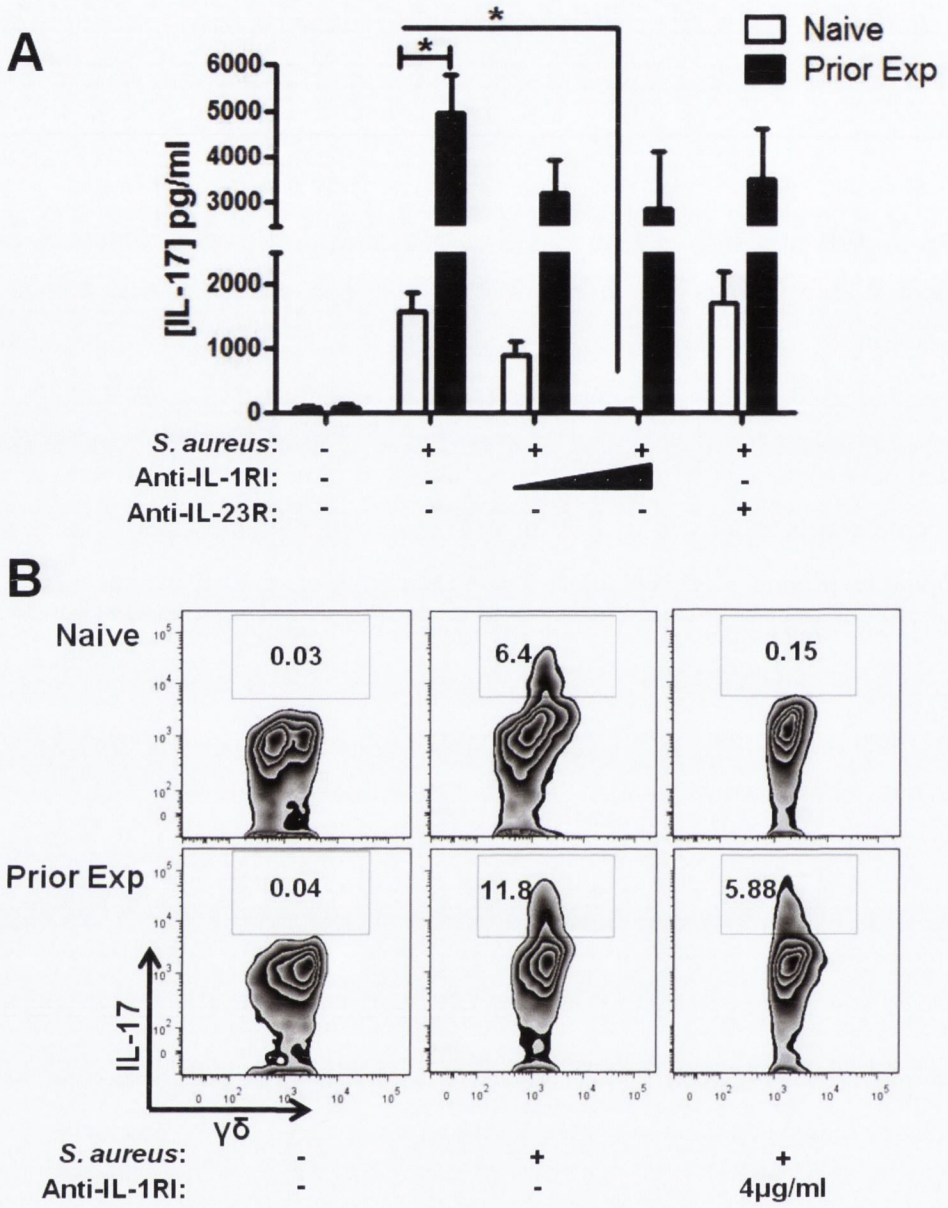


Figure 4.16: IL-1R or IL-23R signalling is dispensable for the enhanced IL-17 response of primed γδ T cells *in vitro*

γδ T cells purified (>98% pure) from prior exposed or naive mice were co-cultured overnight with *S. aureus* infected macrophages (MOI 1:10), in the presence or absence of monoclonal antibodies to IL-1RI (1μg/ml or 4μg/ml) or IL-23R (1μg/ml). Uninfected macrophages were used as a control. IL-17 concentration in supernatants was quantified by ELISA (A). Results expressed as mean±SEM for n=4-5 replicates per group. *$p<0.05$. After 18 h in culture, cells were incubated with Brefeldin A, stained for surface CD3 and γδTCR, and intracellular IL-17, for analysis by flow cytometry (B). Results expressed as representative FACS plots of 4 independent experiments.

4.2.7. Inhibition of MHC II signalling impairs IL-17 production by S. aureus-primed γδ T cells

To further investigate the mechanisms responsible for enhanced IL-17 production by γδ T cells following prior exposure to *S. aureus,* blocking antibodies to MHC II (5μg/ml or 50μg/ml) were added to our co-culture system. Following 18 h of culture, IL-17 expression by γδ T cells was analysed by flow cytometry and IL-17 secretion into the supernatants measured by ELISA.

Preliminary experiments revealed that blocking of MHC II with 5μg/ml anti-MHCII antibody reduced IL-17 production by *S. aureus*-primed γδ T cells, compared to γδ T cells from naive mice (Figure 4.17 A). Unfortunately, this trend was not replicated with a higher dose of anti-MHCII antibody (50μg/ml). As this result represents only a single replicate, these experiments will need to be repeated to assess whether it is conclusive or not. Analysis of IL-17 production by flow cytometry revealed that anti-MHC II blocking antibody (50μg/ml) substantially impaired IL-17 production by *S. aureus*-primed γδ T cells, with IL-17^{+}γδ^{+} T cell frequencies reduced from of 13.2% to 6.1% (Figure 4.17 B). IL-17 production by naive γδ T cell was unaffected by the addition of anti-MHC II blocking antibody at either concentration. This experiment represents a single replicate, however it may indicate that MHC II signalling plays an important role in activation of memory γδ T cells, similar to observations made by Sheridan *et al* (173).

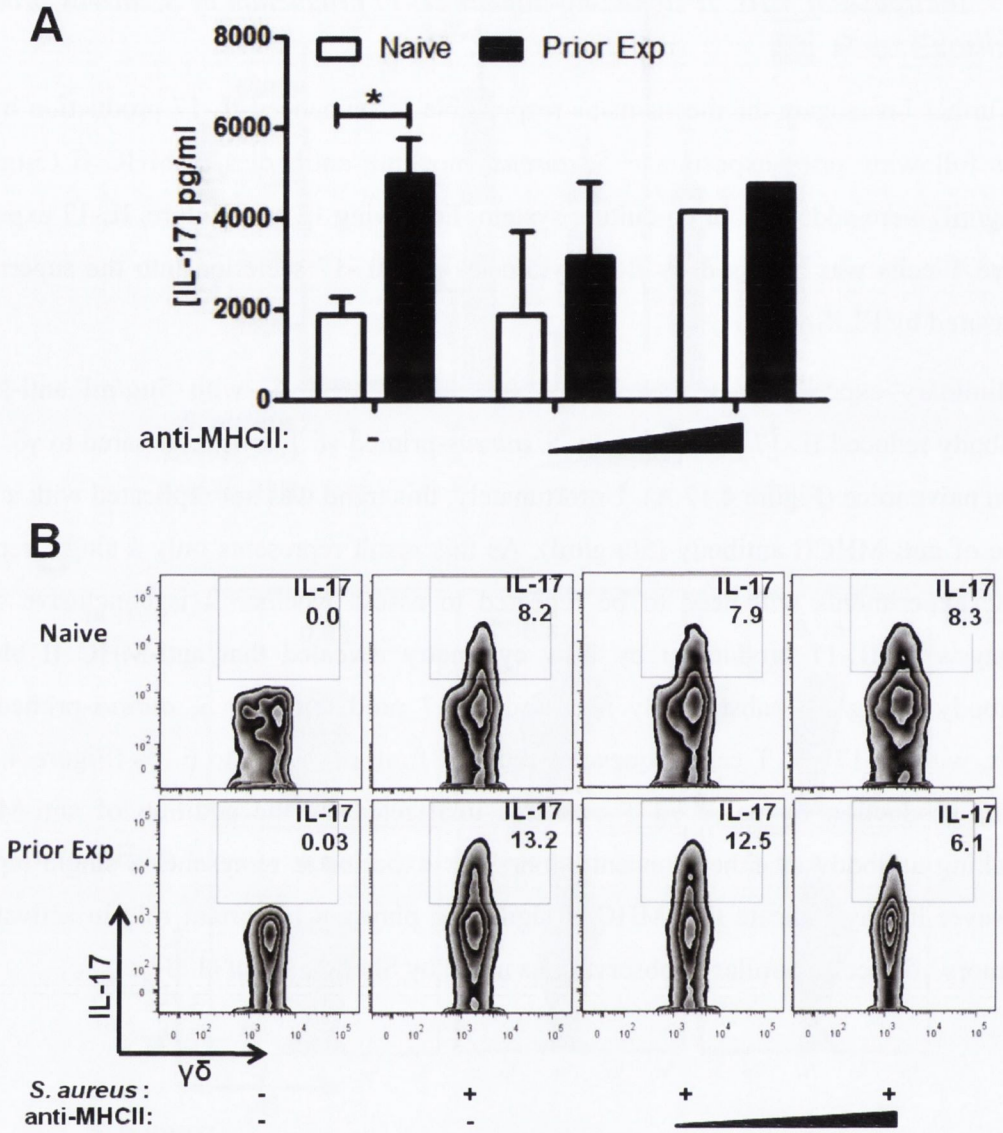


Figure 4.17: Co-culture of *S. aureus*-primed γδ T cells with *S. aureus* infected macrophages in the presence of anti-MHCII impairs IL-17 production

γδ T cells purified (>98% pure) from prior exposed or naive mice were co-cultured overnight with *S. aureus* infected macrophages (MOI 1:10), in the presence of blocking monoclonal anti-MHCII antibody (5μg/ml or 50μg/ml). Uninfected macrophages were used as a control. IL-17 concentration in supernatants was quantified by ELISA (A). Results expressed as mean±SEM. n=1-2 replicates per group. After 18 h in culture, cells were incubated with Brefeldin A, stained for surface CD3 and γδTCR, and intracellular IL-17 for analysis by flow cytometry (B). Results expressed as representative FACS plots of 1-2 replicates.

4.2.8. S. aureus-primed γδ T cells do not respond to E. coli infection with increased IL-17 production

To confirm the specificity of *S. aureus*-primed γδ T cells, γδ T cells isolated from naive or prior exposed mice that were infection free (21 d post final exposure) were cultured with peritoneal macrophages infected with *S. aureus* (MOI 1:10) or *E. coli* (MOI 1:10). Following 18 h of culture, IL-17 expression by γδ T cells was analysed by flow cytometry and IL-17 secretion into the supernatants measured by ELISA.

Interestingly, *E. coli* infected macrophages were capable of producing IL-17, unlike *S. aureus* infected macrophages (Figure 4.18 A). Although, *S. aureus*-primed γδ T cells produced more IL-17 when co-cultured *in vitro* with *E. coli* infected macrophages compared to γδ T cells from naive mice, this increase was non-significant (Figure 4.18 B). As macrophages alone were capable producing IL-17 upon infection with *E. coli*, FACS analysis was carried out to determine the contribution of γδ T cells to IL-17 secretion. Neither naive or *S. aureus*-primed γδ T cells were capable of producing IL-17 upon stimulation with *E. coli* infected macrophages, suggesting that IL-17 detected in the co-culture supernatants was macrophage derived (Figure 4.18 C). In conclusion, prior exposure to *S. aureus* enhances IL-17 production by γδ T cells upon *in vitro* re-exposure to *S. aureus*, but not *E. coli*, suggesting that *S. aureus*-primed γδ T cells respond specifically to staphylococcal antigens.

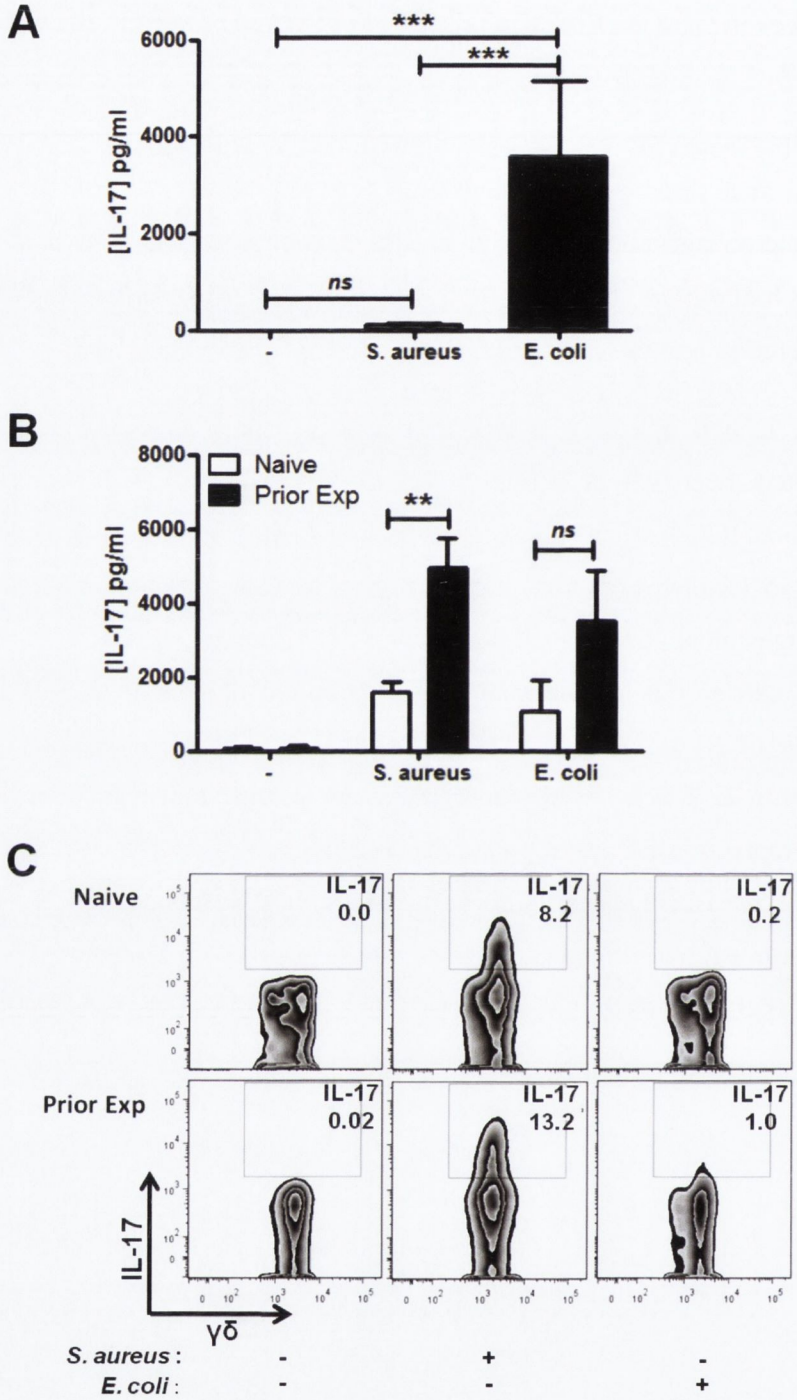


Figure 4.18: Co-culture of *S. aureus*-primed γδ T cells with *E. coli* infected macrophages does not result in IL-17 production

γδ T cells purified (>98% pure) from prior exposed or naive mice were co-cultured overnight with *S. aureus* or *E. coli* infected macrophages (MOI 1:10). IL-17 secretion was quantified by ELISA in the supernatants of infected macrophages alone (A) and of co-cultures with purified γδ T cells (B). Results expressed as mean±SEM. n=3 replicates per group. **p<0.05, ***p<0.001. After 18h in culture, cells were incubated with Brefeldin A, stained for surface CD3 and γδTCR, and intracellular IL-17 for analysis by flow cytometry (C). Results expressed as representative FACS plots of 2 replicates.

4.2.9. Adoptive transfer of S. aureus-primed γδ T cells enhances bacterial clearance during subsequent infection

Previous results demonstrate the accumulation of Vγ4 T cells in the peritoneal cavity as a result of *S. aureus* exposure. Upon re-exposure to the bacterium, these Vγ4 cells contribute substantially to the augmented IL-17 response observed in prior exposed mice (Figure 4.11 and 4.13), which was associated with increased bacterial clearance (Section 3.2.7). To establish if primed γδ T cells alone could enhance clearance of *S. aureus* infection, PECs isolated from prior exposed mice (on d 35; 21 d post final exposure) were purified by cell sorting and $1x10^5$ *S. aureus*-primed γδ T cells were then transferred to naive syngeneic hosts. $1x10^5$ γδ T cell-depleted $CD3^+$T cells (i.e. $CD3^+\gamma\delta^-$ cells) isolated from the same prior exposed mice were transferred into a separate group of naive mice as a control. At 3 h post cell transfer, mice were challenged via i.p. injection with *S. aureus* ($5x10^8$ CFU). At 72 h post infection, the peritoneal cavity was lavaged to assess the local bacterial burden. The kidneys and liver were also isolated to assess bacterial dissemination.

Prior to transfer >90% of purified *S. aureus*-primed γδ T cells were $V\gamma4^+$ (Figure 4.19 A). Transfer of *S. aureus*-primed γδ T cells significantly reduced bacterial burden in the peritoneal cavity (the site of infection), compared to mice that received primed $CD3^+$ T cells depleted of γδ T cells (Figure 4.19 B). Dissemination of bacteria to the kidneys (Figure 4.19 C) and liver (Figure 4.19 D) was also reduced in recipients of *S. aureus*-primed γδ T cells, compared to control mice. These results demonstrate an important protective role for *S. aureus*-primed γδ T cells, specifically $V\gamma4^+$ cells, which display enhanced IL-17 production upon subsequent exposure to the bacterium.

To identify a mechanism of enhanced bacterial clearance induced by transfer of *S. aureus*-primed γδ T cells, the influx of phagocytes into the peritoneal cavity was assessed by flow cytometry 72 h post infection. Macrophage frequencies were significantly enhanced in the peritoneal cavities of mice that received transfers of *S. aureus*-primed γδ T cells compared to $CD3^+\gamma\delta^-$ recipient mice (Figure 4.20 B). Additional analysis of absolute numbers also demonstrated elevated macrophage influx into the peritoneal cavities of mice that received transfers of *S. aureus*-primed γδ T cells (Figure 4.20 C). Conversely, neutrophil proportions were significantly decreased at 72 h post infection in $CD3^+\gamma\delta^+$ recipient mice, compared to mice that received transfers of $CD3^+\gamma\delta^-$ cells (Figure 4.20 D). Analysis of absolute numbers revealed a similar trend with decreased influx of neutrophils detected in γδ T cell recipient mice 72 h post infection (Figure 4.20 E).

In conclusion, these adoptive transfer experiments demonstrated that *S. aureus*-primed γδ T cells (which are primarily Vγ4) are capable of enhancing clearance of *S. aureus* infection possibly through their downstream effects on phagocytes.

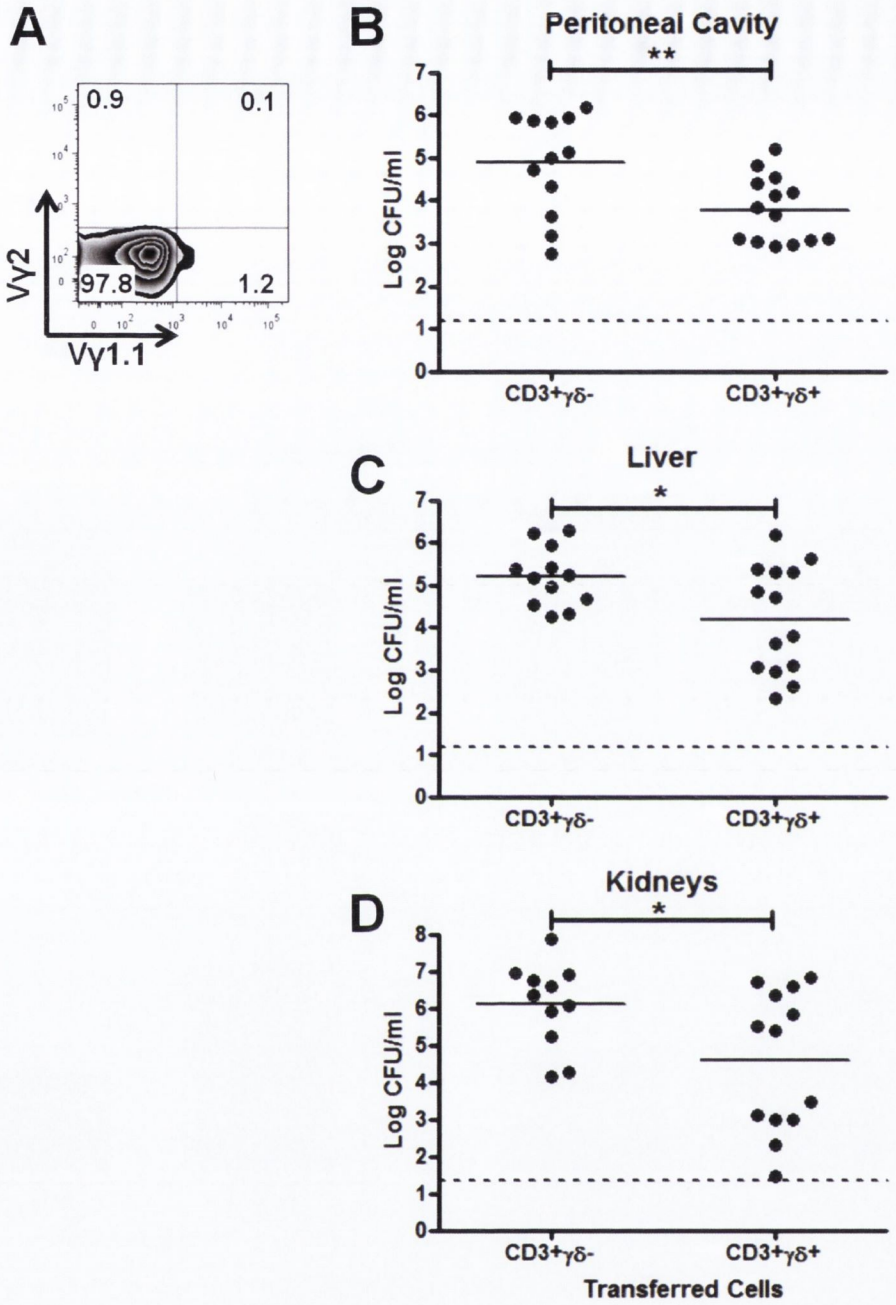


Figure 4.19: Adoptive transfer of *S. aureus*-primed γδ T cells to naive mice enhances bacterial clearance during subsequent infection

γδ T cells were purified (>98% pure) from the peritoneum of prior exposed mice on d 35 (21 d post final exposure), and $1x10^5$ cells transferred via i.p. injection to naïve syngeneic hosts. $1x10^5$ γδ T cell-depleted $CD3^+$ T cells (i.e. $CD3^+\gamma\delta^-$ cells) isolated from the same *S. aureus* exposed mice were transferred to a separate group of naïve mice as a control. Prior to transfer purified $CD3^+\gamma\delta^+$ cells were stained with CD3, γδTCR, Vγ1.1 and Vγ2 for flow cytometry analysis (A). Results expressed as representative FACS plots of n=3. At 3 h post transfer, mice were infected with *S. aureus* via i.p. injection ($5x10^8$ CFU). At 72 h post infection, bacterial burden was assessed in the peritoneal cavity (B), kidneys (C) and liver (D). Results expressed as log CFU/ml with mean indicated by bar. Dashed lines indicate limit of detection. n=12-14 per group. Data pooled from 3 independent experiments. *$p<0.05$, **$p<0.005$.

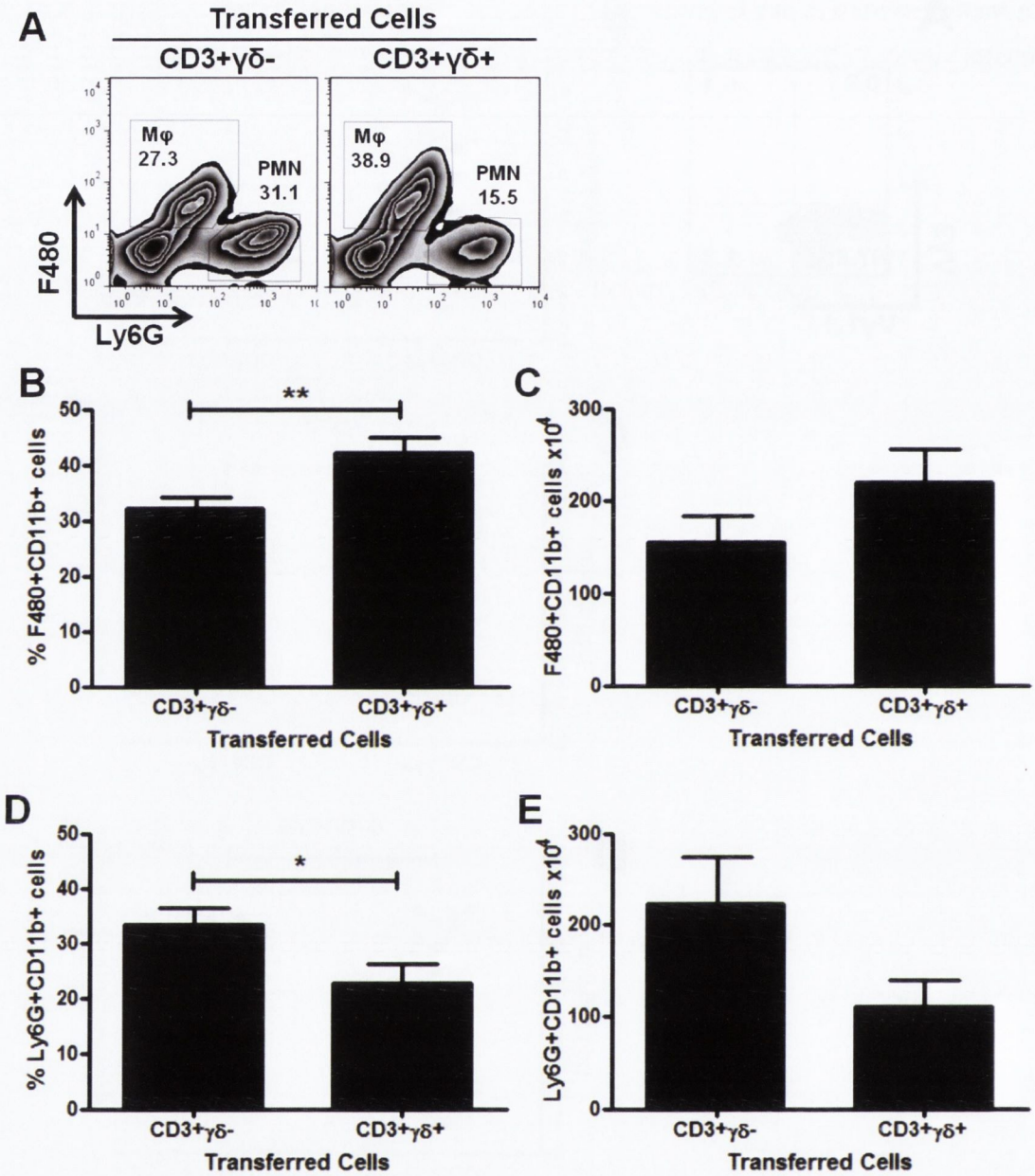


Figure 4.20: Adoptive transfer of *S. aureus*-primed γδ T cells enhances macrophage recruitment during subsequent infection

γδ T cells were purified (>98% pure) from the peritoneum of prior exposed mice on d 35 (21 d post final exposure), and $1x10^5$ cells transferred via i.p. injection to naïve syngeneic hosts. $1x10^5$ γδ T cell-depleted $CD3^+$ T cells (i.e. $CD3^+\gamma\delta^-$ cells) isolated from the same prior exposed mice were transferred to a separate group of naïve mice as a control. At 3 h post transfer, mice were infected with *S. aureus* via i.p. injection ($5x10^8$ CFU). At 72 h post infection, macrophage ($F480^+Ly6G^-$) proportions (B) and absolute numbers (C), and neutrophil ($Ly6G^+F480^-$) proportions (D) and absolute numbers (E) in the peritoneal cavity was assessed by flow cytometry. Results expressed as mean±SEM (B-E), with representative FACS plots of peritoneal $CD11b^+$ cells shown, demonstrating gating strategy (A). n=12-14 per group. Data pooled from 3 independent experiments. *$p<0.05$, **$p<0.005$.

4.3. Discussion

γδ T cells occupy a unique niche in the immune system due to their pleiotropic effector functions, their capacity to recognise distinct phosphoantigens and their preferential localisation at mucosal sites, all of which support a prominent role for γδ T cells in anti-microbial immunity. The present study demonstrates for the first time that exposure to *S. aureus* can prime a subset of IL-17 producing γδ T cells capable of protecting against subsequent staphylococcal infection. Although memory responses by γδ T cells in humans had already been identified (181, 192, 204) , the murine equivalent was lacking. The recent identification of memory γδ T cells in murine models enables further investigation into the potential of targeting these cells in novel vaccine strategies.

There is accumulating evidence for targeting these non-traditional lymphocytes in novel vaccinations strategies. In a rare variant of SCID that results in decreased αβ T cells and increased numbers of γδ T cells, patients can respond effectively to vaccinations against diphtheria, highlighting the capacity of γδ T cell antigen recall responses even in the absence of conventional αβ T cell help (374). Vaccines targeting γδ T cells have shown efficacy in West Nile virus infection, where administration of α-glucans known to promote γδ T cell expansion resulted in attenuated viremia and mortality following lethal infection. Protection was associated with induction of virus specific IgG and IgM and the selective expansion of Vγ1.1 cells (375). γδ T cells are also currently being targeted in novel anti-cancer vaccines (376, 377). Kobayashi *et al* demonstrated that activation and expansion of reactive Vγ9Vδ2 cells *ex vivo* followed by adoptive transfer into a patient with metastatic renal cell carcinoma who then received doses of IL-2 and zoledronate (a drug which enhances accumulation of intracellular IPP) resulted in complete remission of the tumour. This effect was associated with a sharp increase in IFNγ production by the patient's Vγ9Vδ2 cells (378).

Differential expression of Vγ and/or Vδ chains are used to categorise murine γδ T cell subsets, which often display tissue-specific homing and distinct cytokine profiles (139). Employing a model of *S. aureus* induced peritonitis, rapid recruitment of IL-17 producing γδ T cells to the peritoneal cavity was demonstrated, with these cells accumulating in significant numbers as early as 1 h post infection. IL-17 producing γδ T cells were also detected in the MLN, the primary draining lymph node for the peritoneal cavity (379, 380). The rapid response to *S. aureus* infection is characteristic of the innate-like function of γδ T cells. However, the γδ T cell response adapted to *S. aureus* infection over time. At 1 h post infection Vγ1.1 and Vγ2 cells comprised the majority of γδ T cells recruited to the site

of infection. However by 72 h, a large population of Vγ4 cells had accumulated in the peritoneal cavity. Moreover, in a novel model of recurrent peritonitis, the altered composition of resident γδ T cells was maintained up to 3 weeks after final exposure to the bacterium. Significantly, these Vγ4 T cells expanded as a consequence of prior exposure were maintained in the peritoneal cavity and the MLN in the absence of bacteria and represent a population of memory-like cells capable of enhanced IL-17 production upon subsequent exposure.

Vγ1.1 cells have been reported to produce IL-17 in aspergillus infected mice with CGD (335), but are traditionally thought to be more anti-inflammatory with depletion studies indicating increased inflammatory responses during *L. monocytogenes* (147) and coxsackievirus B3 infection (148). Conversely, Vγ2 cell depletion led to reduced inflammatory responses in both of these models (147, 148). Moreover, these same pro-inflammatory Vγ2 cells appear to be pathogenic in autoimmune models and were identified as the predominant γδ T cell subset infiltrating the CNS of mice with experimental autoimmune encephalomyelitis and the joints of mice with collagen-induced arthritis (149, 150). Vγ4 cells have been shown to contribute significantly to anti-bacterial immunity during *L. monocytogenes* (152), *B. subtilis* (153) and *E. coli* infections, where IL-17-producing $V\gamma4^{+}V\delta1^{+}$ T cells control the early recruitment of neutrophils (154-156).

Human γδ T cells, like αβ T cells, are capable of antigen recall and can recognise a bacterial antigen upon re-exposure to the organism, allowing for a more rapid and efficient immune response (181, 192, 204, 381). Murine γδ T cells resembling adaptive αβ T cells and bearing the characteristics of pathogen-specific human γδ T cells have recently been identified in a model of *L. monocytogenes* infection (173). In that study, a population of $CD27^{-}CD44^{+}$ memory γδ T cells were induced in response to a secondary infection via the oral mucosal route, but not the systemic route, suggesting that mucosal priming plays a key role in the expansion of memory γδ T cells in that model. Critically, this was associated with enhanced protection against secondary infection with *L. monocytogenes*, but not *Salmonella enterica* serovar Typhimurium. The authors further demonstrated that the memory γδ T cell population comprised predominantly of Vγ4Vδ1 cells capable of producing elevated levels of IL-17 and IFNγ upon secondary oral infection, largely in an MHC class II-dependent manner. Similarly, Roark *et al.* also demonstrated high levels of CD44 expression on a population of effector memory $V\gamma4^{+}V\delta1^{+}$ cells during inflammation induced by administration of complete Freud's adjuvant (CFA) (174). In the present study *S. aureus*-primed $CD27^{-}V\gamma4^{+}$ γδ T cells were also $CD44^{hi}$ and displayed enhanced cytokine

production upon re-exposure to *S. aureus* both *in vivo* and *in vitro*, reflecting an effector-memory phenotype. In this *S. aureus* induced peritonitis model however, $CD27^-$ Vγ4 T cells secreted only IL-17, and not IFNγ as reported by Sheridan *et al.* (173). This may be possible due to Th1 cells, which are also induced by prior exposure (Section 3.2.9), compensating for IFNγ production. However, in the Sheridan study *Listeria*-specific memory γδ T cells produced greater quantities of IL-17 than IFNγ (173).

Consistent with previous studies (169, 322), IL-1β was found to be indispensable for IL-17 production by γδ T cells during *S. aureus* induced peritonitis in naive mice. Furthermore, increased bacterial dissemination correlated with reduced IL-17 production, impaired chemokine secretion and decreased neutrophil recruitment upon infection of IL-1RI deficient animals. This is reflective of clinical data where patients with defects IL-1R signalling suffer from increased susceptibly to *S. aureus* infections (382). Similarly, IL-1R-blockade with a monoclonal antibody abrogated IL-17 production by γδ T cells from naive mice, in co-culture studies with *S. aureus* infected macrophages *in vitro*. In contrast, *S. aureus*-primed γδ T cell secretion of IL-17, which was significantly elevated compared to that produced by γδ T cells from naive mice, was not reliant on IL-1R signalling. Moreover, neither IL-1β secretion nor IL-1R expression by γδ T cells was increased in prior exposed mice compared to non-exposed mice. As IL-23R signalling was not critical for IL-17 expression by γδ T cells in naive or prior exposed mice, and IL-1α and IL-18 were undetectable in the peritoneal cavity of both groups of mice, it is likely that these *S. aureus*-primed γδ T cells are in fact memory cells capable of rapid recall of staphylococcal antigens upon re-exposure.

Experiments demonstrated that memory γδ T cell responses were *S. aureus*-specific, as exposure to *E. coli* did not result in significantly enhanced IL-17 production by *S. aureus*-primed γδ T cells compared to naive γδ T cells. In addition, preliminary studies suggest that inhibition of MHC II signalling interfered with the ability of memory γδ T cells to respond to bacterial stimulation, suggesting that antigen presentation plays a role in functional γδ T cell memory. Future work aims to repeat these studies to confirm the importance of MHC II signalling in activation memory γδ T cells. The type of antigens recognised by γδTCRs and the importance of antigen presentation in these reactions are less well defined for γδ T cells than for classical αβ T cells. Several reports have demonstrated the capacity of γδ T cells to recognise cell surface expressed ligands; including the classical MHC I and II molecules (383), the non-classical MHC I-related proteins T10 and T22 molecules in mice (384, 385), and MICA/B in humans (386-388), as

well as CD1c in humans (389), and CD1d in both mice and humans (390-392). Other studies have demonstrated the direct recognition of antigens by γδ T cells, such as phosphoantigens (178), herpes simplex virus glycoprotein (393, 394), the algae protein phycoertythrin (181), tetanus toxoid (395, 396), mycobacterial heat shock proteins (397, 398), listeriolysin O (399) and staphylococcal enterotoxin A (SEA) (400). Reports vary greatly on the absolute requirement for antigen presentation in γδ T cell recognition of antigens. The response of human γδ T cells to tetanus toxoid was MHC restricted (395), and phosphoantigen activation of γδ T cells was increased in the presence of monocytes most likely due to enhanced presentation (369, 370). In contrast, there was no APC requirement or MHC restriction in γδ T cell responses to heat shock proteins (401) or listeriolysin O (399) derived peptides. Thus, researchers have postulated that antigen presentation is optional and may be limited to select peptides (402). Other peptides may be presented in a non-MHC restricted manner, possibly involving specialised cell surface molecules, or through non-specific binding between reactive antigens and cells (403), or via direct stimulation of the γδTCR by multivalent antigens, in the absence of any kind of presentation (404, 405). Whilst the precise mechanisms of peptide recognition by the γδ TCR remain to be determined, based on work by Sheridan *et al* (173) and in this current study, MHC II signalling appears to play a key role in the activation of memory γδ T cells, and may even be required for the generation of this adaptive phenotype by these non-traditional lymphocytes. In addition to the γδTCR, γδ T cells express TLRs and NK receptors that allow them to respond to other non-peptide microbial components, and it would be interesting to examine the effects of γδTCR blockade of *S. aureus*-primed cells in the co-culture system to dissect the relative importance of TCR recognition in γδ T cell activation.

A memory response specifically by *S. aureus*-primed γδ T cells, was confirmed by that the fact that adoptive transfer of γδ T cells, but not $CD3^{+}\gamma\delta^{-}$ T cells, from the peritoneum of mice infected with S. *aureus* enhanced bacterial clearance in naive mice upon challenge with *S. aureus*. The increased eradication of bacteria from the peritoneum also resulted in reduced dissemination to peripheral sites, including the kidneys and liver of γδ T cell recipient mice. Prior exposure to *S. aureus* therefore induced a γδ T cell memory phenotype capable of enhancing bacterial clearance upon re-exposure to the bacterium.

Transfer of *S. aureus*-primed γδ T cells drove increased macrophage infiltration to the peritoneal cavity. Conversely, neutrophil influx was decreased at this time point in γδ T cell recipient mice, which was interesting as deficiency in neutrophils is often associated

with exacerbation of *S. aureus* infection (303, 306-308). However, conflicting reports are emerging which suggest that limiting, but not ablating, neutrophil functions may actually enhance bacterial clearance (263, 312), with researchers postulating that *S. aureus* uses its ability to survive within neutrophils as a means of immune evasion and dissemination (315). Thus excessive neutrophil responses may be detrimental to the host. Macrophages, on the other hand, have the capacity to control neutrophil responses through clearance of necrotic neutrophils (32, 35, 36, 406). It is important to note that this phagocyte profile was observed 72 h post infection, and that optimal neutrophil responses are most like observed 3-24 h post infection (as was observed in Section 3.2.10) suggesting a beneficial effect for the resolution of neutrophil responses during the later stages of acute infection. Therefore, the protection conferred by memory γδ T cells is likely mediated by the control of downstream phagocytic cell responses. In line with this previous studies have demonstrated that murine Vγ4Vδ1 cells are important in controlling phagocyte recruitment during *E. coli* induced peritonitis. Early influx of neutrophils, which peaked 24 h post *E. coli* infection, was controlled by IL-17A producing Vγ4Vδ1 cells (155). Infiltration of macrophages peaked after neutrophil responses, 48 h post infection, with the influx of macrophages relying on CC chemokine production by Vγ4Vδ1 cells (157). The authors concluded that Vγ4Vδ1 cells help bridge the gap between neutrophil and macrophage responses during bacterial infection (155, 157). Given that Vγ4 T cells have been shown to be important in several bacterial infections (152-156), these cells possibly represent potent anti-bacterial cells capable of mediating protection through regulating the kinetics of neutrophil and macrophage recruitment.

Overall this study significantly advances the current understanding of cellular immune responses to *S. aureus*, identifying a subset of IL-17 producing γδ T cells that undergo rapid expansion following infection, which are maintained in the peritoneal cavity and MLN. These IL-17 secreting memory γδ T cells are then capable of conferring enhancing bacterial clearance following re-exposure to *S. aureus.* Future studies are now required to identify the specific staphylococcal antigens recognised by γδ T cells that contribute to the generation of memory γδ T cells. Little is known about what staphylococcal antigens may directly activate γδ T cells. Zwitterionic polysaccharides, such as CP8 and wall teichoic acid, have been shown to activate CD4 cells in an MHC II dependent manner (316, 318). Based on preliminary findings within the current study, MHC II signalling appears to play a part in functional γδ T cell memory, thus it is tempting to speculate that these zwitterionic polysaccharides maybe capable of also activating γδ T cells directly in an

MHC II dependent manner. γδTCRs are also capable of directly recognising microbial ligands such as herpes simplex viral glycoprotein (393, 394) and mycobacterial heat shock protein (397, 402). γδ T cells may therefore be capable of identifying structurally similar staphylococcal proteins. In fact human Vγ9Vδ2 cells have been shown to exhibit cytotoxic responses upon γδTCR recognition of SEA presented by HLA class II molecules (400).

It is however important to consider the fact that *S. aureus* is a human pathogen and there are differences between human and mouse γδ T cells. Therefore, studies in humans will be necessary to validate these findings from murine models. Although the phosphoantigen HMBPP is not produced by *S. aureus*, transfer of HMBPP activated γδ T cells protects against *S. aureus* infection in SCID mice (371), suggesting that human γδ T cells do play some role in anti-staphylococcal immunity. If IL-17 secreting memory γδ T cells are induced and found to have a role in anti-staphylococcal immunity in humans, these cells represent a potentially important and novel target for the rational design of future vaccines against *S. aureus*.

Chapter 5

Exposure to staphylococcal antigens expands a population of Th1 cells identifying these cells as possible vaccine targets

5.1. Introduction

During bacterial infection, Th1 derived IFNγ plays a pivotal role in enhancing macrophage effector function, transforming macrophages into potent anti-bacterial cells (61, 62, 65). In addition, IFNγ promotes MHC II and co-stimulatory molecule expression by macrophages, enabling more efficient antigen presentation (59). Classically, Th1 cells were associated with protection against intracellular infections (57-59), but have also been shown to play important roles in immunity against extracellular bacterial infections (74, 345). Conflicting evidence currently exists on the importance of IFNγ signalling in natural anti-staphylococcal immunity, with some studies identifying no role for IFNγ signalling (169), whereas others have demonstrated both detrimental (323, 324) and protective functions (325) for IFNγ responses. Additionally, evidence exists for differential roles of IFNγ signalling during *S. aureus* infection depending on the stage of the disease (326, 327). However, Th1 cells appear to play an important role in acquired immunity, as vaccine induced Th1 cells were capable of protecting against systemic *S. aureus* infection (329). In addition, immunisation with live attenuated *S. aureus* also drove IFNγ production by CD4 T cells upon antigen re-stimulation *in vitro* and *in vivo* (330). Vaccination with viable *S. aureus* 8 weeks prior to challenge with *S. aureus* enhanced clearance of bacteria in immunised mice. However, this effect was lost in IFNγ deficient mice or via administration of anti-IFNγ monoclonal antibody (407). These studies highlight Th1 and IFNγ responses as potential targets for *S. aureus* therapies. In fact, administration of recombinant IFNγ has even been suggested as a possible prophylactic strategy in the prevention of *S. aureus* induced mastitis (408).

Given that whole cell live or killed vaccines largely fail to generate protective immunity against *S. aureus* (409), future anti-*S. aureus* vaccines will likely rely on the selection and combination of staphylococcal antigens capable of inducing robust immunity. *S. aureus* expresses a broad range of virulence factors, including cell wall anchored proteins which are proteins covalently attached to the peptidoglycan layer. These proteins are exposed on the surface of staphylococcal cells and are in direct contact with the host's immune system and as such represent attractive targets for vaccine strategies. Staphylococci deficient in sortase, an enzyme involved in surface protein attachment to the peptidoglycan layer, are unable to display several surface proteins and cannot establish infection in experimental models, indicating the importance of surface proteins in pathogenesis (410). Clumping factor A (ClfA) is a staphylococcal cell wall anchored protein expressed by virtually all *S.*

aureus strains (411). ClfA has been identified as an important virulence factor through its ability to promote bacterial adherence to fibrinogen and complement regulator 1 (411). It is composed of 933 amino acids with residues 40 to 559 composing region A, the fibrinogen-binding domain. ClfA promotes the clumping of *S. aureus* in plasma and adherence of bacteria to blood clots (412-414). It is the dominant fibrinogen-binding protein present on the surface of *S. aureus* in the stationary phase of growth (415, 416). ClfA also plays a role in *S. aureus* immune evasion as it is thought to coat staphylococcal cells in fibrinogen, inhibiting the binding of opsonins and consequently ingestion by phagocytes (417, 418). These properties suggest that ClfA may be a suitable target antigen for vaccine studies.

Injection of mice with the recombinant fibrinogen-binding A domain of ClfA in combination with complete Freund's adjuvant (CFA) induced protective humoral immunity in a model of *S. aureus* induced septic arthritis (411). However, another study demonstrated that protection against *S. aureus* induced by administration of ClfA adjuvanted with alum was dependent on IL-17A, but not on vaccine induced antibodies (367). A bivalent conjugate vaccine composed of ClfA and dPNAG has proven highly immunogenic in mice, rabbits, goats and rhesus monkeys through induction of functional anti-adhesive antibodies and opsonic/protective antibodies against ClfA and dPNAG, respectively (419). Additionally, DNA vaccination with plasmids expressing the fibrinogen-binding domain of ClfA induced strong antibody responses (420). Vaccination with the candidal protein rAls3p-N, which is structurally similar to ClfA, combined with alum, induced protection against systemic *S. aureus* infection in mice through a mechanism that depended upon Th1 and Th17 dependent responses (329). Moreover, reverse vaccinology has also identified ClfA as an important antigen for immunisation strategies, with intramuscular immunisation of mice with ClfA combined with CFA conferring substantial protection against subsequent *S. aureus* infection (421). Given that ClfA has proven immunogenicity in numerous vaccine strategies, it represents a suitable candidate antigen for inclusion in a vaccine, and as such a Pfizer have included ClfA as part of their multivalent vaccine which is currently undergoing pre-clinical studies (422).

Due to the emerging importance of Th1 cells in acquired immunity against *S. aureus* infections, it is likely that future anti-*S aureus* vaccine design will combine antigens with select adjuvants in order to shape vaccine induced cellular immunity by inducing specific T cell phenotypes. Adjuvants are substances that can increase or modulate the immunogenicity of an antigen and are used to induce strong and durable immunity, for

example the adjuvant alum promotes humoral responses and Th2 mediated immunity against co-administered antigens (423). CpG oligodeoxynucleotide (CpG) is a TLR9 agonist comprising of unmethylated CpG motifs. It is capable of promoting DC maturation, antigen presentation, IFNγ production by NK and T cells and production of IgG2a (424, 425), and as a consequence of these abilities is used as an adjuvant to induce Th1 responses. Vaccination with purified *B. pertussis* antigens combined with CpG drove both protective Th1 and Th17 responses against subsequent infection (345). As emerging evidence suggests that Th1 cells and IFNγ responses (329, 330, 407) may play an important role in acquired immunity against *S. aureus*, CpG represents a potentially useful adjuvant to investigate the importance of Th1 induced protection against staphylococcal infection.

Due to the mucosal immune system's front line status within the immune system, vaccines targeting mucosal immune responses have become an attractive strategy for many vaccine designs, such as those against HIV (426). Mucosal vaccination represents a promising immunisation strategy as it can potentially stimulate an immune response both at the mucosae and systemically, as well as having the advantage of being needle-free. As *S. aureus* has a propensity to infect mucosal tissues, namely the skin and lungs (228), mucosal immunisation represents an attractive strategy for future vaccine design by aiming to induce immunity at the point of entry for staphylococci. Consequently, a collaborative project was set up with the Adjuvant Research Group at TCD to develop a mucosal vaccine strategy against *S. aureus*.

The efficacy of mucosal vaccines can be hindered by several factors, one of which is poor antigen uptake across epithelial barriers (427). Microfold (M) cells are specialised epithelial cells of the mucosa-associated lymphoid tissues which function as antigen sampling cells capable of delivering antigen to APCs through selectively endocytosing and transporting antigens across the epithelial barrier. Targeting mucosal vaccines to mucosal M cells represents a promising approach to increase efficient transepithelial vaccine uptake (428). *Ulex europaeus* agglutinin 1 (UEA-1) is a fucose binding lectin, which has the potential to deliver attached antigen to subepithelial DCs by binding to and enhancing transcytosis of antigen loaded nanoparticles across M cells (429, 430). Previous studies have demonstrated that intranasal vaccination with a combination of UEA-1 and HIV peptides in microparticles generated robust systemic and mucosal anti-HIV immune responses (431). Based on these studies, particulate vaccines loaded with staphylococcal

ClfA were targeted to M cells using UEA-1m, a peptidomimetic of UEA-1, with the aim of enhancing transepithelial uptake of ClfA, leading to the induction of both systemic and mucosal immunity to ClfA. QS21 is an active fraction of the established adjuvant Quil A, a potent saponin adjuvant (432), that displays reduced toxicity compared to Quil A (432-434). Saponin based adjuvants enhance antigen uptake by DCs, induce DC activation and drive strong antibody and T cell responses (435). QS21 promoted both cellular and humoral mediated immunity when added to systemic vaccine formulations (436-438). This molecule was also demonstrated to induce both systemic and mucosal immunity to a nasally administered DNA vaccine against HIV (433). Orally administered QS21 also exhibits adjuvant activity by inducing both systemic and mucosal immunity against co-administered antigens (434). These studies suggest that QS21 can exert adjuvant activity when administered by non-parenteral routes. Based on these reports, staphylococcal ClfA was also combined with the adjuvant QS21 in an intranasal vaccination strategy with the aim of inducing both systemic and mucosal immune responses. By targeting the mucosal immune response through selection of novel adjuvants in combination with a staphylococcal antigen, these studies aim to identify if mucosal vaccination represents a promising approach for future anti-*S. aureus* vaccines.

The goal of the current study was to establish that exposure to *S. aureus* induces a population of protective Th1 cells, identifying these cells as potential vaccine targets. Initially, the capacity of *S. aureus* antigen-specific Th1 cells to enhanced clearance during *S. aureus* infections was definitively proven through the use of adoptive transfer studies. Having identified that Th1 cells are capable of mediating increased bacterial elimination, a model vaccine was developed using the staphylococcal antigen ClfA combined with the adjuvant CpG, to drive an antigen-specific Th1 response. Finally, the ability of a mucosal vaccine to induce protective immunity against systemic *S. aureus* infection was investigated through the use of novel adjuvant technology. Overall, the current study aims to establish the validity of targeting type 1 immune responses for future anti-*S. aureus* vaccine strategies.

5.2. Results

5.2.1. In vitro expansion of S. aureus-specific CD4$^+$ Th1 cells.

Previous experiments demonstrated the expansion of Th1 cells upon exposure to *S. aureus*, and suggested that these cells may contribute to protection against subsequent infection (Section 3.2.7 & 3.2.9). To test the ability of *S. aureus*-specific Th1 cells to protect against *S. aureus* infection in naive mice, adoptive transfer experiments were set up. This was achieved by firstly expanding *S. aureus*-specific Th1 (IFNγ^+CD4$^+$) cells *in vitro* and then adoptively transferring these antigen-specific Th1 cells into naive mice prior to challenge with *S. aureus*.

To drive *S. aureus* antigen-specific T cells, groups of mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injections on d 0, 7 and 14. On d 21, the peritoneal cavity was lavaged with PBS and cells from the MLN and spleen isolated. Cells were then polarised *in vitro* by culturing total cells in the presence of heat killed *S. aureus* (10^5 CFU/ml) and rIL-12 (10ng/ml) for 96 h at 37°C, following a method previously published by Ross *et al* (345). Cell supernatants were then collected and secreted cytokines were quantified by ELISA. Significant IFNγ production by peritoneal (Figure 5.1 A) and spleen cells (Figure 5.1 C) was detected, however no IFNγ secretion was detected in the polarised MLN cells (Figure 5.1 B). Minimal levels of IL-17, IL-1β, IL-22 and IL-23 were detected in the cell supernatants. These data demonstrate that upon *in vitro* polarisation, both peritoneal and spleen cells expanded populations of *S. aureus*-specific Th1 cells.

Prior to adoptive transfer, the polarised cells were MACS purified using the PAN T cell isolation kit to allow for the isolation of untouched T cells. Live dead staining was then carried out to ensure that polarised T cells were viable and capable of responding *in vivo* to infection. Analysis of PI staining revealed that a majority (>99%) of CD4 Th1 cells were alive prior to adoptive transfer into naive mice (Figure 5.1 D).

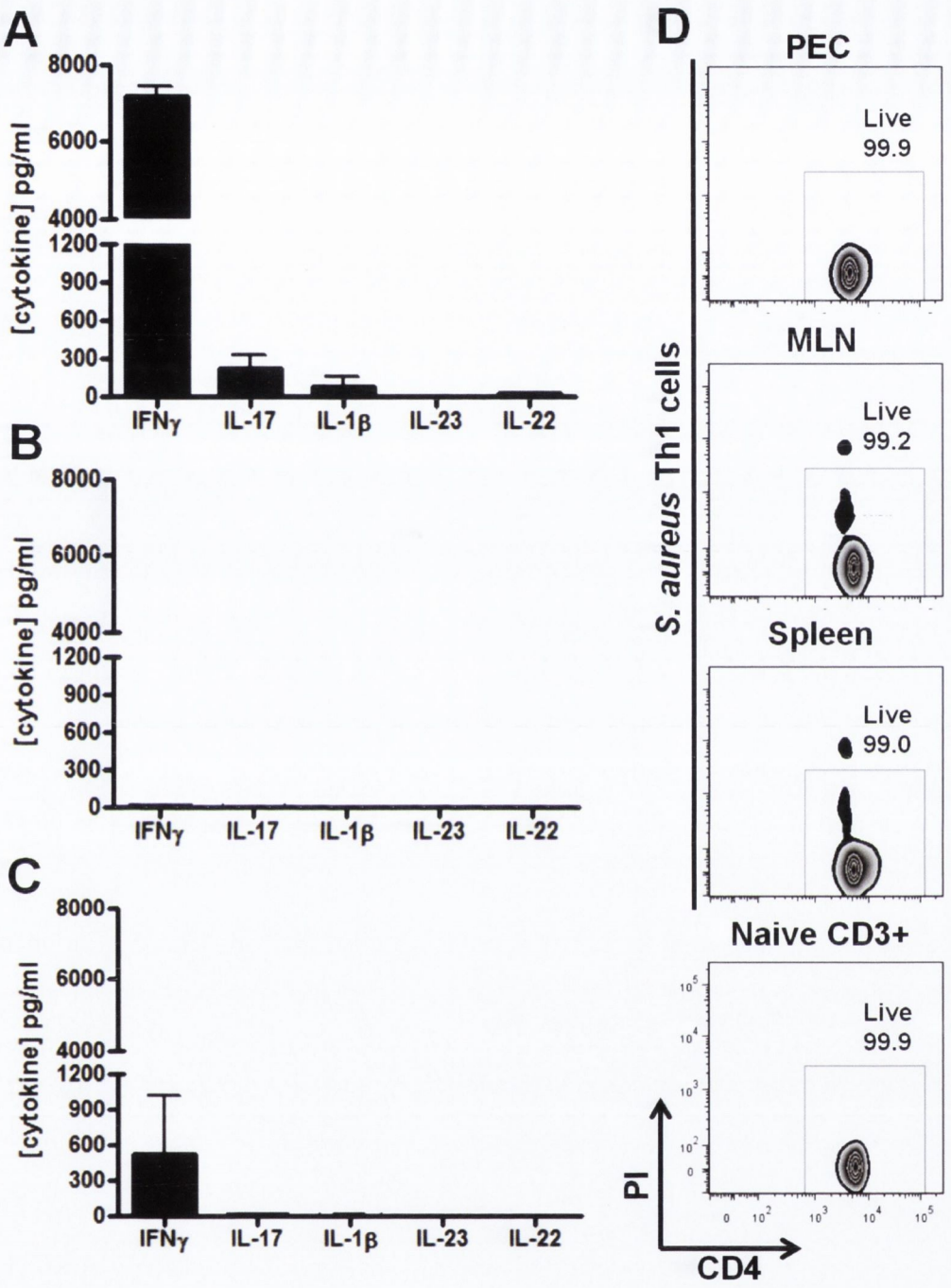


Figure 5.1: Expansion of *S. aureus*-specific Th1 CD4$^+$ cells *in vitro*

Groups of mice were exposed to *S. aureus* (5x10^8 CFU) via i.p. injections d 0, 7 and 14. On d 21 the peritoneal cavity was lavaged with PBS and cells from the MLN and spleen isolated. Cells were polarised *in vitro* using rIL-12 (10ng/ml) and heat killed *S. aureus* (10^5 CFU/ml) for 96 h at 37°C. Cytokines from peritoneal (A), MLN (B) and spleen (C) cell supernatants were analysed using ELISA. The viability of cells was assessed by flow cytometry staining using PI (D). Results expressed as mean±SEM (A-C) with representative FACS plots for PI staining shown (D). Data pooled from 3 independent experiments.

5.2.2. Adoptive transfer of peritoneal S. aureus-specific Th1 cells enhances bacterial clearance during subsequent infection

To test the ability of *S. aureus* antigen-specific Th1 cells, that had originated from PEC, to protect against infection, adoptive transfer studies were carried out in which mice received transfers of $5x10^6$ polarised Th1 cells via i.p. injection. The control group received transfers of $5x10^6$ $CD3^+$ cells isolated from the spleens of naive mice. Previous studies using CFSE labelled T cells revealed that transferred $CD3^+$ T cells were maintained in the peritoneal cavity for up to 24 h post transfer, with 3 h post transfer representing the optimal recovery time point (3 h post transfer 58.8%±13.4 vs. 24 h post transfer 21.4%±12.3 of $CFSE^+$ T cells recovered, n=3). Therefore, at 3 h post cell transfer, mice were challenged with *S. aureus* ($5x10^8$ CFU). At 24 and 72 h post challenge, the peritoneal cavity was lavaged with sterile PBS and peripheral organs isolated to assess the bacterial burden. A significant decrease in bacterial load was observed in the peritoneal cavity (Figure 5.2 A), kidneys (Figure 5.2 B), and spleen (Figure 5.2 D), with a similar trend evident in the liver (Figure 5.2 C), of mice that received transfers of Th1 cells, compared to mice that received naive $CD3^+$ cells. This suggests that transfer of *S. aureus* antigen-specific peritoneal Th1 cells aided in the clearance of *S. aureus* at the local infection site as well as preventing dissemination of the bacteria to peripheral tissues.

As transfer of *S. aureus*-specific peritoneal Th1 cells was associated with increased bacterial elimination upon infection, the effects of Th1 cells on downstream phagocyte responses were assessed to elucidate a mechanism of protection. Local chemokine production and resulting phagocyte influx to the infection site (peritoneal cavity) were compared between mice that received transfers of *S. aureus* antigen-specific peritoneal Th1 cells versus naive $CD3^+$ recipient mice.

Transfer of *S. aureus* antigen-specific Th1 cells did not affect the production of KC (Figure 5.3 A) or MCP-1 (Figure 5.3 C) within the peritoneal cavity during subsequent infection. However, by 3 h post infection a significant increase in MIP-2 production was observed in mice that had received antigen-specific Th1 cells compared to mice that received naive $CD3^+$ cells (Figure 5.3 B). Additionally, RANTES secretion was significantly elevated 3 and 24 h post *S. aureus* infection in mice that received transfers of antigen-specific Th1 cells compared to control mice (Figure 5.3 D).

To establish the effects of local chemokine secretion on downstream phagocyte influx, flow cytometry was used to investigate the infiltration of neutrophils and macrophages to

the infection site. Macrophage proportions (Figure 5.4 B) and absolute numbers (Figure 5.4 C) in the peritoneal cavity were significantly elevated in mice that received *S. aureus*-specific Th1 cells compared to control mice 24 h post infection. Elevated macrophage proportions were maintained until 72 h post infection in *S. aureus*-specific Th1 recipient mice (Figure 5.4 B). As macrophage numbers were significantly elevated in the peritoneal cavity 24 h post infection, the expression of activation markers on the surface of these recruited macrophages was investigated. Substantially increased numbers of MHC II expressing macrophages were detected at this time point in mice that received transfers of antigen-specific Th1 cells (Figure 5.4 D). This suggests increased macrophage infiltration and activation may mediate the enhanced bacterial clearance conferred by *S. aureus* antigen-specific Th1 cells against *S. aureus* infection. Conversely, the proportions (Figure 5.4 E) and absolute numbers (Figure 5.4 F) of neutrophils observed in the peritoneal cavity 72 h post challenge was significantly decreased in mice that received transfers of antigen-specific Th1 cells, compared to mice that received naive $CD3^+$ T cells.

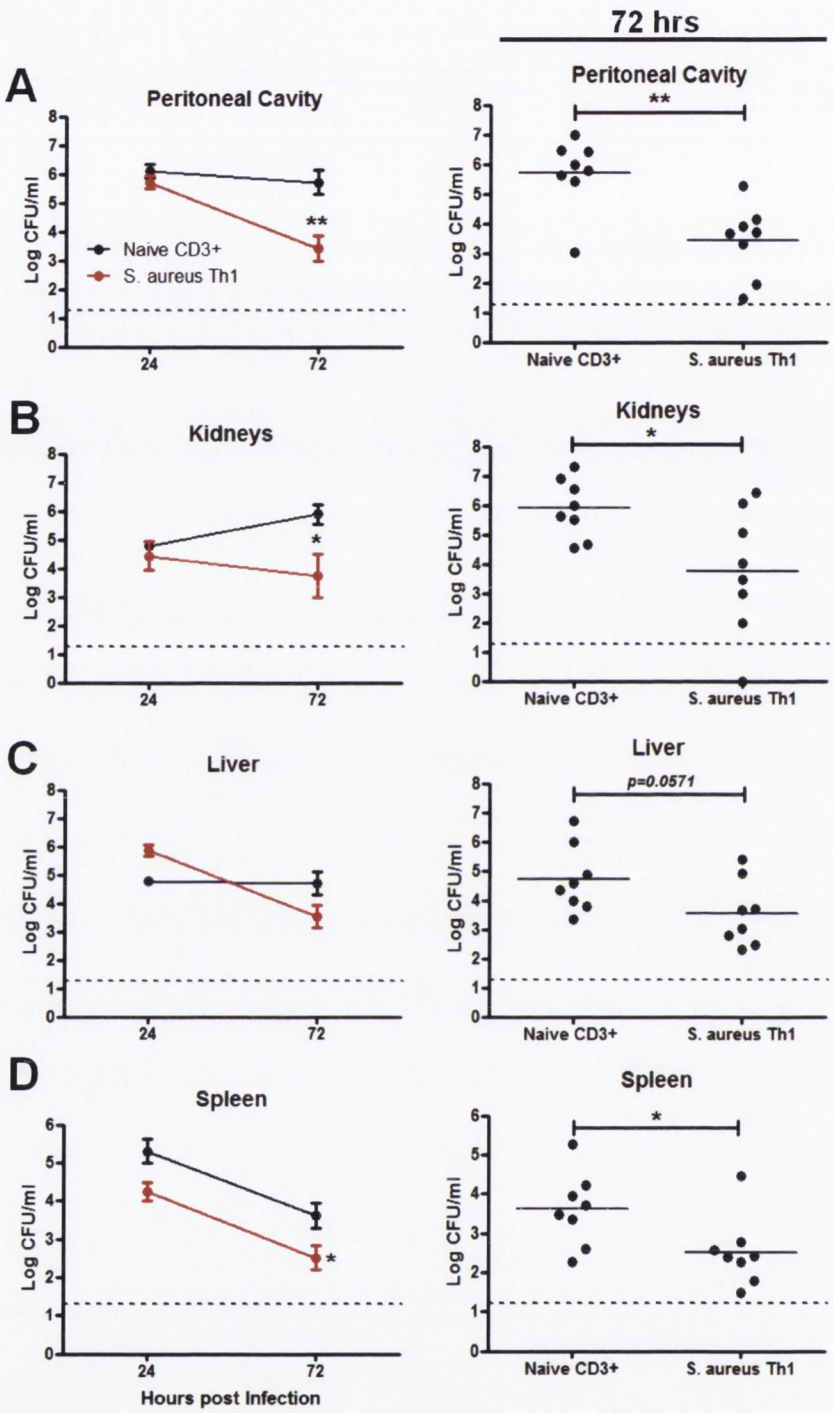


Figure 5.2: Transfer of *S. aureus* antigen-specific peritoneal Th1 cells enhances bacterial clearance during subsequent *S. aureus* infection

Groups of mice received transfers of $5x10^6$ *S. aureus*-specific Th1 cells originating from the peritoneal cavity of previously exposed mice, via i.p injection. Another group of mice received a transfer of $5x10^6$ naive splenic $CD3^+$ cells as a control. At 3 h post transfer both groups of mice were challenged with *S. aureus* ($5x10^8$ CFU) via i.p. injection. At 24 h and 72 h post bacterial challenge the bacterial burden was assessed in the peritoneal cavity (A), kidneys (B), liver (C) and spleen (D). Results expressed as Log CFU/ml of mean±SEM for time course. Bacterial burdens for individual animals are shown for the 72 h time point with mean indicated by bars. 24 h n=4; 72 h n=8. Data pooled from 3 independent experiments.*$p<0.05$, **$p<0.005$.

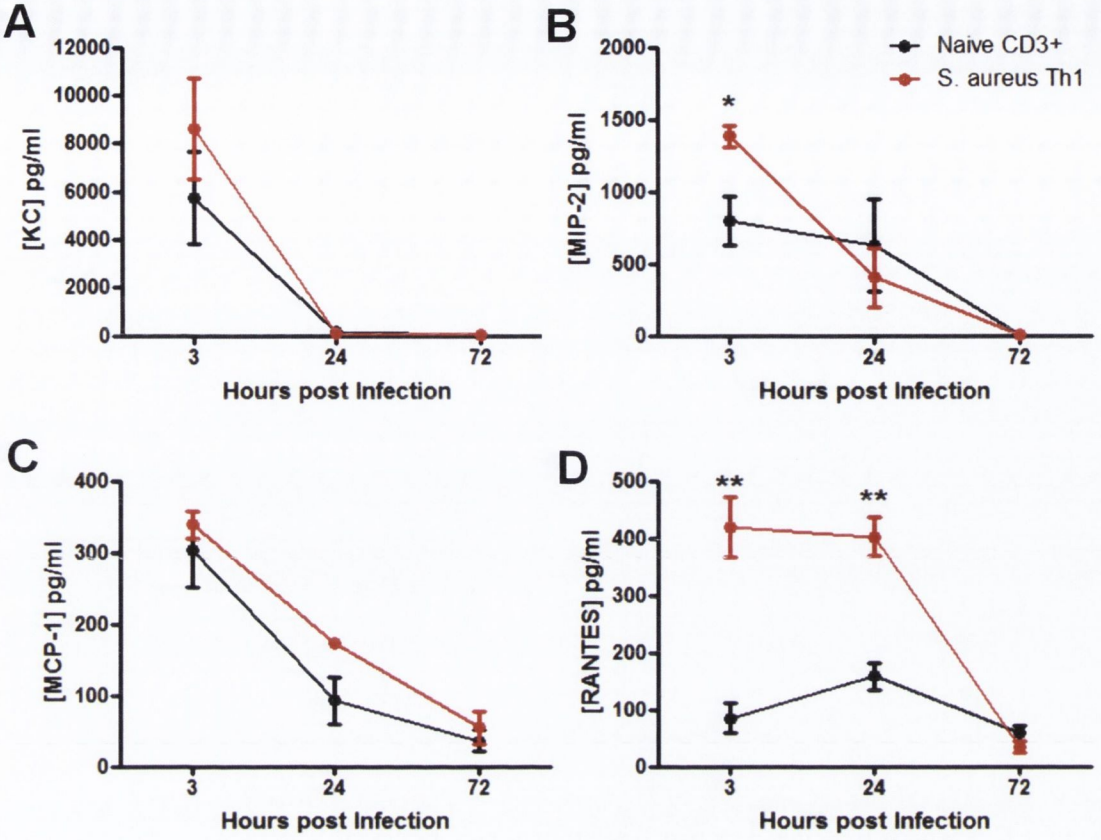


Figure 5.3: Transfer of *S. aureus* antigen-specific peritoneal Th1 cells increases MIP-2 and RANTES secretion during subsequent *S. aureus* infection

Groups of mice received transfers of $5x10^6$ *S. aureus*-specific Th1 cells originating from the peritoneal cavity via i.p injection. Another group of mice received a transfer of $5x10^6$ naive splenic $CD3^+$ cells as a control. At 3 h post transfer mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injections. At indicated time points post bacterial challenge the peritoneal cavity was lavaged with PBS. KC (A), MIP-2 (B) MCP-1 (C) and RANTES (D) production was assessed by ELISA. Results expressed as mean±SEM. 3 h n=5; 24 h n=4; 72 h n=8. Data pooled from 3 independent experiments.*$p<0.05$, **$p<0.005$.

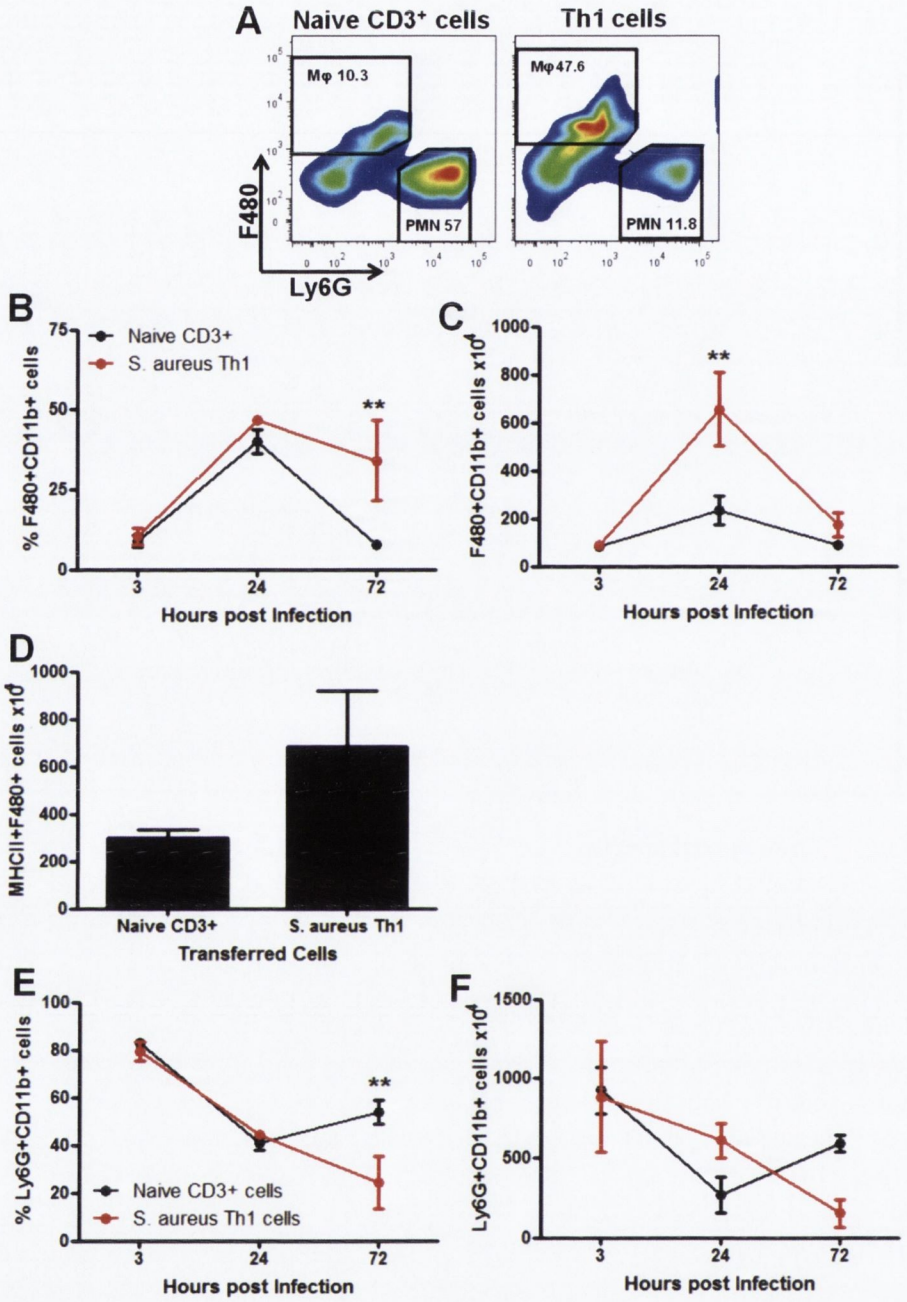


Figure 5.4: Transfer of *S. aureus* antigen-specific peritoneal Th1 cells increases macrophage recruitment and activation during subsequent *S. aureus* infection

Groups of mice received transfers of $5x10^6$ *S. aureus*-specific Th1 cells originating from the peritoneal cavity of prior exposed mice via i.p. injection. A control group of mice received a transfer of $5x10^6$ naive splenic $CD3^+$ cells. At 3 h post transfer mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injection. At indicated time points post challenge, macrophage ($F480^+Ly6G^-$) proportions (B) and absolute numbers (C) and neutrophil ($Ly6G^+F480^-$) proportions (E) and absolute numbers (F) were assessed in the peritoneal cavity by flow cytometry. MHCII expression by infiltrating macrophages was also determined 24 h post infection (D). Results expressed as mean±SEM (B-F) with representative FACS plot of peritoneal $CD11b^+$ cells 72 h post infection shown (A). 3 h n=5; 24 h n=4; 72 h n=8. Data pooled from 3 independent experiments.**$p<0.005$

5.2.3. Adoptive transfer of MLN or splenic S. aureus antigen-specific Th1 cells does not protect against infection in naive mice.

The ability of *S. aureus* antigen-specific Th1 cells that originated from the MLN or spleens of prior exposed mice to protect against infection were also tested. Adoptive transfer studies were carried out in which mice received transfers of $5x10^6$ polarised Th1 cells originating from the MLN and spleen via i.p. injection, with the control group received transfers of $5x10^6$ naive $CD3^+$ cells. At 3 h post cell transfer, mice were challenged with *S. aureus* ($5x10^8$ CFU). At 72 h post challenge, the peritoneal cavity was lavaged with sterile PBS and the organs isolated to assess the bacterial burden. Transfer of *S. aureus*-specific Th1 cells originating from the MLN and spleen did not significantly affect the outcome of *S. aureus* infection. No differences were detected in bacterial loads either at the site of infection or systemically in mice that received MLN or splenic Th1 cell compared to control mice (Figure 5.5). Unsurprisingly, phagocyte influx to the infection site was also unaffected by transfer of MLN or splenic Th1 cells (Figure 5.6).

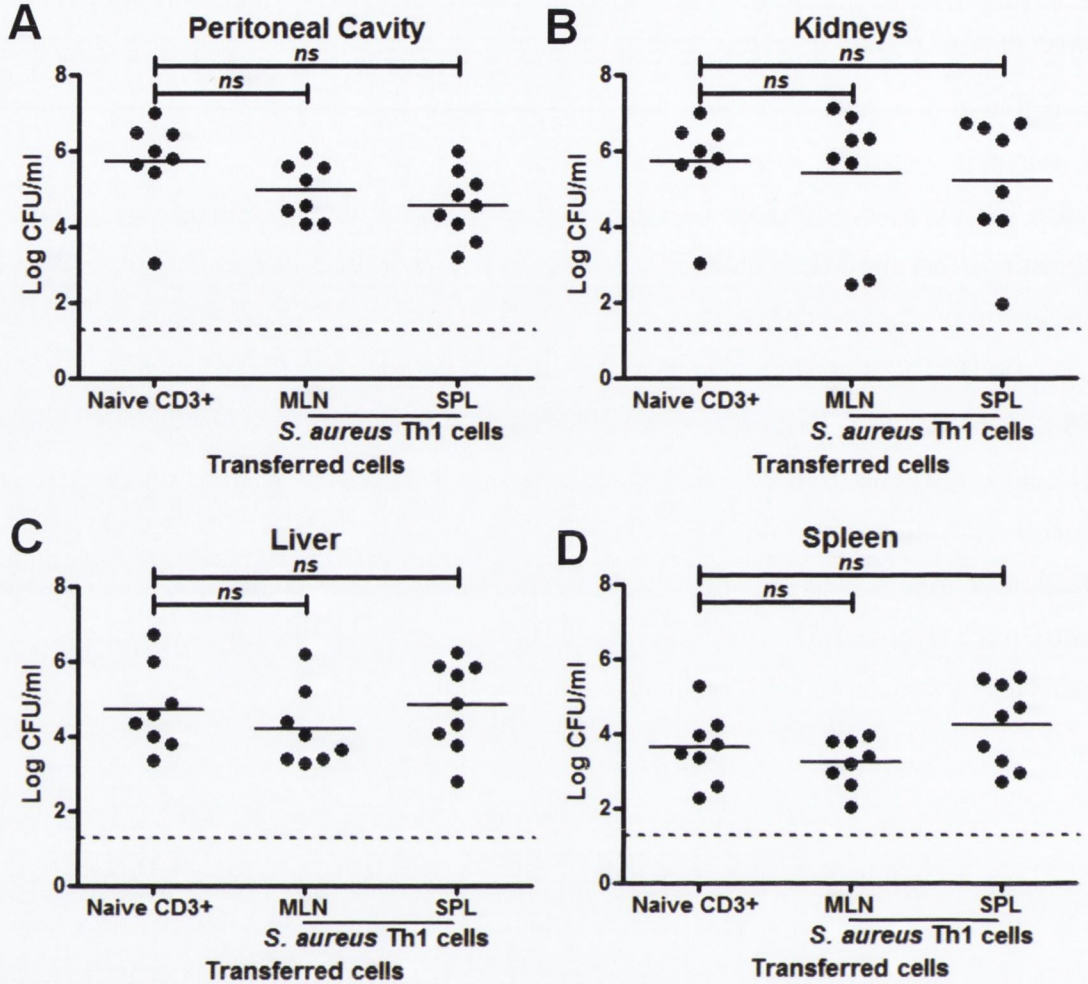


Figure 5.5: Transfer of *S. aureus* antigen-specific Th1 cells originating from the MLN or spleen did not protect against subsequent *S. aureus* infection

Groups of mice received transfers of $5x10^6$ *S. aureus* antigen-specific Th1 cells originating from the MLN or spleens of previously exposed mice via i.p injection. Another group of mice received a transfer of $5x10^6$ naive splenic $CD3^+$ cells as a control. At 3 h post transfer mice were challenged with *S. aureus* ($5x10^8$ CFU) via i.p. injection. At 72 h post bacterial challenge the bacterial burden was assessed in the peritoneal cavity (A), kidneys (B), liver (C) and spleen (D). Results expressed as Log CFU/ml with means indicated by bar. Dashed lines indicate limit of detection. n=8 per group. Data pooled from 2 independent experiments.

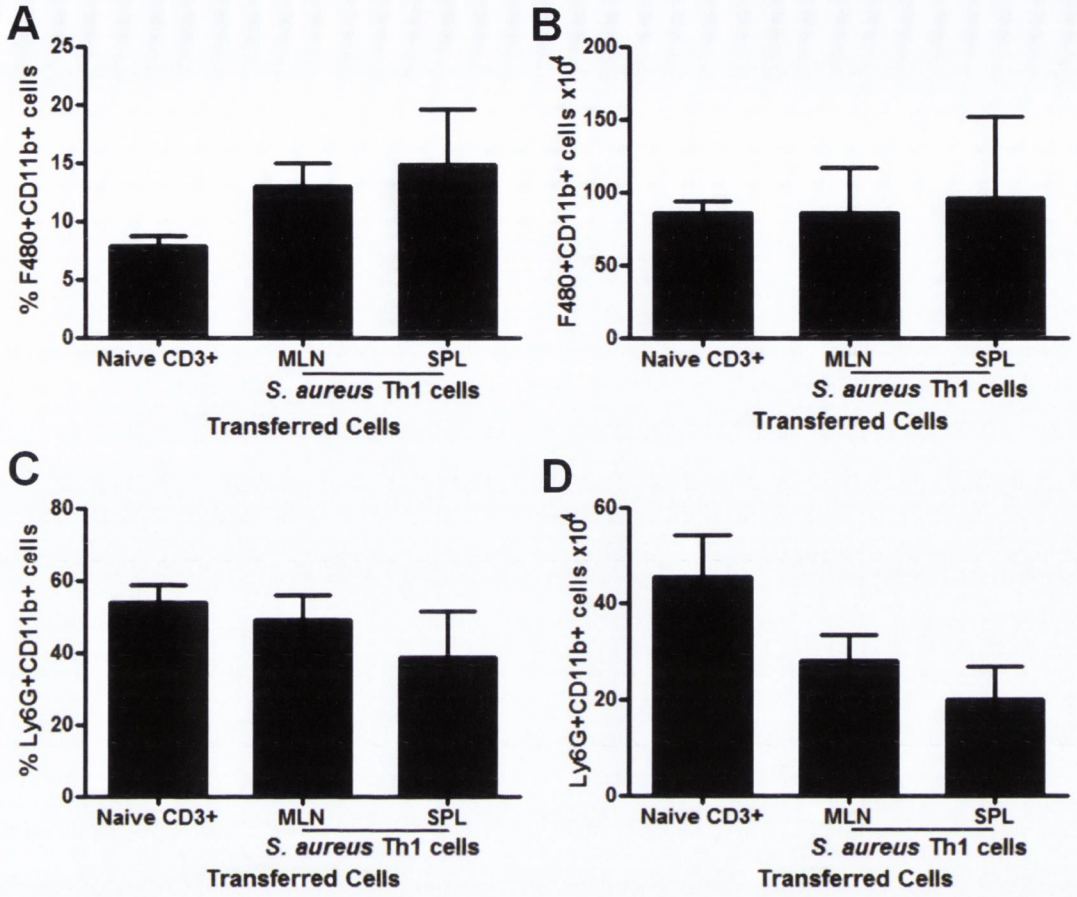


Figure 5.6: Transfer of *S. aureus* antigen-specific Th1 cells originating from the MLN or spleen did not affect phagocyte influx during subsequent *S. aureus* infection

Groups of mice received transfers of $5x10^6$ *S. aureus* antigen-specific Th1 cells originating from the MLN or spleens (SPL) of previously exposed mice via i.p injection. Another group of mice received a transfer of $5x10^6$ naive splenic $CD3^+$ cells as a control. At 3 h post transfer mice were challenged with *S. aureus* ($5x10^8$ CFU) via i.p. injection. At 72 h post infection, macrophage ($F480^+Ly6G^-$) proportions (A) and numbers (B), and neutrophil ($Ly6G^+F480^-$) proportions (C) and numbers (D) in the peritoneal cavity were assessed by flow cytometry. Results expressed as mean±SEM. n=8 per group. Data pooled from 2 independent experiments.

5.2.4. In vitro expansion of S. aureus-specific CD4$^+$ Th17 cells

Previous data demonstrate a protective role for Th1 cells, therefore the ability of *S. aureus* antigen-specific Th17 cells to protect against *S. aureus* infection was also established. This was achieved through the expansion of *S. aureus* antigen-specific Th17 (IL-17$^+$CD4$^+$) cells *in vitro* and transfer of these cells into naive mice.

To drive *S. aureus* antigen-specific Th17 cells, groups of IFNγ$^{-/-}$ mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injections on d 0, 7 and 14. IFNγ$^{-/-}$ animals were used to prevent Th17 cells from switching to Th1 cells, the more preferred phenotype (346, 439). On d 21, the peritoneal cavity was lavaged with PBS and cells from the MLN and spleen were isolated. Cells were then polarised *in vitro* by culturing total cells in the presence of heat killed *S. aureus* (10^5 CFU/ml) with rIL-1β (10ng/ml) and rIL-23 (10ng/ml) for 96 h at 37°C, following a method previously published by Ross *et al* (345). Cell supernatants were then collected and secreted cytokines quantified by ELISA. IL-17 production was detected by polarised peritoneal (Figure 5.7 A), MLN (Figure 5.7 B) and spleen cells (Figure 5.7 C). Unsurprisingly, these cells were incapable of producing IFNγ, with no IL-12 production detected either. Minimal amounts of IL-22 were however observed in the cell supernatants. These data confirmed that upon *in vitro* polarisation, peritoneal, MLN and spleen cells expanded into populations of Th17 cells.

Prior to transfer, cultured cells were MACS purified using the PAN T cell isolation kit to allow for the selection of untouched T cells. Live dead staining was then carried out to ensure that transferred cells were alive and capable of responding *in vivo* to infection. Analysis of PI staining revealed that the majority of Th17 cells (>74%) were alive prior to adoptive transfer into naive animals (Figure 5.7 D).

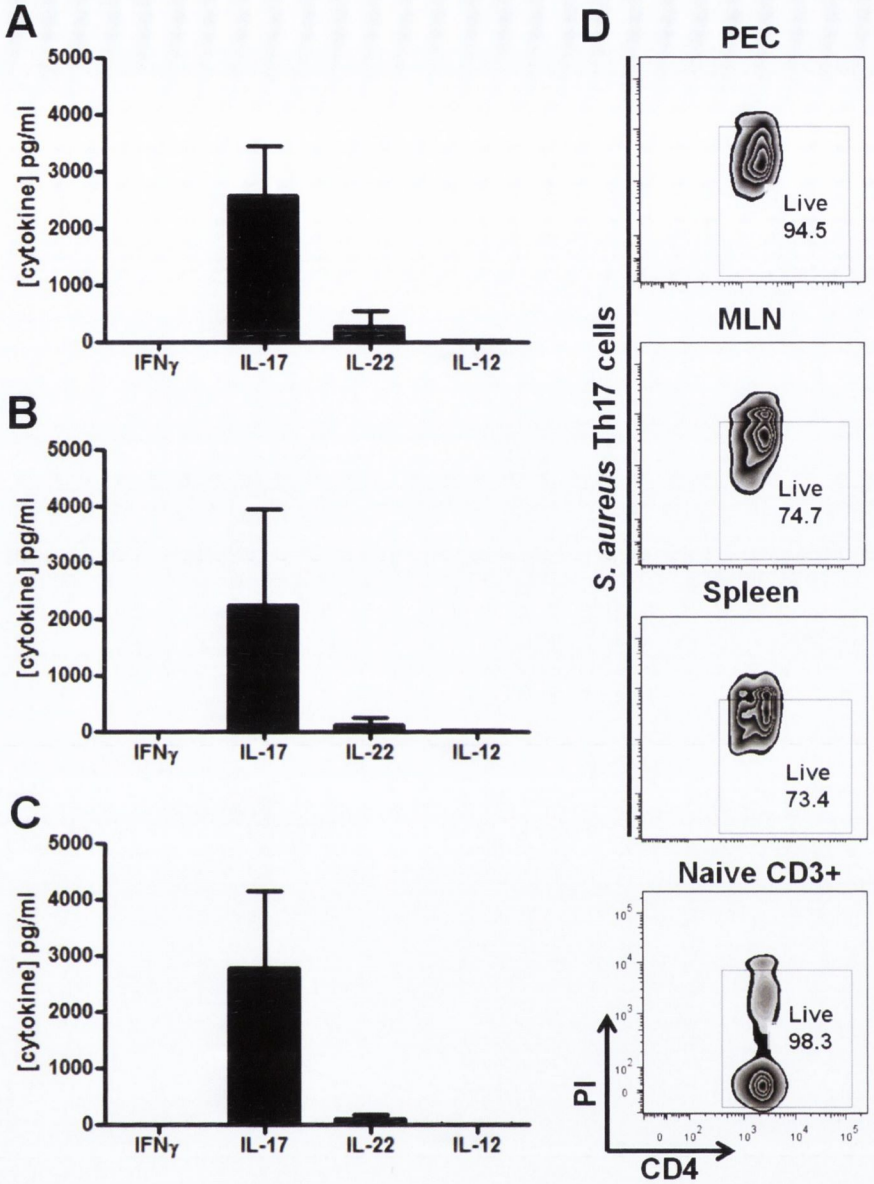


Figure 5.7: Expansion of *S. aureus*-specific Th17 CD4$^+$ cells *in vitro*

Groups of mice were exposed to *S. aureus* (5x10^8 CFU) via i.p. injections d 0, 7 and 14. On d 21 the peritoneal cavity was lavaged with PBS and cells from the MLN and spleen isolated. Cells were polarised *in vitro* using IL-1β and IL-23 (10ng/ml each) and heat killed *S. aureus* (10^5 CFU/ml) for 96 h at 37°C. Cytokines from peritoneal (A), MLN (B) and spleen (C) cell supernatants were analysed using ELISA. The viability of cells was assessed by flow cytometry staining using PI (D). Results expressed as mean±SEM (A-C) with representative FACS plots shown (D). Data pooled from 2 independent experiments.

5.2.5. Adoptive Transfer of S. aureus-specific Th17 cells does not protect against subsequent infection in naive mice

Groups of naive mice received transfers of $5x10^6$ *S. aureus*-specific Th17 cells originating from the peritoneal cavity, MLN or spleen via i.p injection. Another group of mice received a transfer of $5x10^6$ naive $CD3^+$ cells as a control. At 3 h post transfer mice were challenged with *S. aureus* ($5x10^8$ CFU) via i.p. injection. At 72 h post bacterial challenge the bacterial burden in the peritoneal cavity, kidneys, liver and spleen was assessed.

Adoptive transfer of Th17 cells from PEC, MLN or spleen did not significantly affect the outcome of *S. aureus* infection, with no differences detected in the bacterial burden at the infection site (Figure 5.8 A), or in the kidneys (Figure 5.8 B), liver (Figure 5.8 C) and spleen (Figure 5.8 D) 72 h post infection in mice that received transfers of Th17 cells compared to control mice. This may reflect the fact that $CD4^+$ Th17 cells are not the natural source of IL-17 in our model of *S. aureus* induced peritonitis (Section 3.2.9), and upon transfer do not receive the necessary signals from the *in vivo* microenvironment to maintain their phenotype. Unsurprisingly, no differences in phagocyte influx to the infection site were observed between Th17 recipient mice and control mice 72 h post infection (Figure 5.9).

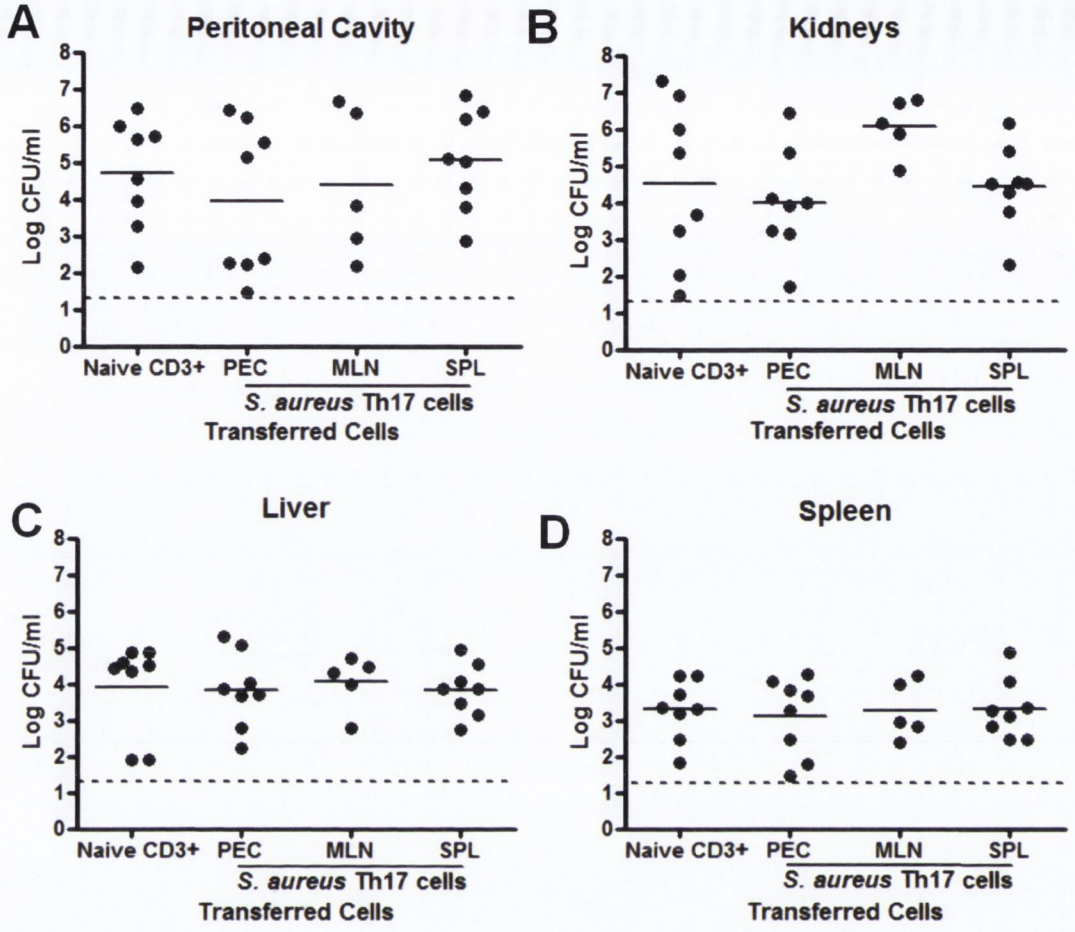


Figure 5.8: Transfer of *S. aureus* antigen-specific Th17 cells does not protect against subsequent *S. aureus* infection

Groups of mice received transfers of $5x10^6$ *S. aureus* antigen-specific Th17 cells originating from the peritoneal cavity (PEC), the lymph (MLN) or the spleen (SPL) via i.p injection. Another group of mice received a transfer of $5x10^6$ naive splenic $CD3^+$ cells as a control. At 3 h post transfer mice were exposed to *S. aureus* ($5x10^8$ CFU) via i.p. injection. At 72 h post bacterial challenge the bacterial burden was assessed in the peritoneal cavity (A), kidneys (B), liver (C) and spleen (D). Results expressed as Log CFU/ml with mean indicated by bar. Dashed lines indicate limit of detection. n=8 for naive $CD3^+$, or PEC and spleen Th17 recipient group, n=5 for MLN Th17 recipient group. Data pooled from 2 independent experiments.

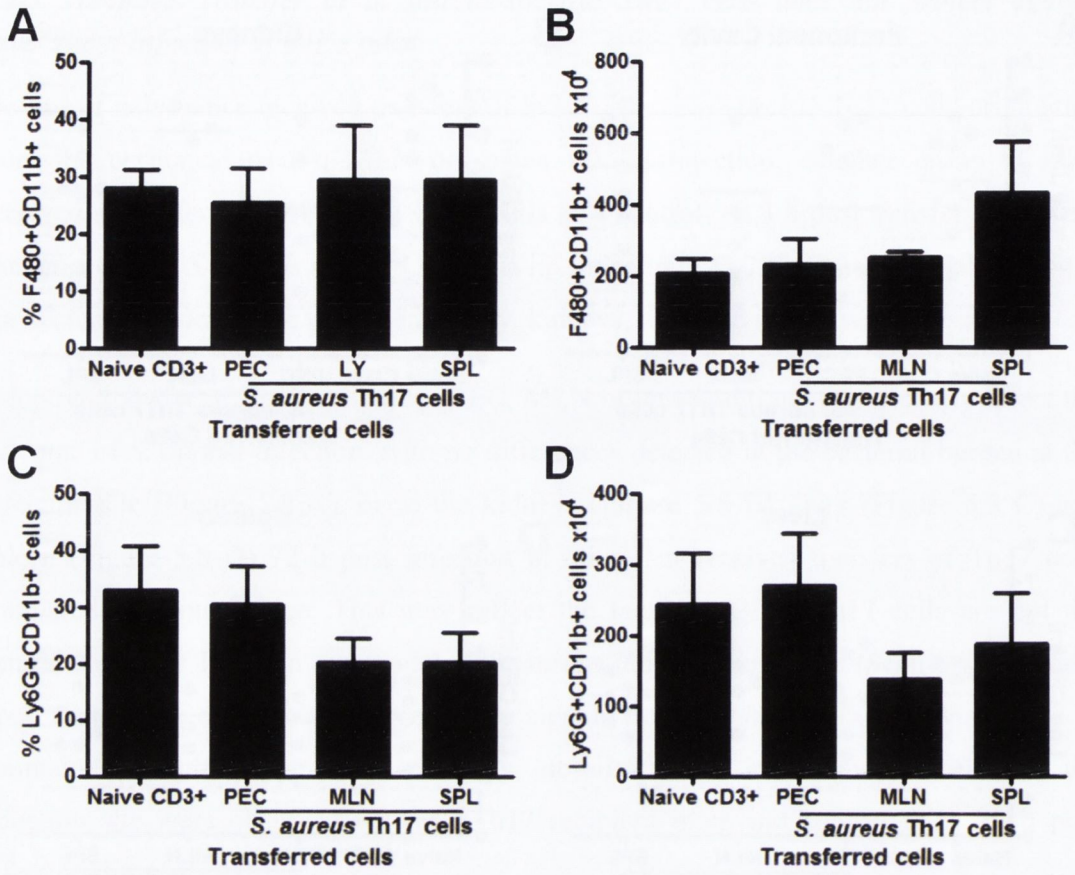


Figure 5.9: Transfer of *S. aureus* antigen-specific Th17 cells did not affect phagocyte influx during subsequent *S. aureus* infection

Groups of mice received transfers of $5x10^6$ *S. aureus* antigen-specific Th17 cells originating from the peritoneal cavity (PEC), the lymph (MLN) or the spleen (SPL) via i.p injection. Another group of mice received a transfer of $5x10^6$ naive $CD3^+$ cells as a control. At 3 h post transfer mice were challenged with *S. aureus* ($5x10^8$ CFU) via i.p. injection. At 72 h post infection macrophage ($F480^+Ly6G^-$) proportions (A) and absolute numbers (B), and neutrophil ($Ly6G^+F480^-$) proportions (C) and absolute numbers (D) in the peritoneal cavity were assessed by flow cytometry. Results expressed as mean±SEM. n=8 for naive $CD3^+$, or PEC and spleen Th17 recipient group, n=5 for MLN Th17 recipient group. Data pooled from 2 independent experiments.

5.2.6. Vaccination with ClfA alone does not protect against S. aureus infection

Having identified that transfer of *in vitro* polarised *S. aureus* antigen-specific Th1 cells protected against infection, the generation of a model vaccine that would specifically induce a protective Th1 response against *S. aureus* infection was explored. This was achieved through vaccination with the staphylococcal antigen, ClfA, previously shown to be immunogenic in numerous studies (367, 411, 420, 421), combined with the TLR9 agonist, CpG, an adjuvant known drive Th1 responses towards antigens (424, 425).

Prior to vaccination of antigen combined with adjuvant, initial studies confirmed that vaccination with antigen alone, i.e. ClfA only, was incapable of inducing protection against *S. aureus* infection. Groups of mice were vaccinated via the subcutaneous (s.c) route with ClfA alone (1μg/mouse), or with vehicle (PBS) alone on d 0, 14 and 28. On d 35, mice were challenged with *S. aureus* ($5x10^8$CFU) via i.p. injection. At 72 h post infection, the peritoneal cavity was lavaged with PBS, and the kidneys and liver isolated to assess the bacterial burden. Analysis revealed that vaccination with antigen alone was not capable of mounting a protective immune response, as no significant differences in the local bacterial burden were observed between ClfA vaccinated mice and control mice (Figure 5.10 A). In addition, immunisation did not limit the spread of infection, with comparable bacterial loads observed between the two groups in the kidneys (Figure 5.10 B) and liver (Figure 5.10 C) at 72 h post infection.

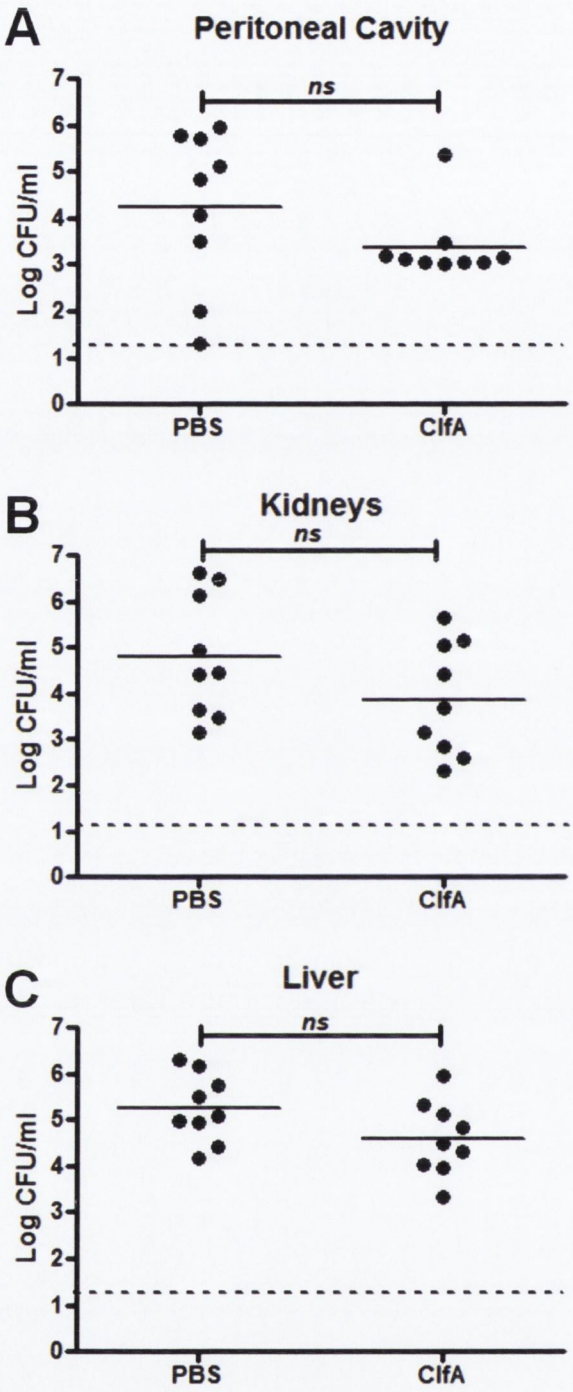


Figure 5.10: Vaccination with ClfA alone does not protect against *S. aureus* infection

Mice were vaccinated with ClfA (1μg/mouse) via s.c. injection on d 0, 14 and 28. On d 35 mice were challenged with *S. aureus* ($5x10^8$ CFU) via i.p. injection alongside a control group of non-immunised mice. At 72 h post infection the bacterial burden was assessed in the peritoneal cavity (A), kidneys (B) and liver (C). Results expresses as Log CFU/ml with means indicated by bar. Dashed lines indicate limit of detection. n=8 per group. Data pooled from 2 independent experiments.

5.2.7. Vaccination with ClfA+CpG induces IFNγ and IL-17 production by leukocytes upon in vitro stimulation with ClfA

Having identified that vaccination with antigen alone, i.e. ClfA alone, was incapable of mounting a protective immune response against *S. aureus*, the effects of combining ClfA with the Th1 inducing adjuvant CpG, were investigated. Groups of mice were vaccinated via the s.c route with CpG (50μg/mouse) alone, CpG combined with ClfA (1μg/mouse), or with PBS alone on d 0, 14 and 28. On d 63 (5 weeks after final immunisation) the inguinal lymph nodes (ILN) and spleens were isolated. Total isolated cells were stimulated *in vit*ro with ClfA (10μg/ml) or anti-CD3 and PMA as a positive control. FACS staining was carried out on cells 6 h post stimulation. Cell supernatants were taken 72 h post stimulation for analysis of secreted cytokines by ELISA.

Vaccination with ClfA+CpG drove a marked increase in IFNγ (Figure 5.11 A) and IL-17 (Figure 5.11 B) secretion by ILN cells following *in vitro* re-stimulation with ClfA in comparison to ILN cells from CpG treated mice or control mice. Cells isolated from the spleens of mice vaccinated with ClfA+CpG produced increased amounts of IFNγ upon re-exposure to ClfA *in vitro*, compared to splenic cells from CpG treated mice or control mice (Figure 5.11 C). However re-stimulation with ClfA had did not induce IL-17 production by spleen cells isolated from mice vaccinated with ClfA+CpG (Figure 5.11 D).

As CpG is known to drive Th1 responses and induce isotype switching to IgG2a (424, 425), antibody responses induced by immunisation with ClfA+CpG were investigated. IgG2a titres were significantly elevated in the sera of mice immunised with ClfA+CpG compared to control mice (Figure 5.12 A). Critically, these antibodies were functional, with sera from ClfA+CpG vaccinated mice significantly inhibiting the binding of *S. aureus* to fibrinogen via ClfA (Figure 5.12 B), thus confirming that antibodies present were specifically ClfA neutralising. Taken together, vaccination with ClfA+CpG drove cellular response mediated by both IFNγ and IL-17, as well as a humoral response against ClfA.

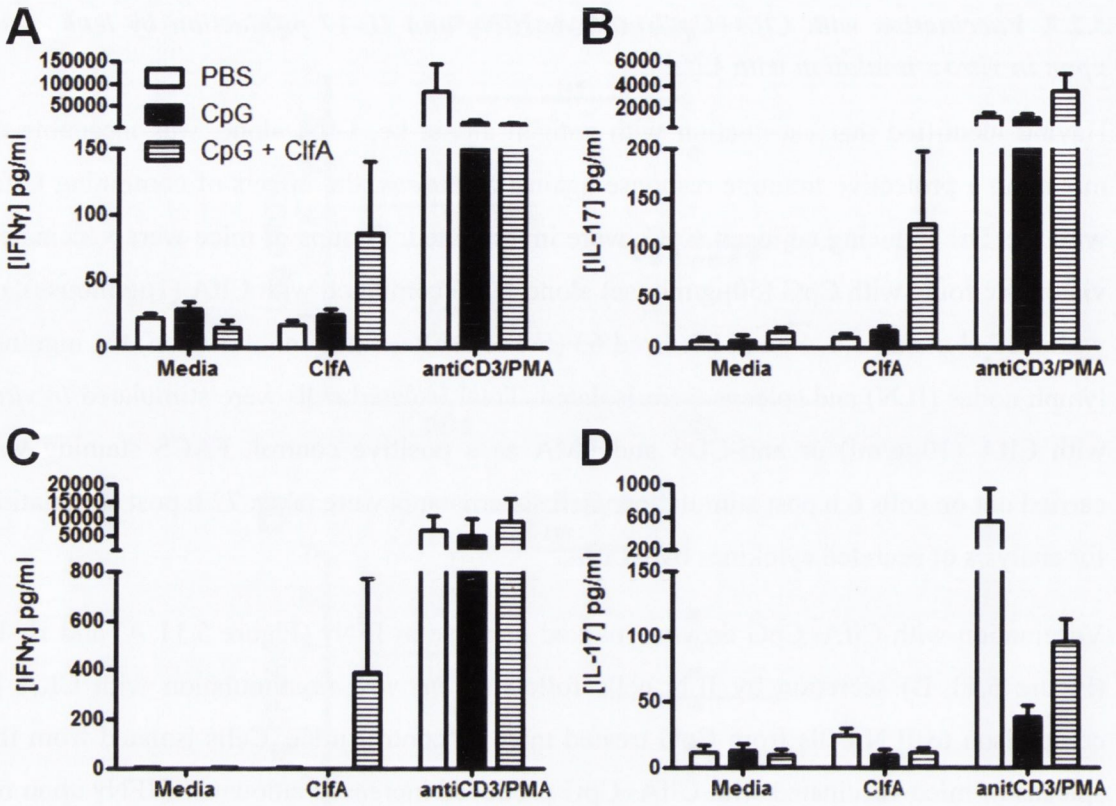


Figure 5.11: Vaccination with CpG and ClfA increases cytokine secretion ILN and spleen cells upon *in vitro* re-stimulation with ClfA

Mice were vaccinated with CpG (50μg/mouse) or CpG+ClfA (1μg/mouse) via s.c. injection on d 0, 14 and 28. On d 63 cells were isolated from the ILN (A, B) and spleens (C, D) of immunised mice and re-stimulated *in vitro* with ClfA (10μg/ml) for 72 h at 37°C. Cells isolated from the ILN and spleens of non-immunised mice were used as a negative control. After 72 h cell supernatants were collected to assess IFNγ (A, C) and IL-17 (B, D) secretion by ELISA. Results expressed as mean±SEM. n=5 per group.

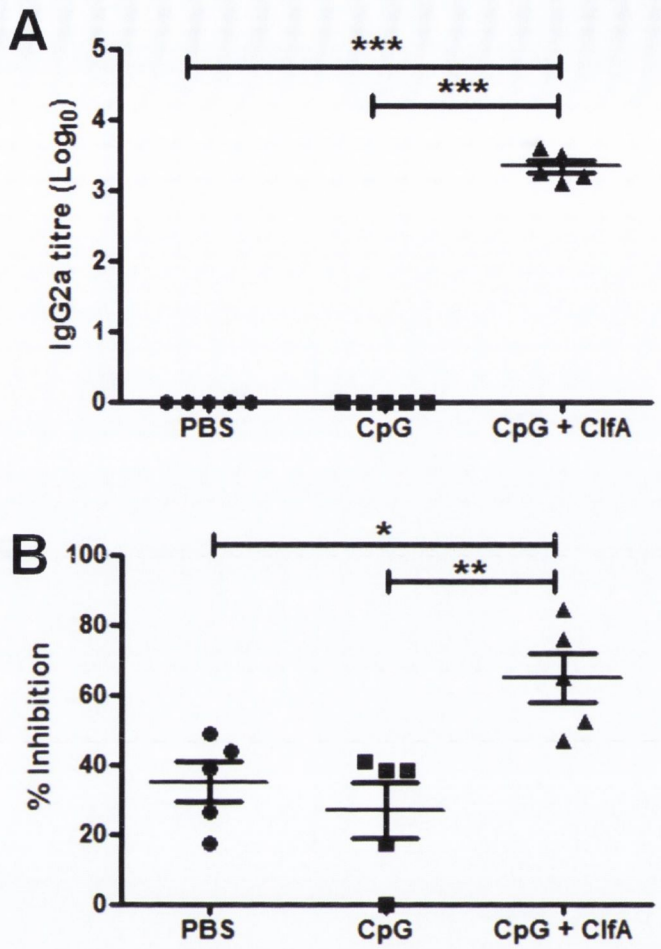


Figure 5.12: Vaccination with ClfA+CpG increases anti-ClfA serum antibody responses

Mice were vaccinated with CpG (50μg/mouse) or CpG+ClfA (1μg/mouse) via s.c. injection on d 0, 14 and 28. On d 63 sera was collected from vaccinated mice. Sera from non-immunised mice were used as a negative control. IgG2a titres were determined using ELISA (A). Neutralising antibodies were determined by measuring the ability of serum to inhibit staphylococcal adherence to fibrinogen via ClfA. Adherence was expressed as a percentage of bacterial adherence in the absence of serum and percentage inhibition was determined by subtracting the percentage value from 100 (B). Results expressed as mean±SEM. n=5 per group. *$p<0.05$, **$p<0.005$, ***$p<0.001$

5.2.8. Vaccination with CpG+ClfA enhances bacterial clearance during subsequent S. aureus infection

To test the ability of ClfA+CpG induced immunity to protect against *S. aureus* infection, groups of mice were vaccinated via the s.c route with CpG (50μg/mouse) alone, CpG combined with ClfA (1μg/mouse), or with vehicle (PBS) alone on d 0, 14 and 28. On d 63 (5 weeks post final immunisation) mice received an i.p. injection of *S. aureus* ($5x10^8$ CFU). At 24 and 72 h post infection the peritoneal cavity was lavaged to determine the bacterial burden and the organs isolated to assess the dissemination of infection.

Vaccination with ClfA+CpG enhanced the clearance of *S. aureus* infection. Bacterial burden was significantly reduced in the peritoneal cavity of mice vaccinated with ClfA+CpG compared to non-immunised or CpG treated mice (Figure 5.13 A). A significant decrease (~2.5 log) in the bacterial load was also observed in the kidneys of ClfA+CpG immunised mice (Figure 5.13 B). In the liver, a similar trend was evident with a decrease of 0.5 log CFU/ml detected in the ClfA+CpG vaccinated group (Figure 5.13 C).

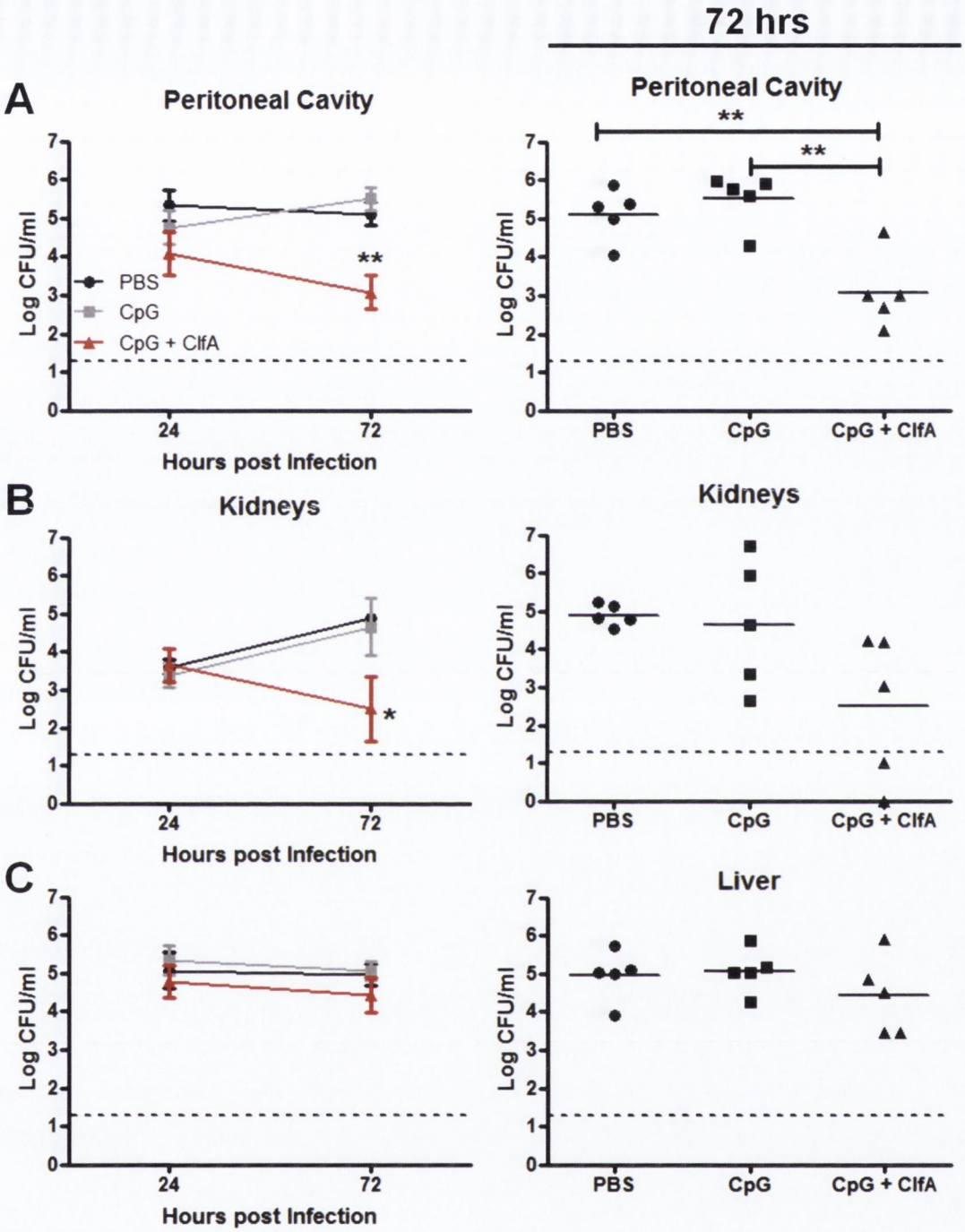


Figure 5.13: Vaccination with ClfA+CpG enhances bacterial clearance during *S. aureus* infection.

Mice were vaccinated with CpG (50μg/mouse) or CpG+ClfA (1μg/mouse) via s.c. injection on d 0, 14 and 28. On d 63 mice were challenged with *S. aureus* ($5x10^8$ CFU) via i.p. injection alongside a control group of non-immunised mice. At 24 h and 72 h post infection the bacterial burden was assessed in the peritoneal cavity (A), kidneys (B) and liver (C). Results expresses as mean Log CFU/ml of mean±SEM for time course. Bacterial burdens for individual animals are shown for the 72 h time point with means indicated by bars. Dashed lines indicate limit of detection. n=5 per group. ** $p<0.05$

5.2.9. Enhanced bacterial clearance mediated by vaccination with ClfA+CpG is associated with the induction of Th1 immunity

As vaccination with ClfA+CpG enhanced bacterial clearance during *S. aureus* infection, the type of immune response induced by immunisation was investigated. Intracellular flow cytometry staining was carried out on cells isolated from the peritoneal cavity, MLN and spleens of non-immunised mice and mice that were vaccinated with CpG alone or ClfA+CpG at 24 and 72 h post *S. aureus* infection.

Vaccination with ClfA+CpG led to elevated Th1 frequencies at the infection site 24 and 72 h post infection, compared to non-immunised mice (Figure 5.14 B). Analysis of absolute numbers of Th1 cells in ClfA+CpG vaccinated mice revealed a significant increase in Th1 cells 72 h post infection (Figure 5.14 C). A similar trend was evident in the MLN with significantly elevated proportions of Th1 cells observed in ClfA+CpG vaccinated mice compared to control mice 24 h post infection (Figure 5.14 D). By 72 h post infection, absolute numbers of Th1 cells were enhanced in MLN of ClfA+CpG vaccinated mice (Figure 5.14 E). Vaccination with ClfA+CpG did not significantly affect Th1 frequencies (Figure 5.14 F) or numbers (Figure 5.14 G) in the spleen.

An increase in peritoneal $IFN\gamma^+CD8^+$ cell proportions (Figure 5.15 B) and absolute numbers (Figure 5.15 C) was also observed 72 h post infection in ClfA+CpG immunised mice compared to control groups. Similarly, IFNγ producing CD8 cell frequencies were significantly enhanced in the MLN of ClfA+CpG vaccinated mice compared to control mice, 24 h post infection (Figure 5.15 D), with analysis of absolute numbers revealing a similar trend at this time point (Figure 5.15 E). Although analysis of splenic $IFN\gamma^+CD8^+$ T cell frequencies revealed no significant trend between ClfA+CpG vaccinated mice and control groups (Figure 5.15 F). However, significantly elevated numbers of $IFN\gamma^+CD8^+$ cells were observed in the spleens of ClfA+CpG immunised mice (Figure 5.15 G).

Overall, these results reveal that vaccination with ClfA in combination with the adjuvant CpG significantly increased IFNγ production by CD4 and CD8 T cells, both at the site of infection and in the draining MLN, a profile characteristic of type 1 immunity. It appears that during the course of infection, vaccine induced Th1 and $IFN\gamma^+CD8^+$ cells drain from the MLN to the peritoneal cavity between 24 and 72 h post infection where they mediate enhanced bacterial clearance of *S. aureus* infection. Of note, vaccination with ClfA+CpG had no significant effect on the production of IFNγ by γδ T cells (data not shown).

IL-17 production by CD4 and CD8 cells from vaccinated mice was also investigated. Although proportions of peritoneal Th17 cells were not significantly affected by vaccination with ClfA+CpG (Figure 5.16 B), analysis of absolute numbers revealed significantly elevated Th17 cell numbers in the peritoneal cavity of ClfA+CpG vaccinated mice compared to non-immunised mice 72 h post infection (Figure 5.16 C). Vaccination with ClfA+CpG did not significantly affect MLN Th17 cell frequencies (Figure 5.16 D) or numbers during *S. aureus* infection (Figure 5.16 E). Significantly increased Th17 cell proportions (Figure 5.16 F) and numbers (Figure 5.16 G) were detected in spleens of ClfA+CpG vaccinated mice compared to control groups 72 h post infection. Overall however, vaccination with ClfA+CpG did not drive as strong a Th17 response at the infection site as it did Th1, for example $4x10^4$ peritoneal Th1 cells vs. 0.25 $x10^4$ peritoneal Th17 cells were detected in ClfA+CpG vaccinated mice 72 h post infection, with a similar trend was observed in the MLN and spleen.

Vaccination with ClfA+CpG did not significantly affect IL-17^+CD8^+ T cells frequencies or numbers in the peritoneal cavity (Figure 5.17 B & C) or MLN (Figure 5.17 D & E) during *S. aureus* infection. Analysis of IL-17 production by CD8 T cells however did reveal significant increases in IL-17^+CD8^+ cell frequencies (Figure 5.17 F) and numbers (Figure 5.17 G) in the spleens of ClfA+CpG vaccinated mice compared to control mice 24 and 72 h post infection. Again, this IL-17 response by splenic $CD8^+$ T cells was not as strong the IFNγ response by these cells ($27.3x10^4$ splenic IFNγ^+CD8^+ cells vs. 11.0 $x10^4$ splenic IL-17^+CD8^+ cells in ClfA+CpG vaccinated mice 72 h post infection).

Overall, vaccination with ClfA+CpG did not drive as robust an IL-17 response during *S. aureus* infection as it did an IFNγ response. However, significant increases in IL-17 responses by αβ T cells were detected in the spleens of ClfA+CpG immunised mice suggesting a partial, albeit lesser, role for these cell types in vaccine mediated immunity against *S. aureus* infection.

To determine if the cytokine producing cells expanded in the vaccinated mice exhibited a memory phenotype, expression of the memory markers CD44 and CD62L by CD4 and CD8 cells was assessed. Effector memory cells were identified by their $CD44^{hi}CD62L^{hi}$ profile. Vaccination with ClfA+CpG did not significantly affect effector memory CD4 proportions (Figure 5.18 B) in the peritoneum, however the absolute numbers of $CD44^{hi}CD62L^{hi}$ $CD4^+$ T cells were significantly increased in ClfA+CpG vaccinated mice compared to control mice 72 h post infection (Figure 5.18 C). Frequencies of effector

memory CD4 cells detected in the MLNs were significantly increased in CpG+ClfA vaccinated mice 24 h post infection (Figure 5.18 D), with analysis of absolute numbers revealing a similar trend 72 h post infection (Figure 5.18 E). Vaccination with CpG+ClfA significantly increased both the frequency (Figure 5.18 F) and numbers (Figure 5.18 G) of CD4 effector memory cells in the spleens 72 h post infection. No differences in expression of effector memory markers were observed on CD8 T cells from CpG+ClfA vaccinated mice compared to control mice either in the peritoneal cavity, MLN or spleen (data not shown).

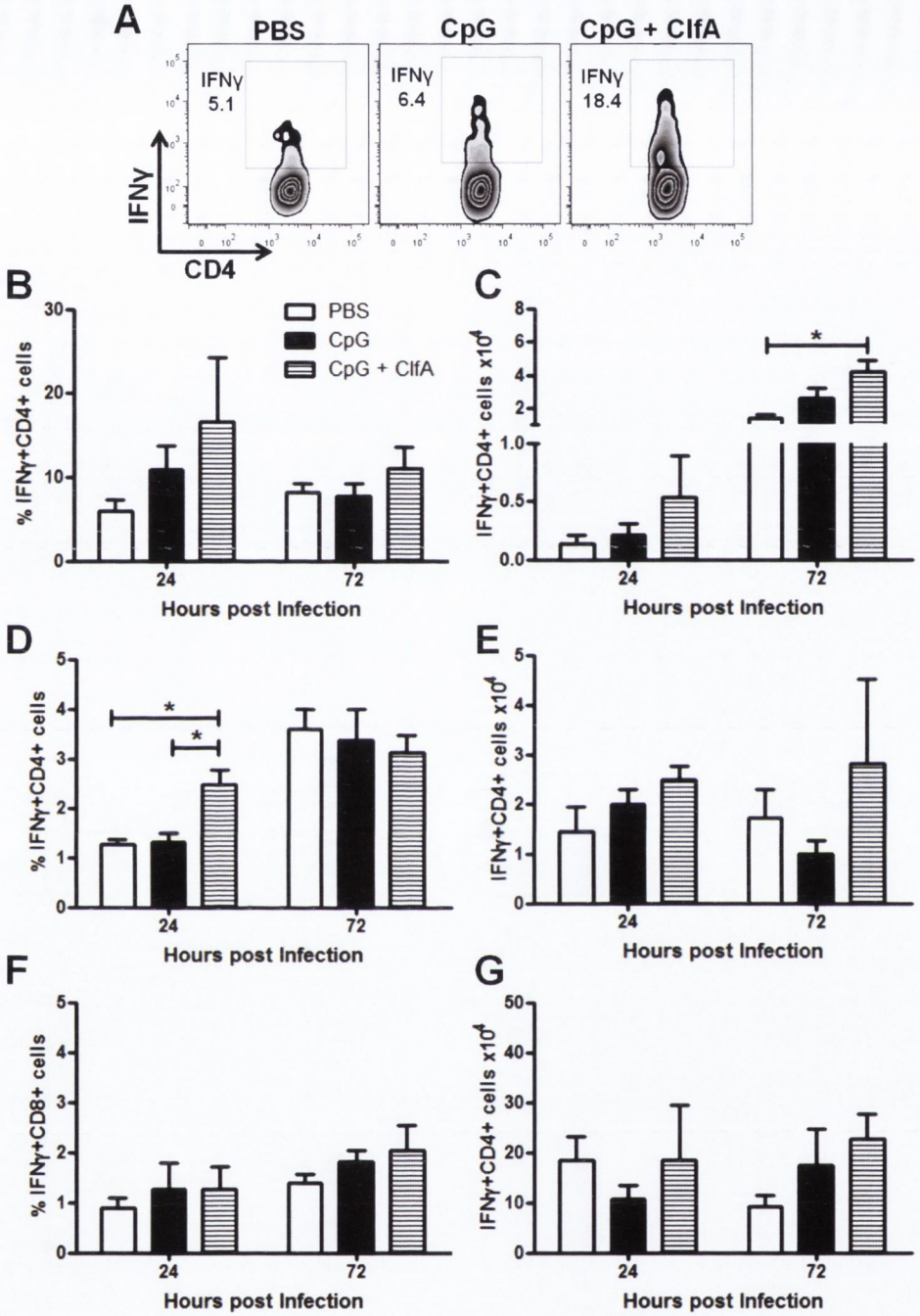


Figure 5.14: Vaccination with ClfA+CpG increases IFNγ production by CD4$^+$ T cells during *S. aureus* infection

Mice were vaccinated with CpG (50μg/mouse) or CpG+ClfA (1μg/mouse) via s.c. injection on d 0, 14 and 28. On d 63 mice were challenged with *S. aureus* (5x10^8 CFU) via i.p. injection alongside a control group of non-immunised mice. At 24 h and 72 h post infection cells were isolated from the peritoneal cavity (B, C), MLN (D, E) and spleen (F, G) of vaccinated and non-vaccinated mice to assess the proportions (B, D, F) and absolute numbers (C, E, G) of IFNγ$^+$CD4$^+$ T cells. Results expressed as mean±SEM (B-F) with representative FACS plots from PEC 72 h post infection shown (A). n=5 per group. *$p<0.05$

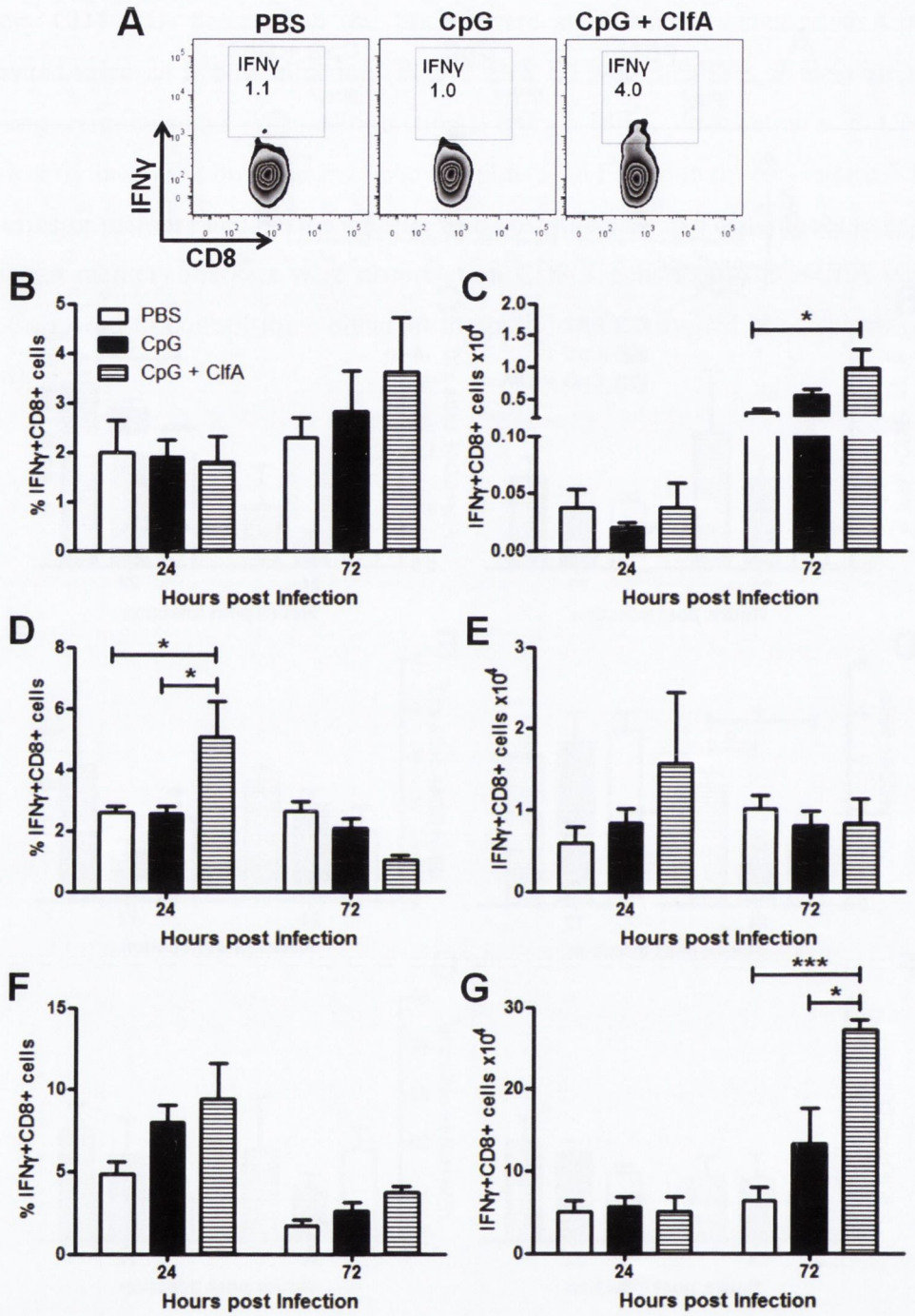


Figure 5.15: Vaccination with ClfA+CpG increases IFNγ production by $CD8^+$ T cells during *S. aureus* infection

Mice were vaccinated with CpG (50μg/mouse) or CpG (50μg/mouse) and ClfA (1μg/mouse) via s.c. injection on d 0, 14 and 28. On d 63 mice were challenged with *S. aureus* ($5x10^8$ CFU) via i.p. injection alongside a control group of non-immunised mice. At 24 h and 72 h post infection cells were isolated from the peritoneal cavity (B, C), MLN (D, E) and spleen (F, G) of vaccinated and non-vaccinated mice to assess the proportions (B, D, F) and absolute numbers (C, E, G) of $IFN\gamma^+CD8^+$ T cells. Results expressed as mean±SEM (B-F) with representative FACS plots from PEC 72 h post infection shown (A). n=5 per group. *$p<0.05$, ***$p<0.001$

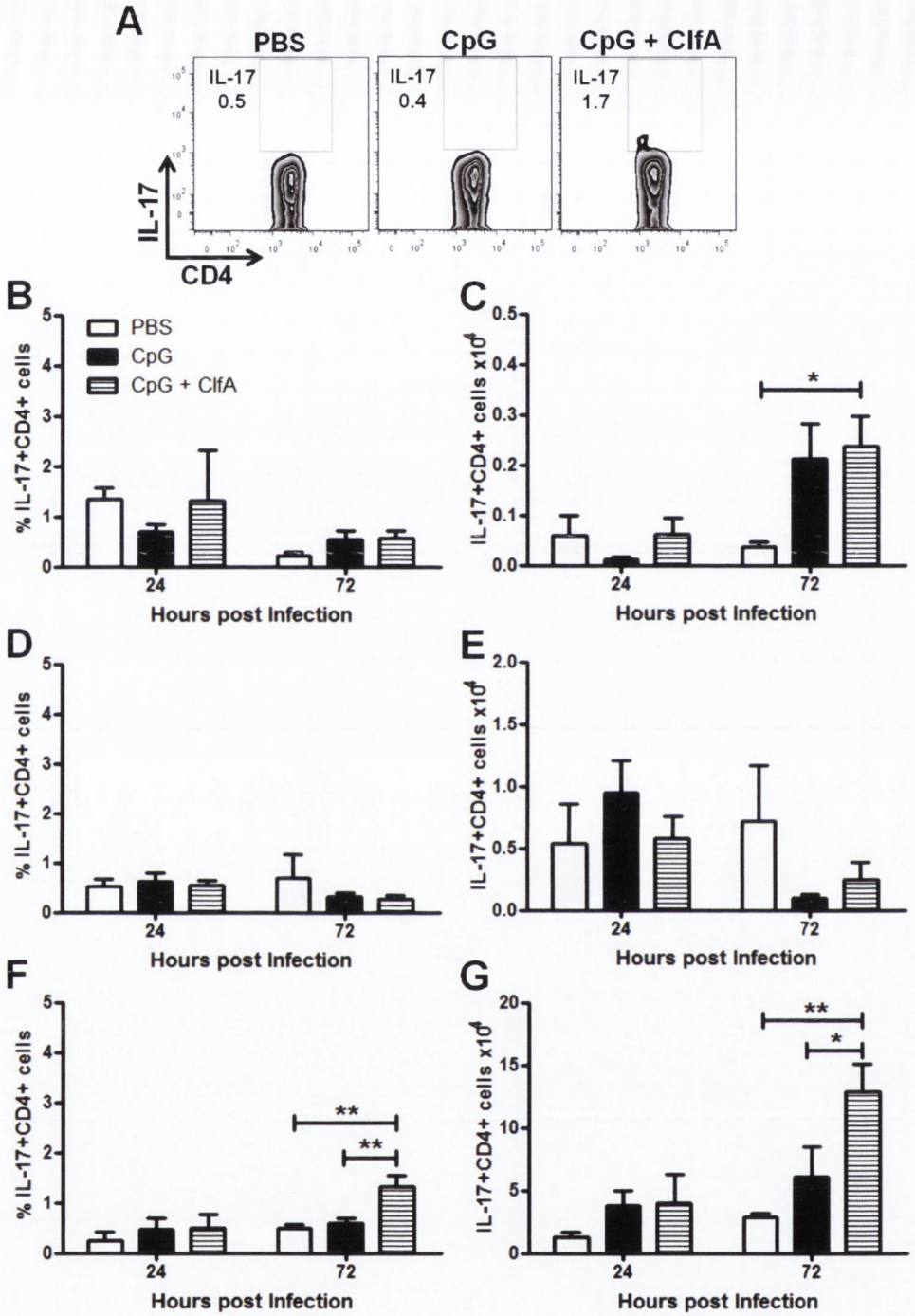


Figure 5.16: Vaccination with ClfA+CpG increases IL-17 production by $CD4^+$ T cells in the peritoneal cavity and spleen, but not MLN, during *S. aureus* infection

Mice were vaccinated with CpG (50μg/mouse) or CpG+ClfA (1μg/mouse) via s.c. injection on d 0, 14 and 28. On d 63 mice were challenged with *S. aureus* ($5x10^8$ CFU) via i.p. injection alongside a control group of non-immunised mice. At 24 h and 72 h post infection cells were isolated from the peritoneal cavity (B, C), MLN (D, E) and spleen (F, G) of vaccinated and non-vaccinated mice to assess the proportions (B, D, F) and absolute numbers (C, E, G) of $IL\text{-}17^+CD4^+$ T cells. Results expressed as mean±SEM (B-F) with representative FACS plots from splenic cells 72 h post infection shown (A). n=5 per group. *$p<0.05$, **$p<0.005$

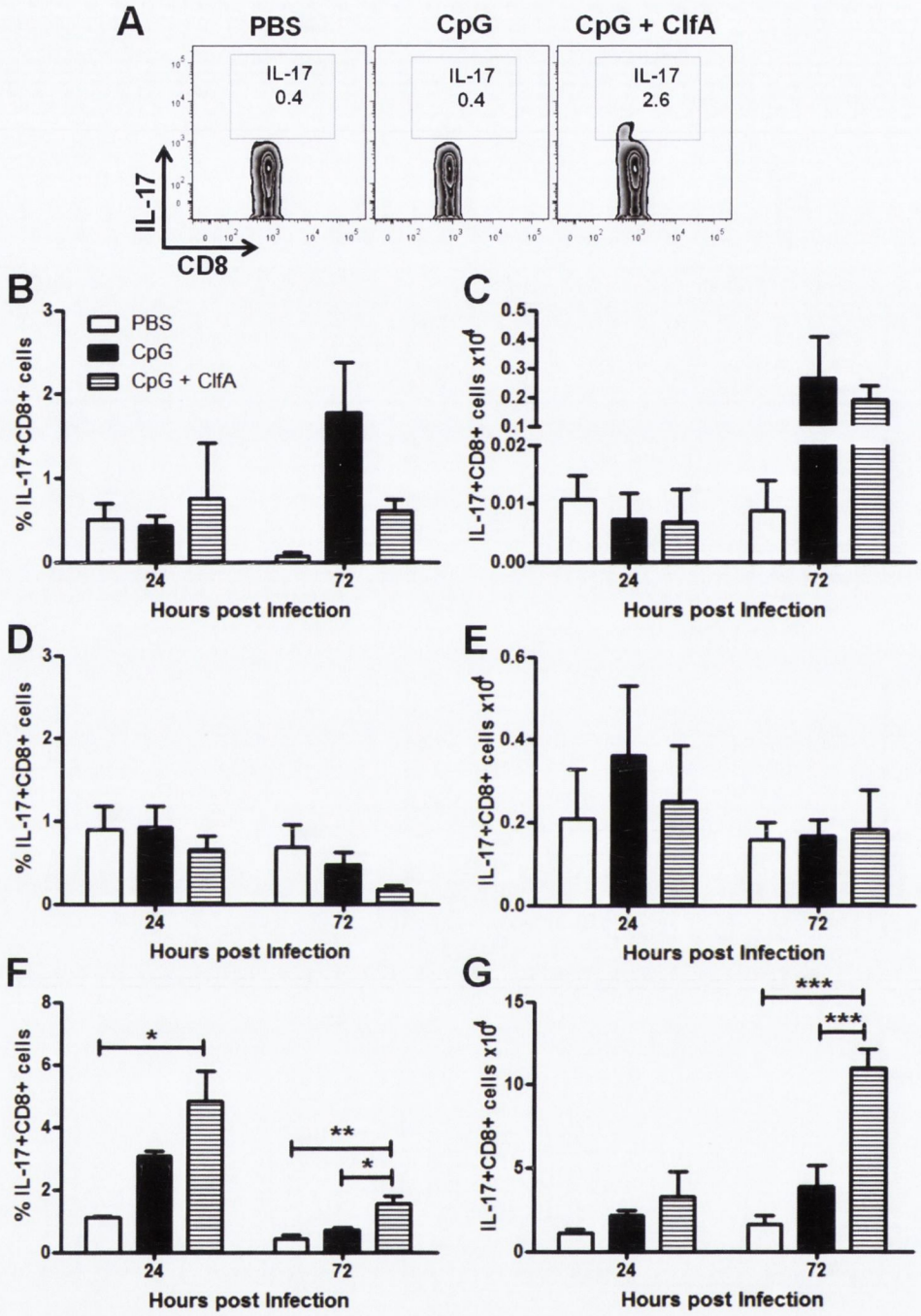


Figure 5.17: Vaccination with ClfA+CpG increases IL-17 production by CD8$^+$ T cells in the spleen, but not the peritoneal cavity or MLN during *S. aureus* infection

Mice were vaccinated with CpG (50µg/mouse) or CpG+ClfA (1µg/mouse) via s.c. injection on d 0, 14 and 28. On d 63 mice were challenged with *S. aureus* ($5x10^8$ CFU) via i.p. injection alongside a control group of non-immunised mice. At 24 h and 72 h post infection cells were isolated from the peritoneal cavity (B, C), MLN (D, E) and spleen (F, G) of vaccinated and non-vaccinated mice to assess the proportions (B, D, F) and absolute numbers (C, E, G) of IL-17$^+$CD4$^+$ T cells. Results expressed as mean±SEM (B-F) with representative FACS plots from splenic cells 72 h post infection shown (A). n=5 per group. *p<0.05, **p<0.005, ***p<0.001

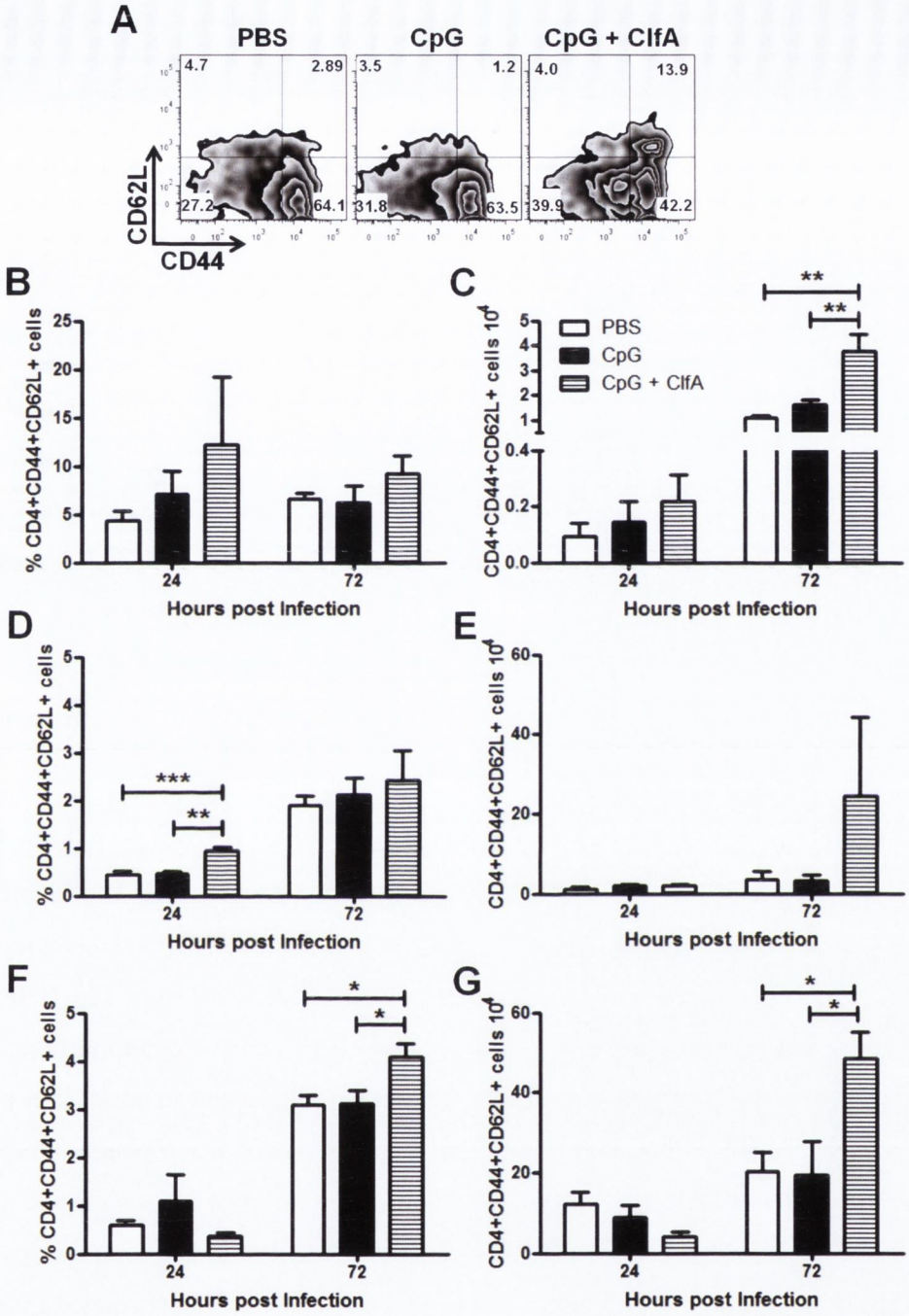


Figure 5.18: Vaccination with ClfA+CpG drove enhanced effector memory CD4+T cell accumulation during *S. aureus* infection

Mice were vaccinated with CpG (50µg/mouse) or CpG+ClfA (1µg/mouse) via s.c. injection on d 0, 14 and 28. On d 63 mice were challenged with *S. aureus* ($5x10^8$ CFU) via i.p. injection alongside a control group of non-immunised mice. At 24 h and 72 h post infection cells were isolated from the peritoneal cavity (B, C), MLN (D, E) and spleen (F, G) of vaccinated and non-vaccinated mice to assess the proportions (B, D, F) and absolute numbers (C, E, G) of $CD4^+$ T cell that were $CD44^{hi}CD62L^{hi}$. Results expressed as mean±SEM (B-D) with representative FACS plots from PEC 72 h post infection shown (A). n=5 per group. *p<0.05, **p<0.005

5.2.10. Vaccination with ClfA+CpG enhanced phagocyte activation during S. aureus infection

The enhanced bacterial clearance induced by vaccination with ClfA+CpG was associated with enhanced type 1 immunity during *S. aureus* infection. The effects of vaccine induced type 1 responses on downstream events were assessed to elucidate a mechanism of protection. Chemokine production and resulting phagocyte influx to the infection site were compared 24 and 72 h post infection between non-immunised mice and mice vaccinated with CpG alone or ClfA+CpG.

There was no significant difference in KC, MIP-2, MCP-1 or RANTES secretion between ClfA+CpG vaccinated mice and control groups at 24 and 72 h post infection (data not shown). These chemokines likely play important roles during the acute phases of *S. aureus* infection, i.e. 3 h post infection, and investigation at this time point may reveal more robust trends. Vaccination with ClfA+CpG did not affect the influx of macrophages (Figure 3.19 B) or neutrophils (Figure 3.19 C) into the peritoneal cavity compared to non-immunised mice during *S. aureus* infection.

Phagocyte activation marker expression and activation status of the phagocytes recruited to the infection site in ClfA+CpG vaccinated mice was investigated. Analysis revealed that although phagocyte influx was unaffected by ClfA+CpG vaccination, the phagocytes present in ClfA+CpG vaccinated mice displayed increased activation. Using 123-DHR to measure ROS present within the cells (334), significantly elevated proportions of macrophages with ROS activity was detected in ClfA+CpG vaccinated mice at 72 h post infection compared to control groups (Figure 5.20 A). Analysis of macrophage numbers with ROS activity did not reflect this, with no trend observed between vaccinated and control groups (Figure 5.20 B). Similarly, a significant increase in the frequencies of neutrophils with ROS was detected in both CpG treated and ClfA+CpG vaccinated groups compared to non-immunised controls 72 h post infection (Figure 5.20 C). Analysis of neutrophil numbers with ROS activity reflected this, with a marked increase in the numbers of neutrophils with ROS activity detected in CpG and ClfA+CpG treated groups 72 h post infection (Figure 5.20 D).

Although CpG treatment is associated with enhanced antigen presentation through up-regulation of co-stimulatory ligands (425), proportions of MHC II expressing macrophages were not significantly affected by vaccination with CpG+ClfA (data not shown). However, analysis of MFI revealed a marked increase in MHC II expression by macrophages from

CpG+ClfA vaccinated mice 72 h post infection compared to control groups (Figure 3.21 A), suggesting an upregulation of MHC II molecules on individual macrophages upon vaccination with CpG+ClfA. Analysis of neutrophil activation markers revealed significantly enhanced CXCR2 expression in mice vaccinated with ClfA+CpG compared to control groups (Figure 5.21 B & C), a profile which is related to augmented neutrophil trafficking to infection sites (440). Taken together, these data suggest that enhanced bacterial clearance mediated by vaccination is not due to a simple increase in phagocyte influx to the infection site, but rather an enhancement of phagocyte effector functions

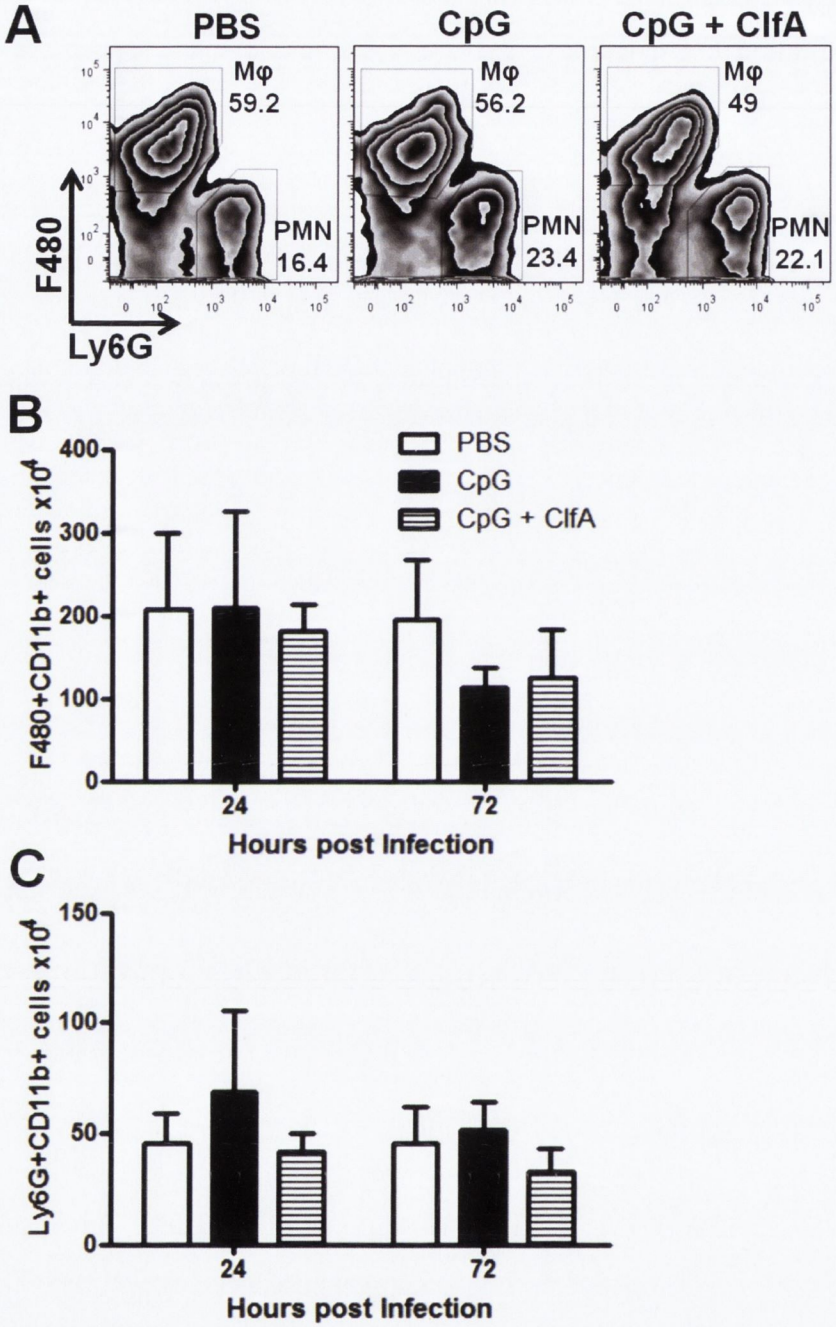


Figure 5.19: Vaccination with ClfA+CpG does not affect phagocyte influx to the peritoneal cavity during *S. aureus* infection

Mice were vaccinated with CpG (50μg/mouse) or CpG+ClfA (1μg/mouse) via s.c. injection on d 0, 14 and 28. On d 63 mice were challenged with *S. aureus* ($5x10^8$ CFU) via i.p. injection alongside a control group of non-immunised mice. At 24 h and 72 h post infection cells were isolated from the peritoneal cavity to assess the numbers of macrophages ($F480^+Ly6G^-$) (B) and neutrophils ($Ly6G^+F480^-$) (C). Results expressed as mean±SEM (B, C) with representative FACS plots of $CD11b^+$ PEC cells 72 h post infection shown (B). n=5 per group.

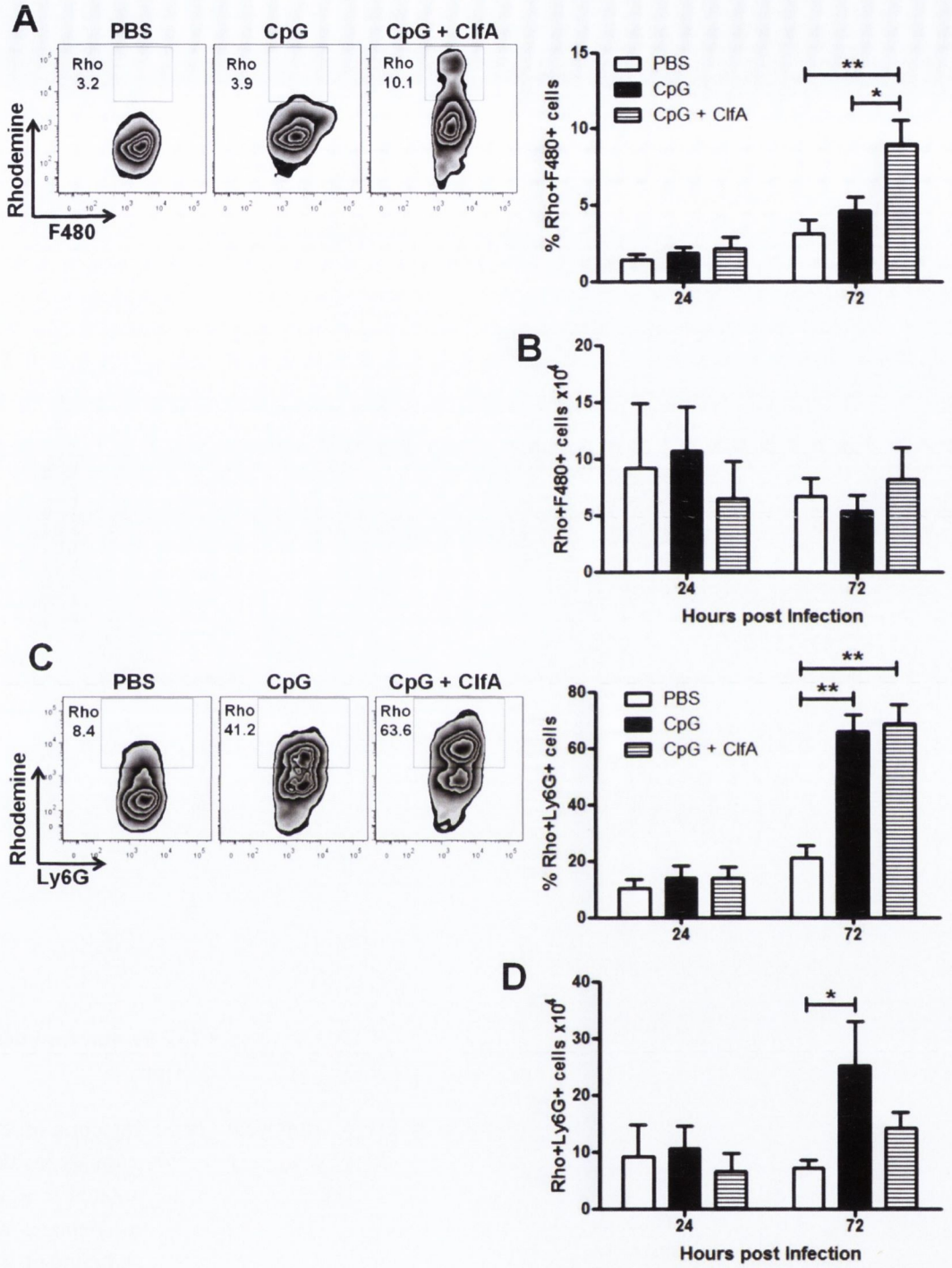


Figure 5.20: Vaccination with ClfA+CpG enhances phagocyte ROS activity during *S. aureus* infection

Mice were vaccinated with CpG (50μg/mouse) or CpG+ClfA (1μg/mouse) via s.c. injection on d 0, 14 and 28. On d 63 mice were challenged with *S. aureus* ($5x10^8$ CFU) via i.p. injection alongside a control group of non-immunised mice. At 24 h and 72 h post infection cells were isolated from the peritoneal cavity for flow cytometry staining. ROS activity in macrophages proportions (A) and numbers (B), and ROS activity in neutrophil proportions (C) and numbers (D) was detected by rhoedemine (Rho) positive cells. Results expressed as mean±SEM, with representative FACS plots from PEC 72 h post infection (A, C). n=5 per group.

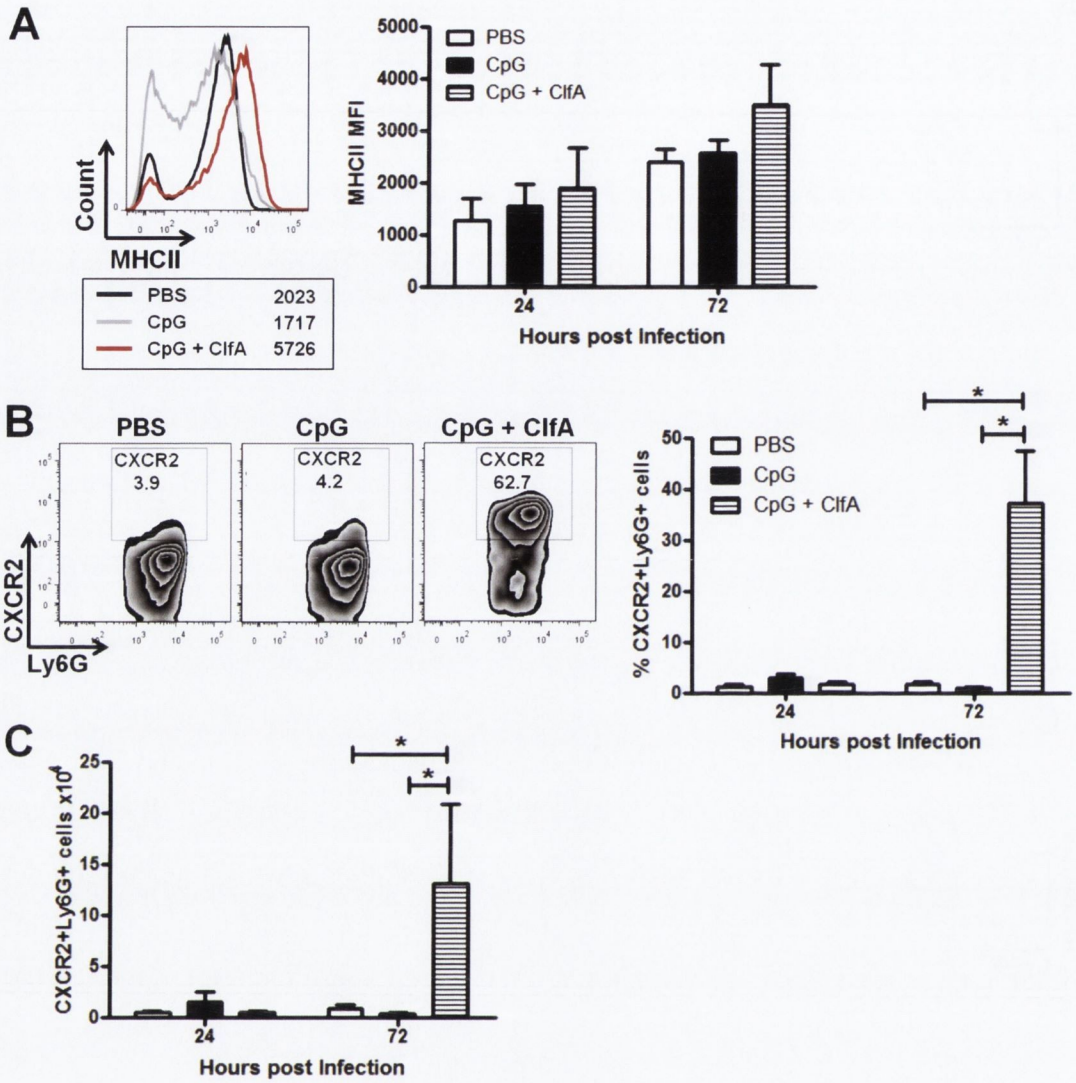


Figure 5.21: Vaccination with ClfA+CpG does not affect MHCII expression by macrophages, but does enhance CXCR2 expression by neutrophils during *S. aureus* infection

Mice were vaccinated with CpG (50μg/mouse) or CpG+ClfA (1μg/mouse) via s.c. injection on d 0, 14 and 28. On d 63 mice were challenged with *S. aureus* ($5x10^8$ CFU) via i.p. injection alongside a control group of non-immunised mice. At 24 h and 72 h post infection cells were isolated from the peritoneal cavity for flow cytometry staining. The MFI of MHC II expression by macrophages was assessed by flow cytometry (A). CXCR2 expression by neutrophil proportions (B) and numbers (C) was also assessed. Results expressed as mean±SEM, with representative histogram (A) and FACS plots (B) from PEC 72 h post infection shown. n=5 per group.

5.2.11. Vaccination with UEA-1m targeted, ClfA loaded nanoparticles induces cellular immunity in the absence of humoral immunity

Collaborative work was carried out with the Adjuvant Research Group at TCD utilising novel adjuvant technology to investigate the validity of mucosal vaccination as an anti-*S. aureus* vaccine strategy. Initial work by the Adjuvant Research Group established that UEA-1m targeted nanoparticles loaded with ClfA could promote antigen-specific T cells in the absence of an antibody response (Figure 5.22 & 23). Groups of mice were vaccinated intranasally with either UEA-1m targeted, ClfA loaded nanoparticles or ClfA+QS21 on d 0, 14 and 28. On d 35, splenic cells were isolated from vaccinated mice and stimulated *in vitro* with ClfA (10μg/ml). Cells isolated from non-vaccinated mice were used as a control.

In vitro re-stimulation with ClfA induced increased IFNγ by splenic $CD4^+$ (Figure 5.22 B) and $CD8^+$ (Figure 5.22 C) T cells isolated from mice vaccinated with UEA-1m targeted, ClfA loaded nanoparticle vaccinated mice, compared to control mice and QS21 vaccinated mice. Similarly, re-exposure to ClfA *in vitro* resulted in increased IL-17 secretion by splenic $CD4^+$ (Figure 5.22 E) and $CD8^+$ (Figure 5.22 F) T cells isolated from mice vaccinated with UEA-1m targeted, ClfA loaded nanoparticles. Analysis of antibody titres in these mice revealed that anti-ClfA antibodies were undetectable in sera from mice vaccinated with UEA-1m targeted, ClfA loaded nanoparticles (Figure 5.23 A). Conversely, high titres of ClfA specific IgG (Figure 5.23 A) and neutralising antibodies (Figure 5.23 B), capable of blocking adherence of *S. aureus* to fibrinogen via ClfA, were induced by vaccination with ClfA+QS21.

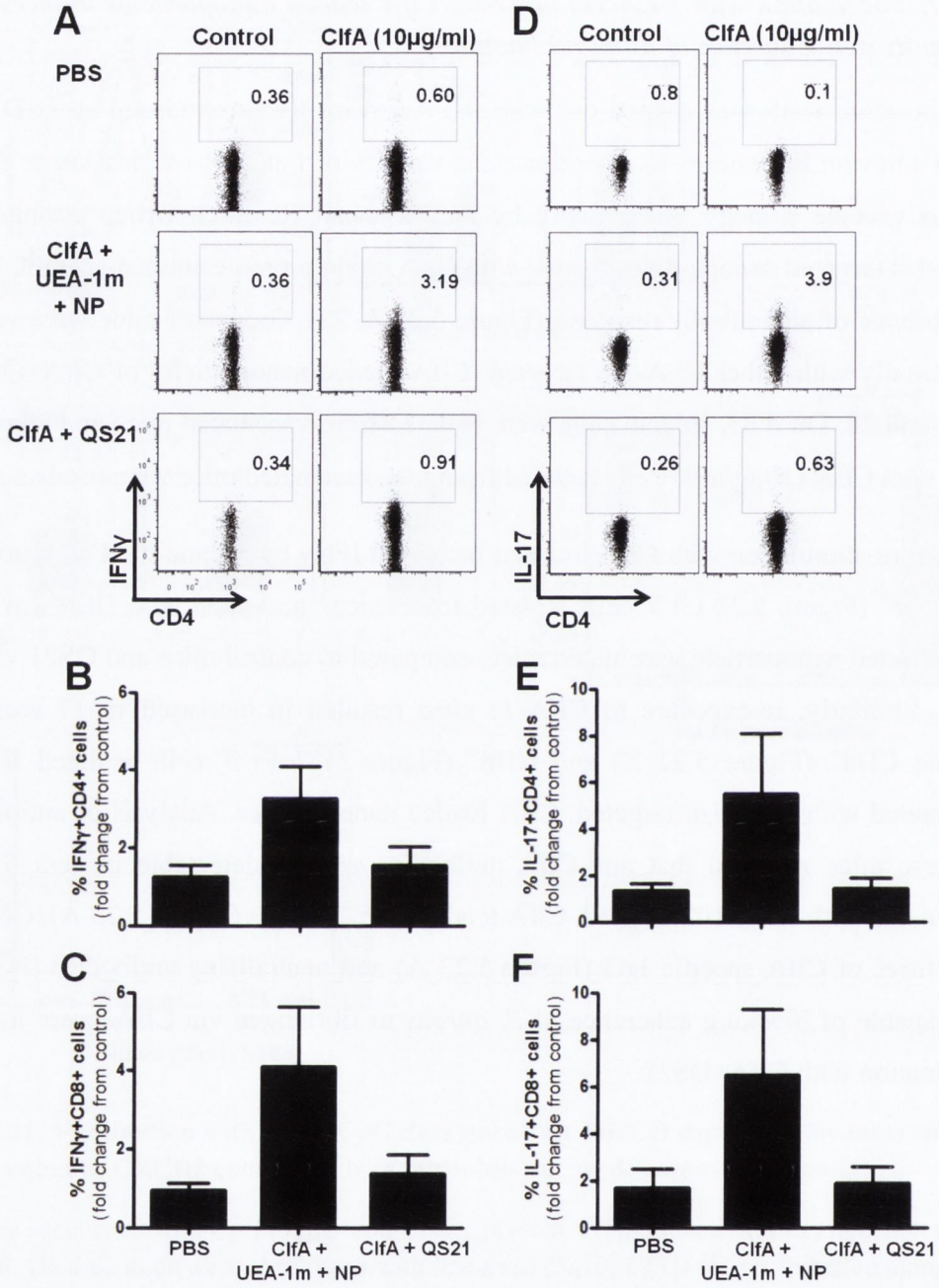


Figure 5.22: Intranasal vaccination with UEA-1m targeted, ClfA loaded nanoparticles induces IFNγ and IL-17 secretion by T cells upon *in vitro* re-stimulation with ClfA

Mice were immunised intranasally on d 0, 14 and 28 with UEA1-m (10µg/mouse) targeted, ClfA (2µg/mouse) loaded nanoparticles (NP) or with ClfA (2µg/mouse) and QS21 (10µg/mouse). On d 35 spleen cells from vaccinated and non-immunised mice were isolated and stimulated *in vitro* with ClfA (10µg/ml) for 6 h at 37°C. IFNγ production by $CD4^+$ (B) and $CD8^+$ (C) T cells and IL-17 production by $CD4^+$ (E) and $CD8^+$ (F) T cells was assessed by flow cytometry. Results expressed as mean±SEM of fold change from un-stimulated control, with representative FACS plots of IFNγ (A) and IL-17 (D) production by CD4 T cells shown. n=5 per group. ***Experiments performed by Dr. Karen Misstear, Adjuvant Research Group, School of Biochemistry and Immunology, TCD.***

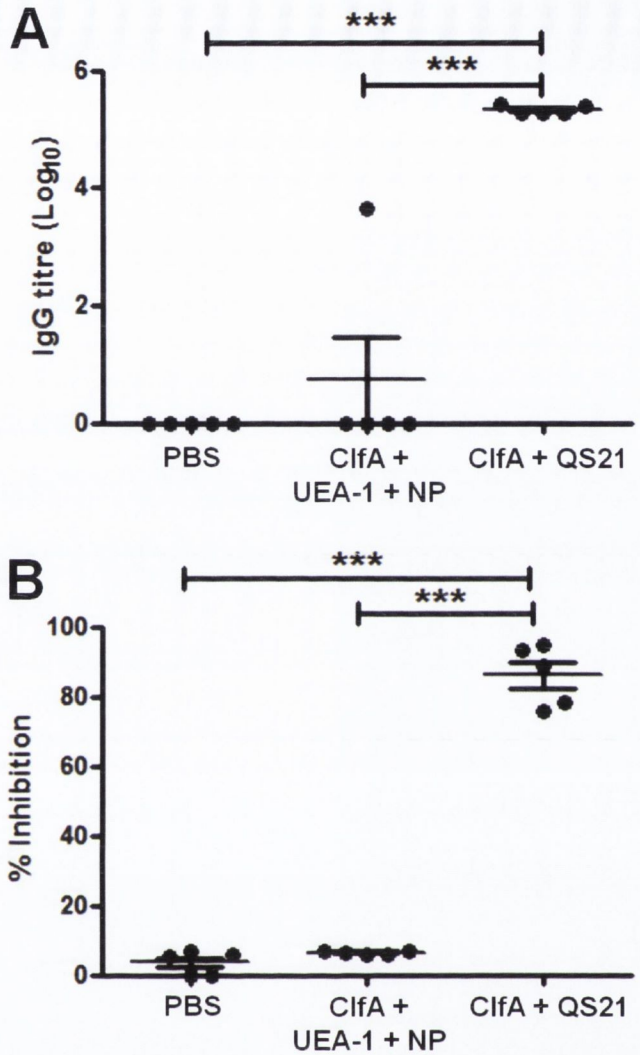


Figure 5.23: Intranasal vaccination with UEA-1m targeted, ClfA loaded nanoparticles did not induce an anti-ClfA humoral response

Mice were immunised intranasally on d 0, 14 and 28 with UEA1-m (10μg/mouse) targeted, ClfA (2μg/mouse) loaded nanoparticles (NP) or with ClfA (2μg/mouse) and QS21 (10μg/mouse). On d 35 sera was collected for analysis of ClfA specific IgG titres by ELISA (A). Neutralising antibodies were determined by measuring the ability of serum to inhibit staphylococcal adherence to fibrinogen via ClfA. Adherence was expressed as a percentage of bacterial adherence in the absence of serum and percentage inhibition was determined by subtracting the percentage value from 100 (B). Results expressed as mean±SEM. n=5 per group. ***$p<0.001$. ***Experiments performed by Dr. Karen Misstear, Adjuvant Research Group, School of Biochemistry and Immunology, TCD and Prof. Joan Geoghegan, Staphylococcal Pathogenesis Group, Department of Microbiology, TCD.***

5.2.12. Vaccination with UEA-1m targeted, ClfA loaded nanoparticles enhances bacterial clearance upon S. aureus infection independently of an antibody response

Having determined that UEA-1m targeted, ClfA loaded nanoparticles could drive anti-ClfA cellular immunity in the absence of a humoral response, the ability of this vaccination strategy to protect against systemic *S. aureus* infection was established. Groups of mice were vaccinated intranasally with either UEA-1m targeted, ClfA loaded nanoparticles or ClfA+QS21 on d 0, 14 and 28. Mice were then challenged with *S. aureus* via i.p. injection ($5x10^8$ CFU) on d 35. At 24 h and 72 h post challenge the peritoneal cavity was lavaged with sterile PBS and the organs isolated to assess the bacterial burden.

At 72 h post challenge, the bacterial burden at the infection site was significantly reduced in mice that were vaccinated with UEA-1m targeted, ClfA loaded nanoparticles compared to non-immunised controls (Figure 5.24 A). A similar but non-significant trend was observed in the mice vaccinated with ClfA+QS21. Dissemination of bacteria to peripheral sites was analysed by quantifying bacterial burden in the kidneys and spleen. In the kidneys, both groups of vaccinated mice had significantly reduced bacterial burden compared to non-immunised mice (Figure 5.24 B). In the spleen, mice vaccinated with ClfA+QS21 had a significant decrease in bacterial burden compared to the control, with a similar trend evident in mice vaccinated with UEA-1m target, ClfA loaded nanoparticles (Figure 5.24 C).

Having established that vaccination resulted in enhanced elimination of *S. aureus* infection, the mechanism of protection was determined. ClfA-specific serum antibody titres, induced by immunisation with ClfA+QS21, remained stable during the course of infection, with high titres of IgG (Figure 5.25 A) and neutralising antibodies still present 72 h post infection (Figure 5.25 B). This humoral response was completely undetectable in animals vaccinated with the UEA1m targeted, ClfA loaded nanoparticles.

In addition to driving T cell responses (Figure 5.22), immunisation with UEA-1m targeted ClfA loaded nanoparticles resulted in enhanced macrophage recruitment to the peritoneal cavity at 72 h post infection compared to non-immunised mice (Figure 5.26 B). Furthermore, the frequency of MHC II expressing macrophages was significantly enhanced in mice vaccinated with UEA-1m targeted ClfA loaded nanoparticles compared to non-immunised and ClfA+QS21 vaccinated mice (Figure 5.26 C). Infiltration of neutrophils into the peritoneal cavity was also significantly increased at 72 h post infection in mice that were vaccinated with UEA-1m targeted ClfA loaded nanoparticles (Figure

5.26 D). Conversely, immunisation with the QS21-adjuvanted vaccine did not affect phagocyte recruitment or activation (MHC II expression on macrophages) to the peritoneum compared to control mice.

In conclusion, this study demonstrates for the first time that a mucosal vaccine, which exclusively drives cellular immunity in the complete absence of a humoral immune response, is capable of enhancing clearance of *S. aureus* infection, through the induction of antigen-specific T cells that control downstream effector phagocytes.

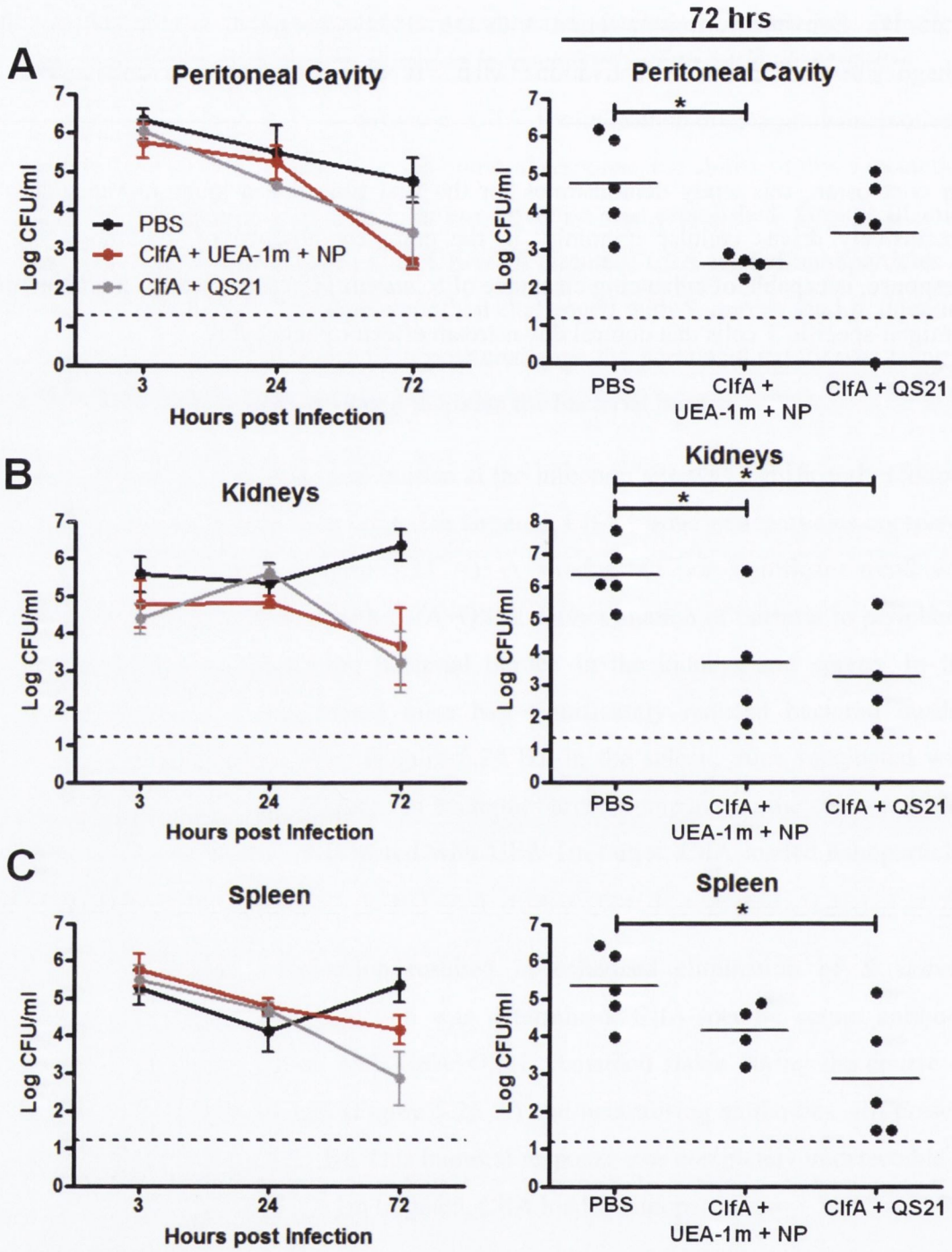


Figure 5.24: Intranasal vaccination with UEA-1m targeted, ClfA loaded nanoparticles enhances bacterial clearance of *S. aureus* infection

Mice were immunised intranasally on d 0, 14 and 28 with UEA1-m (10μg/mouse) targeted, ClfA (2μg/mouse) loaded nanoparticles (NP) or with ClfA (2μg/mouse) and QS21 (10μg/mouse). On d 35 vaccinated and non-vaccinated mice were challenged with *S. aureus* ($5x10^8$ CFU) via i.p. injection. At indicated time points post infection, the bacterial burden of the peritoneal cavity (A), kidneys (B) and spleens (C) was determined. Results expressed as mean±SEM Log CFU/ml for time course. Bacterial burdens for individual animals are shown for the 72 time point with mean indicated by bars. n=5 per group. *$p<0.05$

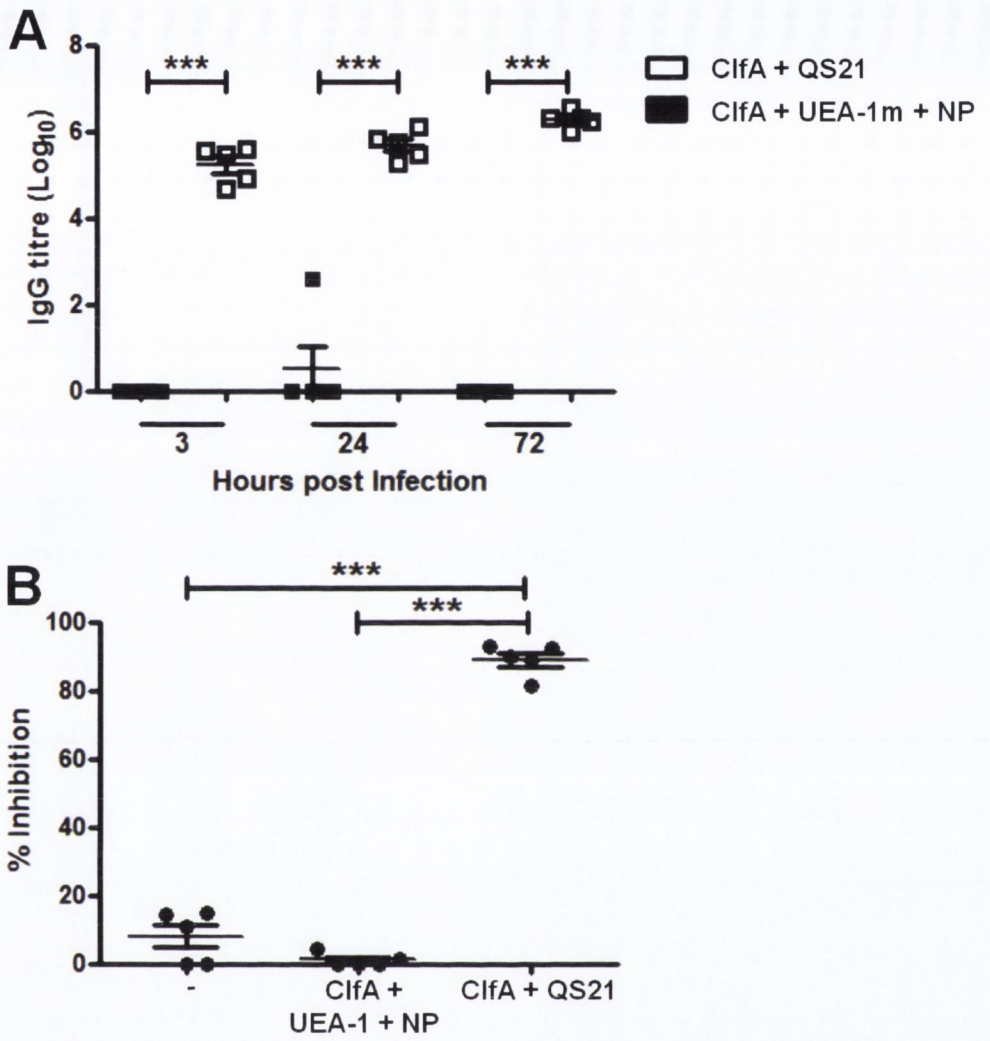


Figure 5.25: Intranasal vaccination with UEA-1m targeted, ClfA loaded nanoparticles does not induce a humoral response during *S. aureus* infection.

Mice were immunised intranasally on d 0, 14 and 28 with UEA1-m (10μg/mouse) targeted, ClfA (2μg/mouse) loaded nanoparticles (NP) or with ClfA (2μg/mouse) and QS21 (10μg/mouse). On d 35 vaccinated and non-vaccinated mice were challenged with *S. aureus* ($5x10^8$ CFU) via i.p. injection. At indicated time points post infection sera was collected for analysis of ClfA specific IgG titres by ELISA (A). At 72 h post infection neutralising antibodies were determined by measuring the ability of serum to inhibit staphylococcal adherence to fibrinogen via ClfA. Adherence was expressed as a percentage of bacterial adherence in the absence of serum and percentage inhibition was determined by subtracting the percentage value from 100 (B). Results expressed as mean±SEM. n=5 per group. ***$p<0.001$. ***Experiments performed by Dr. Karen Misstear, Adjuvant Research Group, School of Biochemistry and Immunology, TCD and Prof. Joan Geoghegan, Staphylococcal Pathogenesis Group, Department of Microbiology, TCD***

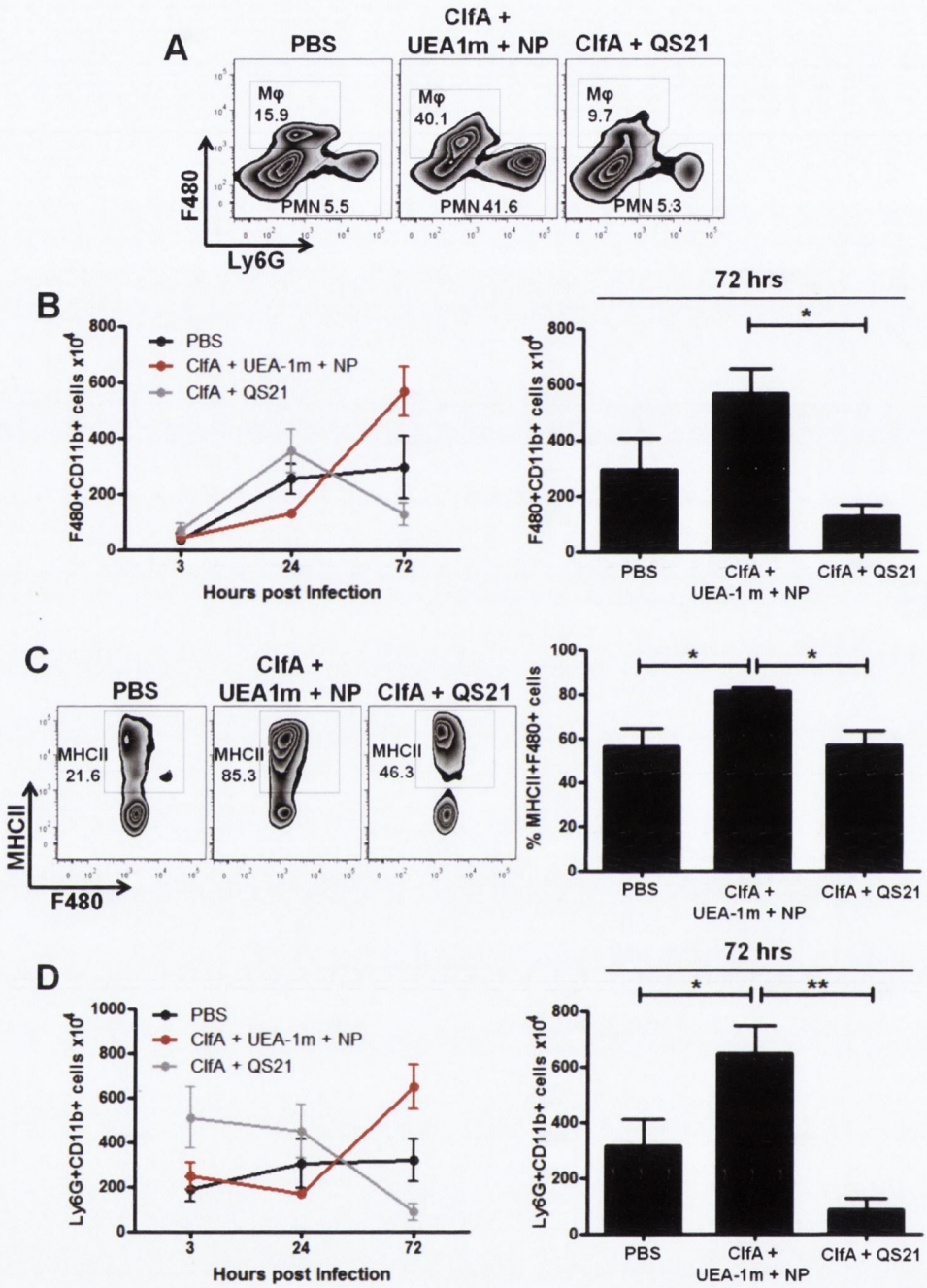


Figure 5.26: Intranasal vaccination with UEA-1m targeted, ClfA loaded nanoparticles enhances phagocyte influx and activation during subsequent *S. aureus* infection

Mice were immunised intranasally on d 0, 14 and 28 with UEA1-m (10μg/mouse) targeted, ClfA (2μg/mouse) loaded nanoparticles (NP) or with ClfA (2μg/mouse) and QS21 (10μg/mouse). On d 35 vaccinated and non-vaccinated mice were challenged with *S. aureus* ($5x10^8$ CFU) via i.p. injection. At indicated time points post infection PECs were isolated to analyse the phagocyte influx. Macrophage numbers ($F480^+Ly6G^-$) (B) were determined by flow cytometry. At 72 h post infection MHCII expression by macrophages was established (C). Neutrophil numbers ($Ly6G^+F480^-$) (D) were also determined flow cytometry. Results expressed as mean±SEM for time course and 72 h post infection, with representative FACS plots of $CD11b^+$ cells demonstrating gating strategy (A) and $MHCII^+F480^+$ cells (C) from PEC 72 h post infection shown. n=5 per group. *p<0.05, **p<0.005.

5.3. Discussion

Conflicting evidence currently exists on the importance of IFNγ signalling in natural anti-staphylococcal immunity (169, 323-327). However, several reports have identified a protective role for IFNγ signalling in acquired immunity against *S. aureus* infections (329, 330, 407). The studies outlined in Chapter 3 using the prior exposure model suggest a protective role for *S. aureus* antigen-specific IFNγ producing T cells during *S. aureus* induced peritonitis. Expanding on this, adoptive transfer of *S. aureus* antigen-specific Th1 cells to naive mice enhanced bacterial clearance of *S. aureus* infection. Interestingly, this protective response was only induced when Th1 cells originating from the peritoneal cavity of prior exposed mice were transferred, but not cells from the MLN or spleens of prior exposed mice. Analysis of cytokine secretion by the polarised Th1 cells revealed that T cells isolated from the peritoneal cavity and polarised *in vitro* under Th1 conditions produced substantially greater quantities of IFNγ (7205.84 pg/ml) compared to polarised MLN (9.37 pg/ml) and spleen cells (516.6 pg/ml), suggesting why these PECs were most efficient at enhancing staphylococcal elimination upon transfer. During *S. aureus* induced peritonitis, peritoneal Th1 cells represent the most abundant source of IFNγ, as MLN and spleen T cells produce minimal IFNγ in this model (Section 3.2.9), and as such these peritoneal cells may be more readily polarised *in vivo* to produce greater quantities of IFNγ. Given that total cells were stimulated in the *in vitro* cultures, the peritoneal APCs may represent a more activated phenotype, compared to those originating from the MLN and spleen, as these cells were isolated from the site where infection took place. Therefore, these peritoneal APCs may be better equipped to present antigens within the heat killed *S. aureus* suspension to CD4 cells, thereby driving greater IFNγ production by CD4 cells within the peritoneal cultures.

Adoptive transfer of *S. aureus*-specific Th1 cells that originated in the peritoneal cavity correlated with a significant increase in local MIP-2 and RANTES secretion in the peritoneal cavity upon infection. Interestingly, this trend is similar to that observed in prior exposed mice during *S. aureus* infection (Section 3.2.8). This increase in MIP-2 correlated with a marked increase in neutrophils in Th1 recipient mice 24 h post infection. By 72 h post infection however, neutrophil influx was significantly decreased in mice that received transfers of Th1 cells. This suggests that initially, the downstream effects of Th1 cells resulted in enhanced neutrophil recruitment, followed by a more rapid contraction of the neutrophil response. Conversely, infiltration of macrophages to the infection site was significantly increased 72 h post infection compared naive CD3 recipient mice. This

enhanced macrophage influx may be due to elevated RANTES signalling, which has been documented to induce macrophage recruitment (360, 361). Taken together, these results reveal that Th1 induced protection is associated with a controlled neutrophil response and enhanced macrophage recruitment.

Controlled neutrophil influx may actually benefit the host during *S. aureus* infections, as previous studies have correlated high neutrophil numbers with increased bacterial burden (263, 312, 323). This may be in part due to *S. aureus*' ability to survive intracellularly within neutrophils as a means of immune evasion and dissemination (315). Macrophages may be protective against *S. aureus* induced peritonitis through active phagocytosis of *S. aureus* directly, as well as controlling neutrophil responses through the ability of these cells to clear apoptotic neutrophils, culminating in maximised bacterial clearance whilst simultaneously moderating inflammation (35, 36). Transfer of Th1 cells was also associated with enhanced macrophage activation, as increased numbers of MHC II expressing macrophages were detected in Th1 recipient mice. This upregulation of MHC II could contribute to anti-*S. aureus* immunity by facilitating greater antigen presentation of staphylococcal peptides, leading to activation of additional T cells. Given that elevated macrophage influx and activation was associated with protection, it appears that macrophages play an important role in Th1 mediated anti-staphylococcal immunity.

Adoptive transfer of *S. aureus* antigen-specific Th17 cells to naive mice did not protect against *S. aureus* infection. This was somewhat surprising, as defects in Th17 responses are associated with increased susceptibility to *S. aureus* infections in humans (94, 95). Furthermore, murine studies have suggested a protective role for Th17 cells in vaccine induced immunity (329). However in our model of *S. aureus* induced peritonitis, γδ T cells represent the natural source of IL-17, rather than $CD4^+$ Th17 cells (Section 3.2.9). It is therefore likely that upon transfer of Th17 cells to the peritoneal cavity of naive mice, the environment within the peritoneal cavity could not provide the necessary cell stimuli required to maintain a Th17 phenotype, leading to Th17 cell anergy or apoptosis. In conjunction with IL-1 and IL-23 signalling, which are known to stimulate IL-17 production by γδ T cells (149), Th17 cells require antigen presentation, TGF-β and IL-6 interactions to produce IL-17 (84, 441). It is possible that these interactions did not occur within the peritoneum upon transfer, and the Th17 cells underwent apoptosis.

Having identified that Th1 cells were important in enhancing clearance of *S. aureus* infection in naive mice, a model vaccine that would drive Th1 cell responses was

developed. This was achieved through selection of a specific adjuvant known to drive Th1 immunity. The TLR9 agonist, CpG, has been previously shown to polarise Th cell responses to Th1 and away from Th2 cell responses (424, 425). Indeed, vaccination with pertussis antigens in combination with alum induced Th2/Th17 responses, whereas co-administration of antigens with CpG instead of alum shifted this response to a more protective Th1/Th17 response (345). ClfA is an attractive antigen for anti-*S. aureus* vaccines due to its expression on the bacterial surface, facilitating interactions with the host immune system, and its expression by virtually all *S. aureus* strains, potentially allowing for vaccine induced, antigen-specific responses to be protective across a diverse range of strains. In addition, ClfA is also involved in immune evasion (417, 418), thus efficient targeting of this molecule by vaccine induced immunity could potentially hinder staphylococcal immune evasion. Consequently, ClfA has demonstrated immunogenicity in numerous experimental vaccines (329, 367, 411, 420), and is currently being targeted in a multivalent vaccine under development by Pfizer (422).

Subcutaneous vaccination with ClfA+CpG induced antigen-specific IFNγ and IL-17 production upon antigen re-exposure, and drove increased IgG2a titres, which CpG is known to promote (424, 425), and neutralising antibodies. Upon *S. aureus* infection, vaccine induced protection was associated with significantly elevated levels of Th1 and $IFN\gamma^{+}CD8^{+}$ T cells in ClfA+CpG immunised mice. Although CpG is classically thought to induce a Th1 phenotype, it has also been reported to polarise Th17 responses alongside Th1 (345, 442). In this study, vaccination with CpG also drove IL-17 responses upon infection, albeit to a lesser extent than vaccine induced IFNγ responses, with IFNγ producing T cells dominating the cellular immune response. It is therefore likely that vaccine induced protection in this model is predominantly mediated by the CpG induced type 1 response against ClfA. To clarify this, future studies will administer anti-IFNγ antibodies upon infection of vaccinated mice to test if neutralisation of IFNγ responses abrogates vaccine induced protection.

Vaccination with CpG+ClfA induced expansion of CD4 effector memory cells, although a similar trend was not evident within the CD8 population. This suggests that CD4 effector memory cells may be responsible for driving the increased CD8 T cell responses observed in these mice, as opposed to the expansion of ClfA-specific memory CD8 T cells. Indeed, Th1 cells have been found to aid CD8 T cell mediated protection against *L. monocytogenes* (73, 443). It is generally accepted that *Listeria*-specific CD4 T cells protect through an

IFNγ mediated activation of macrophages, whereas *Listeria*-specific CD8 T cells do so via perforin mediated cytolysis (444, 445). In this model of subcutaneous vaccination and subsequent *S. aureus* challenge, vaccine induced ClfA-specific Th1 cells may contribute to the activation of cytotoxic CD8 T cells, which then aid in resolution of infection through clearance of intracellularly infected cells. This would enable eradication of staphylococci which exploit the intracellular environment as a means of immune evasion (315, 353), although further studies are required to investigate whether this vaccination strategy is capable of driving cytotoxic CD8 T cell responses.

Given that IFNγ signalling is known to enhance respiratory burst and the formation of ROS within phagocytes (64), ClfA+CpG vaccine induced type I immunity was associated with enhanced ROS accumulation in macrophages and neutrophils. As administration of CpG has also been documented to facilitate antigen presentation through up-regulation of co-stimulatory molecules (425), ClfA+CpG vaccine drove increased MHC II expression by individual macrophages. In addition, IFNγ has also been shown to induce enhanced expression of co-stimulatory ligands and MHC complexes (59), and it's possible that enhanced IFNγ production by CD4 and CD8 T cells could synergise with the effects induced by CpG, leading to further MHC II upregulation by macrophages. Future studies aim to look at the effects of vaccination with ClfA+CpG on other co-stimulatory ligands expressed by APCs, such as CD80 and CD86, which CpG has also been shown to up-regulate (446). Taken together, CpG+ClfA vaccine induced type I immunity which mediated enhanced clearance of *S. aureus* through increasing phagocyte activation. Of note, in this experiment the interval period between final boost and bacterial challenge was 5 weeks. This extended period was chosen because preliminary data using an interval period of 1 week indicated that CpG was capable of inducing short-term protection, possibly due to priming of innate cells.

Given that *S. aureus* has a propensity to infect mucosal tissues (228), and to colonise the anterior nares (242), intranasal vaccine strategies targeting the mucosal immune system were developed in an attempt to induce immunity at the site of entry for staphylococci. Intranasal vaccination with UEA-1m targeted, ClfA loaded nanoparticles expanded antigen-specific IFNγ and IL-17 producing CD4 and CD8 T cells in the spleens of immunised mice. Notably, no cellular responses were induced when mice were immunised with ClfA conjugated to an alternative adjuvant, QS21, previously shown to induce both cellular and humoral immunity when combined with other vaccination strategies (436-

438). In contrast, high titres of ClfA-specific IgG and neutralising antibodies were only induced by vaccination with ClfA+QS21 but not with UEA-1m targeted, ClfA loaded nanoparticles. Consequently, these vaccination strategies provided a mechanism to dissect the relative importance of humoral versus cellular immunity for anti-*S. aureus* vaccine induced protection. Importantly, these studies revealed that induction of cellular immunity alone in the complete absence of any humoral response, by vaccination with UEA-1m targeted, ClfA loaded nanoparticles, was sufficient to generate protective immunity against *S. aureus*. This protection, mediated by cellular immunity, was associated with enhanced phagocyte influx to the infection site, in addition to elevated MHC II expressing macrophages. This complements previous publications which demonstrated that vaccine induced immunity relied exclusively on T cell responses (320), specifically on Th1 and Th17 driven recruitment and activation of downstream phagocytes (329).

This targeted mucosal vaccine strategy provides a valuable tool for dissecting what constitutes protective immunity against *S. aureus*, and could also potentially be employed to dissect the importance of cellular immunity for other mucosal bacterial infections where Th1 and Th17 responses are known to be important, such as *B. pertussis* (345) and *K. pneumoniae* (74). Of note, intranasal vaccination with UEA-1m targeted, ClfA loaded nanoparticles induced a greater bacterial clearance than subcutaneous vaccination with ClfA conjugated to CpG, possibly due to *S. aureus*' propensity to infect mucosal tissues (228). This highlights mucosal vaccination as an attractive strategy for future anti-*S. aureus* vaccine designs, by promoting protective immune responses at the entry point for invading staphylococci. Successful mucosal vaccines have already been approved for the prevention of polio (447), *Samonella typhi* (448), *Vibrio Cholera* (448), rotavirus (449) and influenza virus (450), and are currently been considered in vaccine design against other pathogens, such as HIV, which specifically target the mucosa for entry (426).

Whilst traditional immunisation strategies targeting antibody responses have been pivotal in controlling or eradicating many serious infectious diseases, these conventional methods have repeatedly failed in the case of *S. aureus*. Targeting type 1 immunity through the use of adjuvants has already shown promise in improving anti-cancer (451), anti-fungal (452) and anti-parasite (453) vaccine efficacy and could potentially be extremely beneficial in the design of vaccines against bacteria, like staphylococci, capable of both extracellular and intracellular survival. In fact, numerous reports have identified the capacity of *S. aureus* to survive intracellularly as a means of immune evasion (263, 296-298, 300, 315,

353) and eradication of intracellular *S. aureus* may only be achieved through efficient triggering of type 1 immunity, known to be important for immunity against other intracellular bacterial infections (57-59, 128, 129, 131, 444). Inducing type 1 immunity in an anti-*S. aureus* vaccine could be achieved by a combination of antigens co-administered with appropriate adjuvants, which would shape the antigen-specific cellular immune response through induction of specific T cell subsets.

It is also important to consider how combining different cellular responses may benefit vaccine design. Ross *et al* demonstrated that inducing synergy between Th1 and Th17 cellular responses, through the use of select adjuvants, generated more efficient prophylactic protection against *B. pertussis* than driving Th2 and Th17 responses (345). This highlights not only the importance of cellular immunity in driving vaccine induced protection against bacterial infections, but also the significance of inducing the "correct type" of cellular immunity to induce full prophylactic protection. In order to induce specific T cell phenotypes, adjuvant selection will be of utmost importance in shaping vaccine induced cellular responses to produce the most effective forms of immunity for specific pathogens. Although CpG provided a valuable tool to induce and investigate Th1 responses both in this study and in the report by Ross *et al* (345), it is not licensed for human use. Currently, Alum, MF59 (Novartis) and AS03 (GSK) are the only adjuvants licensed for human use, highlighting the urgent need for the discovery and development of novel adjuvants capable of directing discrete T cell subsets and suitable for clinical use.

A limiting factor of this investigation is the use of a single antigen; in reality, multiple virulence factors will need to be simultaneously targeted to combat *S. aureus*' complex pathogenicity. Due to the number of staphylococcal virulence factors, all of which have multiple roles and redundant functions (454), the feasibility of targeting a single antigen to induce immunity is doubtful, as has been painstakingly demonstrated by the numerous failures of anti-*S. aureus* vaccine trials. Studies have demonstrated that combining several antigens increases vaccine efficacy compared to immunisation with individual antigens (421, 455). Therefore multivalent vaccines will be important in circumventing the diverse range of staphylococcal virulence factors (454).

Taken together, the results of this study highlight the importance of Th1 responses in both natural and vaccine induced immunity to *S. aureus* and suggest that the enhancement of Th1 responses is an important strategy to explore in advancing current *S. aureus* immunisation design. In addition, this study utilised novel adjuvant technology that

induced a purely cellular response, to categorically prove the importance of targeting cellular immunity for future anti-*S. aureus* vaccine designs. In conclusion, this study established the feasibility of a protective anti-*S. aureus* vaccine which specifically targeted T cell responses and contributes substantially to the growing literature on the importance of T cells in vaccine induced immunity against *S. aureus* infection.

Chapter 6

General Discussion

The treatment of staphylococcal infections is becomingly increasingly difficult due to the prevalence of antibiotic resistant strains, such as MRSA. The MRSA epidemic is an increasing problem in Western Europe and North America (230, 231), with MRSA strains rapidly becoming resistant to even the newest forms of antibiotics (268-270, 456). Alternative approaches to standard antibiotic therapies are therefore urgently required. Future success in the treatment of staphylococcal infections will likely rely upon the development of anti-*S. aureus* vaccines. Although substantial efforts have been employed to develop these vaccines (277, 457), thus far, all passive and active immunisation strategies have failed to show efficacy in human trials. There are several potential reasons for this, not least of which is a lack of understanding of the fundamental correlates of protective immunity against *S. aureus* infections in humans (336).

Immunology will play a vital role in establishing the correlates of anti-staphylococcal immunity and in informing the design of next generation vaccines against *S. aureus*. Traditionally, vaccines against *S. aureus* have targeted the humoral arm of immunity, however recently evidence suggests that induction of humoral immunity alone may not be sufficient to mediate full prophylactic protection (336). The absolute requirement of humoral immunity for protection against *S. aureus* infections is further brought into question as patients with cellular disorders including defects in T cells (305), particularly Th17 cells (94, 95, 305), or those with neutrophil disorders (302-304), are at increased risk of developing staphylococcal infections, whereas B cell or antibody deficiency in patients do not demonstrate increased susceptibility to *S. aureus* infections (301). Similarly, mice with cellular defects in T cells (165, 169, 320, 322), or neutrophil (263, 306-308) and macrophage (310, 311) functions suffer from exacerbated *S. aureus* infection, suggesting that cellular responses play a prominent role in anti-staphylococcal immunity.

Given the importance of cellular immunity for host resistance to *S. aureus* infection, it is now widely accepted that the development of strategies targeting the adaptive cellular response, i.e. T cells, should be explored in anti-*S. aureus* vaccine design. This concept is supported by the critical functions of T cells in anti-bacterial immunity, which include induction of antibody isotype switching and control of phagocytic cell responses. Phagocytes, in turn, work in concert with humoral responses to mediate opsonophagocytosis. In fact, generating antibody responses alone severely limits their capacity to protect if sufficient phagocytes are not recruited and/or activated to facilitate optimal opsonophagocytic killing of invading staphylococci. Therefore, it is unlikely that

antibody mediated responses should be the primary goal of vaccination against *S. aureus*. Rather, the identification of antigenic targets which induce potent T cell immunity responsive to a broad array of staphylococcal strains will likely be advantageous. Precise understanding of what drives "appropriate" cellular immunity to staphylococcal infection will provide the immunological framework for the design of these vaccines (458, 459).

Before particular T cell phenotypes can be targeted by vaccination the discrete T cell populations capable of inducing site-specific protection and natural immunity to *S. aureus* need to be identified. This thesis aimed to address this question by developing a novel model of recurrent peritonitis to investigate the effects of long term exposure on anti-staphylococcal cellular immunity. Specifically, the goal was to identify T cell subsets capable of inducing enhanced clearance upon re-exposure to *S. aureus*. Using this model, prior exposure to *S. aureus* was shown to mediate increased bacterial eradication upon subsequent infection (Table 6.1). This enhanced clearance was associated with an increase in Th1, IFNγ^{+}CD8^{+} (Figure 6.1 A) and IL-17$^{+}\gamma\delta^{+}$ T cell (Figure 6.1 B) responses and appeared to rely upon the abilities of these T cells to regulate the recruitment and activation of downstream phagocytes (Figure 6.1 D & E). Prior exposure to *S. aureus* also induced elevated antibody titres (Figure 6.1 C), akin to observations made in colonised humans, suggesting that humoral immunity may be contributing to overall protection in this model. To establish the absolute requirement of humoral immunity in mediating bacterial clearance against *S. aureus* infections a mucosal vaccination strategy capable of inducing a purely cellular response was employed. Intranasal vaccination with UEA-1m targeted, ClfA loaded nanoparticles drove protective cellular immunity against systemic *S. aureus* infection, independently of an antibody response (Table 6.1) (340). This example of vaccine induced immunity in the complete absence of an antibody response definitively proves the critical role of T cells in anti-staphylococcal immunity.

Interestingly, γδ T cell responses were elevated upon re-exposure to staphylococcal antigens during subsequent infection, suggesting for the first time that γδ T cells may be capable of recall memory responses against *S. aureus*. A recent report has revealed that protective memory γδ T cells were induced upon enteric *L. monocytogenes* infection (173). Consistent with this study, *S. aureus* memory γδ T cells were Vγ4 T cells exhibiting a CD44^{hi}CD27^{-} profile and produced increased amounts of IL-17 upon re-infection. Importantly, IL-1R and IL-23R signalling was disposable for driving IL-17 production by *S. aureus*-primed γδ T cells. As was observed by Sheridan *et al* (173), preliminary work

revealed that inhibition of MHC II signalling impaired γδ T cell responses to antigen re-stimulation suggesting antigen presentation plays a role in activation of memory γδ T cells. However further work is required to definitively establish this, and also to examine the potential contribution of other presentation molecules, such as CD1d, in memory γδ T cell activation. Moreover, the memory γδ T cells observed in this study were *S. aureus*-specific and did not respond to stimulation with *E. coli*, although additional studies with alternative bacterial species are required to confirm this specificity. Most work to date on γδ T cell responses in *S. aureus* infection has been carried out in murine models (165, 169, 322, 460), and of course *S. aureus* is a human pathogen. However, one study has demonstrated that transfer of HMBPP activated human γδ T cells protected SCID mice against subsequent *S. aureus* infection (371). Furthermore, human γδ T cells were shown to recognise and lyse SEA coated target cells (400). These studies support the hypothesis that γδ cells may play a role in human anti-staphylococcal immunity, although further studies are required to fully characterise the contribution of human γδ T cell responses in host resistance to *S. aureus* infection.

Although conflicting evidence currently exists on the importance of IFNγ signalling in natural anti-staphylococcal immunity (169, 323-327), several reports have identified a protective role for IFNγ responses in acquired immunity against *S. aureus* infections (329, 330, 407). This study examined the protective effects of IFNγ producing Th1 cells during natural immunity upon a single exposure to *S. aureus*, or in acquired immunity via repeated exposure or vaccination, within the same model of *S. aureus* induced peritonitis. Results revealed IFNγ was not induced upon a single exposure to *S. aureus*. However, repeated exposure drove the expansion of antigen-specific IFNγ producing Th1 cells, which when adoptively transferred enhanced clearance of *S. aureus* infection, suggesting that IFNγ plays an important role in generating protective acquired immunity in this model. Furthermore, induction of antigen-specific Th1 cells via vaccination with the staphylococcal antigen ClfA (currently a target in pre-clinical vaccine trials (422)) adjuvanted with CpG in a s.c. vaccine, or with UEA-1m targeted nanoparticles in an intranasal vaccine, mediated elevated elimination of *S. aureus* infection (Table 6.1), highlighting the importance of Th1 mediated responses in vaccine induced immunity. Of note, IFNγ producing CD8 T cells were also increased in the prior exposure, s.c. vaccine and intranasal vaccine models suggesting an important role for these cells alongside Th1 cells. Evidence already exists on the ability of Th1 cells and CD8 T cells to work in tandem to clear infection in a model of *L. monocytogenes,* where Th1 cells were found to

aid CD8 T cell mediated protection (73, 443). In this model of infection it is generally accepted that protection by *Listeria*-specific CD4 T cells is IFNγ mediated, whereas *Listeria*-specific CD8 T cells mediate protection by cytolysis of infected cells (444, 445). Together this suggests that type 1 immunity may be beneficial against *S. aureus* infection, possibly through mediating clearance of persisting intracellular staphylococci (315, 353) via killing of intracellularly infected cells, although further studies are required to confirm this.

Importantly, in the current study, a synergistic type 1/IL-17 response appeared to be involved in protection against *S. aureus* infection both in the prior exposure model and in the s.c. and intranasal vaccine models. This is reflective of data published by Lin *et al* which demonstrates the importance of vaccine induced Th1/Th17 cells in mediating protection against systemic *S. aureus* infection (329). However, in this current study no role for Th17 cells was detected in the prior exposure model, possibly due to the abundant production of IL-17 by γδ T cells. In the context of vaccination with ClfA+CpG minor Th17 responses were observed, with type 1 immunity comprising dominating the vaccine induced immune response. Intranasal vaccination with UEA-1m targeted, ClfA loaded nanoparticles drove antigen-specific Th17 cells within the spleen, suggesting that mucosal vaccination is an attractive strategy for induction of Th17 responses. Collectively, these studies suggest that targeting both these axis by immunisation could potentially enhance vaccine efficacy. Accumulating evidence within the literature is reflective of this, with reports proposing that efficient protection against invading bacteria, particularly at mucosal sites, requires synergy of both Th1 and Th17 immunity (74, 345, 354, 355). This current study and others therefore support the concept that vaccine induced IL-17 responses could provide surveillance in the peripheral tissues, such as the mucosa, where they naturally predominate. Most pathogens gain entry through these tissues, therefore upon infection, a rapid IL-17 response would be triggered at the inoculation site. This response would then drive a robust Th1 response, ultimately culminating in protection against bacteria capable of both intracellular and extracellular survival.

In the current study, prior exposure to *S. aureus* significantly enhanced phagocyte (predominantly neutrophils) recruitment, to the infection site 3 h post subsequent infection (Figure 6.1 D). This is similar to other studies using *E. coli* induced peritonitis where early phagocyte responses were dominated by neutrophils (155). It is likely that the enhanced MIP-2 secretion observed in prior exposed mice mediated this enhanced recruitment (362).

After phagocyte influx peaked 3 h post infection, subsequent contraction of neutrophil and macrophage numbers was observed. However, this decrease in numbers was associated with enhanced activation of residual phagocytes 12-24 h post infection (Figure 6.1 E). Adoptive transfer of either purified memory γδ T cells or *in vitro* expanded antigen-specific Th1 cells, conferred protection against *S. aureus* infection through their downstream effects on macrophage recruitment and activation 72 h post infection. These macrophage responses, induced by transfer of *S. aureus*-specific T cells, were observed after initial neutrophil responses to acute infection (i.e. 72 h post infection), and were associated with decreased neutrophil numbers and enhanced bacterial clearance at this time point (Figure 6.1 F). These studies support a new paradigm based on accumulating evidence which suggests that limiting, but not ablating, neutrophil responses is beneficial to the host upon *S. aureus* infection (263, 312, 323). It is possible that in addition to direct phagocytosis of staphylococci, macrophages aid in the resolution of inflammation through control of neutrophil responses (24, 25, 32-36). Together these studies stress the importance of T cells in controlling the process of phagocyte recruitment, activation and clearance, culminating in maximised bacterial clearance. Further work characterising the kinetics of phagocyte responses optimal for clearance of *S. aureus* infection needs to be carried out to assess the relative importance of neutrophils versus macrophages throughout the course of infection, i.e. are neutrophils essential for initial control of acute infection, whereas macrophage play a more important role later on. In addition, these experiments would place particular emphasis on the specific effects of T cell derived cytokines on both the recruitment and activation of phagocytes.

The results presented in this thesis support the notion that vaccination strategies should strive to target specific T cell subsets, thus tailoring the induced immune response to the specific pathogen e.g. activation of Th1 and CD8 T cells for protection against intracellular bacteria or the Th1/Th17 axis for protection against extracellular bacteria. Specific targeting of distinct T cell subsets can be controlled by adjuvant selection. Work by Ross *et al.* demonstrated that by using select adjuvants they could tailor their *B. pertussis* vaccine to drive either an antigen-specific Th1/Th17 phenotype by using CpG as an adjuvant or a Th2/Th17 response through use of alum (345). Vaccine induced Th1/Th17 cells mediated greater protection upon *B. pertussis* infection than Th2 and Th17 cells specific for the same antigens. In addition, enhanced vaccine induced protection against hepatitis B virus was achieved when alum was switched to a more effective adjuvant AS04 (a combination of alum and the TLR4 agonist, monophosphoryl lipid A), which facilitated macrophage

responses leading to T cell activation alongside the induction of humoral immunity (461, 462). In the current study, selection of the Th1 inducing adjuvant CpG drove protective type 1 immunity upon *S. aureus* infection. In addition, the selection of the adjuvants aimed at enhancing transepithelial antigen uptake in a mucosal vaccine strategy, induced protective systemic immunity against *S. aureus* infection. Therefore, the selection of appropriate adjuvants can be a powerful tool for enhancing vaccine efficacy through dictating and directing the nature of the antigen-specific T cell responses.

A successful anti-*S. aureus* vaccine will likely comprise of multiple antigens combined with appropriate adjuvant selection, enabling for the efficient induction of both humoral immunity and specific T cell responses, which would act in concert to protect against a broad array of staphylococcal strains (Figure 6.2). Evidence from this study suggests that a successful anti-*S. aureus* vaccine may need to generate both type 1 immunity (i.e. Th1 and CTL CD8 T cells) and IL-17 producing cells. Upon infection vaccine induced T cells would become activated to produce pro-inflammatory cytokines (such as IFNγ and IL-17) the downstream effects of which would promote phagocyte recruitment to the infection site. Vaccine induced CD8 T cells may also aid in the clearance of intracellularly infected cells, eliminating a survival niche exploited by staphylococci (315, 353). The induction of non-traditional lymphocytes such as γδ T cells capable of IL-17 production, may allow for rapid initiation of the immune response, and could provide protection at staphylococcal entry sites in the mucosal tissues. Upon phagocyte egression to the infection site these cytokines are also capable of enhancing the phagocyte's bactericidal properties, e.g. enhancing RNS and ROS production (64, 365). IL-17 is also capable of inducing AMP production (93), potentially aiding in both the eradication of extracellular and phagocytosed bacteria. Generation of antibodies could neutralise virulence factors and toxins. Vaccine induced Th1 responses could also drive isotype switching to IgG2a (66), an important serotype for opsonification, which in the presence of activated phagocytes aids in opsonophagocytic killing of the invading bacterium. Therefore induction of both arms of immunity would drive more efficient clearance of invading staphylococci, by circumventing the many diverse mechanisms that *S. aureus* uses to evade the immune response (454).

With the identification of murine memory γδ T cells (173, 463), the potential of driving protective vaccine induced responses by these non-traditional lymphocytes can now be explored. Some insights into protective memory γδ T cell responses have already been

made in both human and animal models. Patients with a rare variant of SCID, resulting in the predominance of γδ T cells over αβ T cells, respond effectively to vaccinations against diphtheria, highlighting the protective role of γδ T cell recall responses in vaccine-induced immunity (374). Emerging evidence is revealing that vaccines specifically targeting γδ T cells can be efficacious. Vaccine induced γδ T cell expansion resulted in reduced viremia and mortality upon lethal infection with West Nile Virus (375). Moreover, treatment with HMBPP and IL-2 resulted in prolonged accumulation of γδ T cells in the lungs of macaques and correlated with ameliorated lung lesion formation upon infection with *Yersinia pestis* (464). γδ T cells are also currently being targeted in novel anti-cancer vaccines involving *in vitro* activation and expansion of autologous Vγ9Vδ2 cells through phosphoantigen stimulation, followed by transfusion back into the patients, where these activated Vγ9Vδ2 cells kill transformed cells (377). In order to fully harness the powerful anti-bacterial effects of γδ T cells for *S. aureus* vaccines, novel staphylococcal antigens and γδ T cell inducing adjuvants will need to be identified. It will also be important to consider the routes of vaccine administration optimal for induction of memory γδ T cells, given the propensity of γδ T cells to home to mucosal sites.

The prior exposure model used throughout this study arose from observations by Wertheim *et al*, which revealed that *S. aureus* colonised patients had better survival rates upon infection compared to non-carriers (254), suggesting that long-term exposure to the entire bacterium impacted upon host immunity. In order to replicate this more elegantly *in vivo*, future work aims to develop novel models of extended *S. aureus* nasal colonisation in germ-free mice to expand upon this current study's identification of protective T cell expansion induced by sustained exposure to *S. aureus* via colonisation, as opposed to infection. To translate the findings of the murine γδ T cell studies to the clinical setting, the importance of γδ T cells in anti-staphylococcal immunity in humans must be validated. Future studies aim to address this by characterising γδ T cells in the PBMCs of *S. aureus* bacteraemic patients. Given that γδ T cells are more prominent in mucosal tissues as oppose to the circulation, γδ T cell responses present at alternative sites, such as the mucosae, will also need to be determined. *Listeria*-specific Vγ4 memory cells were only induced upon mucosal infection with *L. monocytogenes*, not by systemic infection, suggesting that mucosal priming plays a key role in the induction of memory γδ T cells (173). As γδ T cells preferentially localise to mucosal sites, it would be interesting to examine if mucosal vaccination is capable of inducing anti-staphylococcal γδ T cell responses, given this pathogens propensity to infect mucosal tissues (228). Expanding on

the evidence of protective type 1 immunity against *S. aureus* infection, further work characterising human Th1 and CD8 T cells within the PBMCs of *S. aureus* bacteraemic patients will also be carried out, with particular focus on demonstrating whether these cells are capable of antigen recall responses or exhibit a memory phenotype.

In summary, this thesis has successfully contributed to the fundamental understanding of T cell responses during systemic *S. aureus* infection. The work strongly supports the importance of T cell responses in protection against *S. aureus* and serves as a platform for future studies to identify the precise staphylococcal antigens capable of generating memory T cell responses. Moreover, this study may facilitate further investigation into anti-bacterial memory γδ T cells, particularly into the potential of targeting these non-traditional lymphocytes in future vaccine strategies. In addition, this work suggests that mucosal vaccination is a promising immunisation strategy and that this system should be evaluated for future anti-*S. aureus* vaccine design.

Table 6.1: Summary of Results

	Antigenic Exposure	**Reduction in bacterial load**	**Increase in Antibody Titre**	**Increase in Neutralising Antibodies**
	Prior Exposure	+	+	+
Vaccination with	**ClfA**	-	+	*No data available*
	ClfA + CpG	+	+	+
	ClfA + UEA1m + NP	+	-	-
	ClfA + QS21	+	++	++

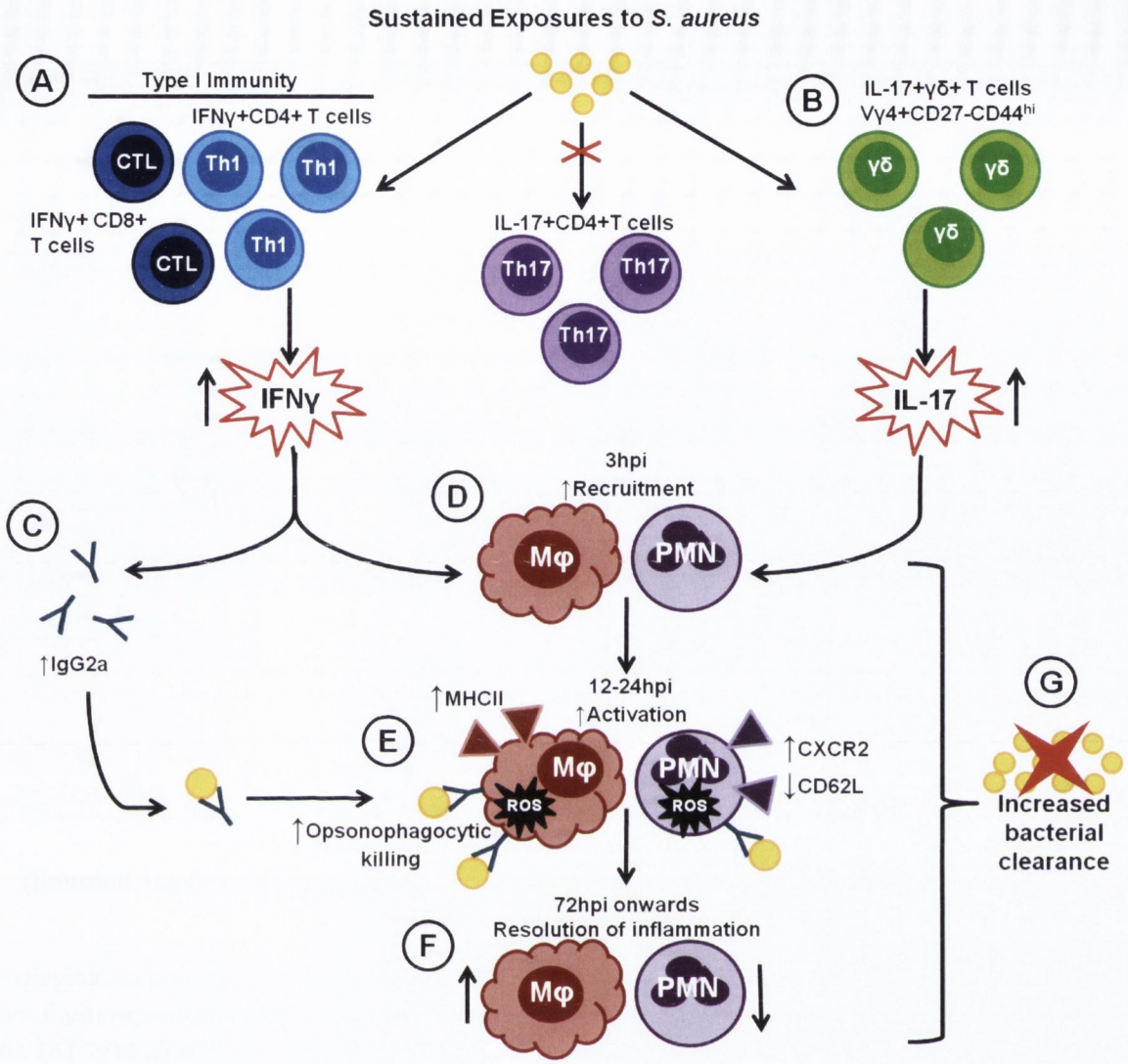


Figure 6.1: Prior exposure to *S. aureus* protects against subsequent infection through T cell mediated control of phagocyte responses

Prior exposure to *S. aureus* increases IFNγ production by CD4 and CD8 T cells (A), as well as enhancing IL-17 secretion by γδ T cells (B). The downstream effects of IFNγ are associated with increased IgG2a antibody titres (C). Both IFNγ and IL-17 responses play roles in enhancing macrophage and neutrophil recruitment to the infection site during the early phases of *S. aureus* infection in prior exposed mice (D). Elevated recruitment is then followed by contraction of phagocyte numbers and increased activation of residual phagocytes 12-24 h post infection (hpi) in prior exposed mice (E). These activated phagocytes are responsible for actively clearing *S. aureus* from the infection site. Critically both Th1 and γδ T cell responses were associated with increased macrophages and decreased neutrophils 72 h post infection, suggesting that these T cells aid in the resolution of inflammation through macrophage control of neutrophil responses (F). Altogether these responses culminate in maximised bacterial clearance from the local infection site whilst minimising dissemination to peripheral sites (G).

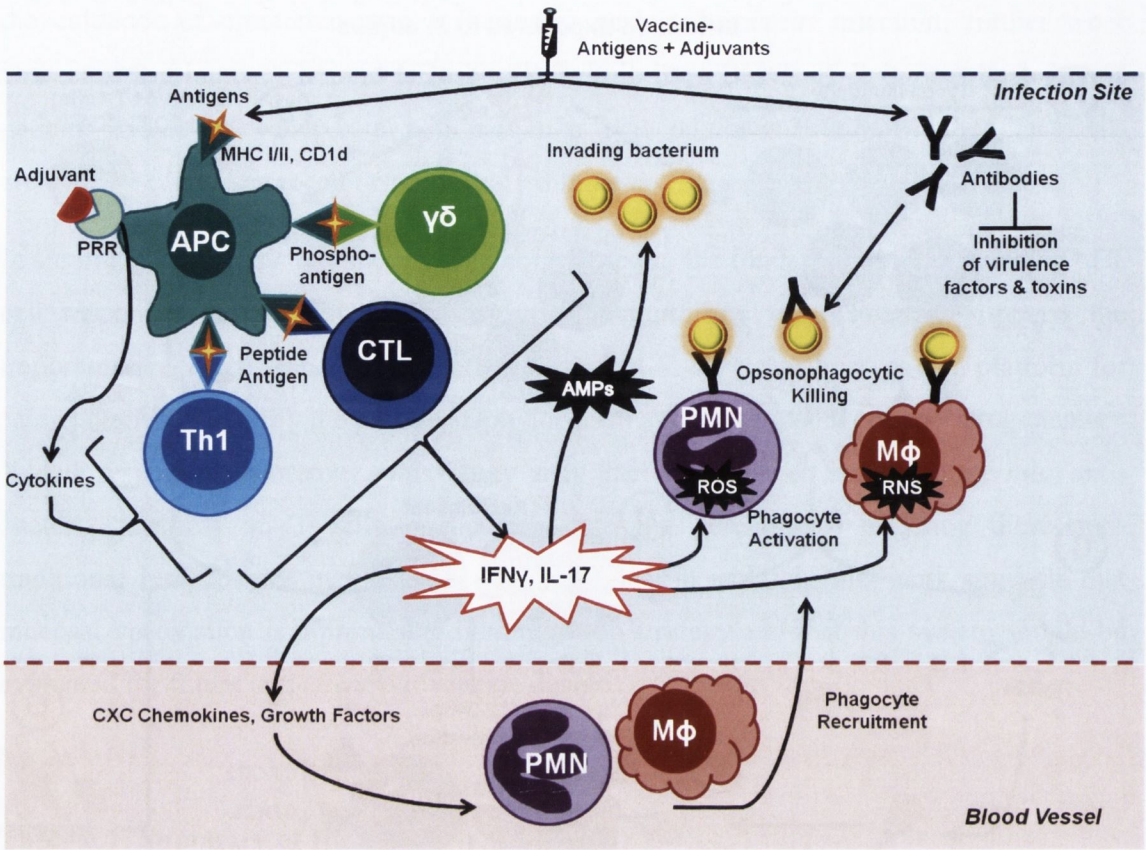


Figure 6.2: An ideal anti-*S. aureus* vaccine would induce antigen-specific cellular immunity which would complement humoral responses upon infection

Vaccine induced cellular immunity could be generated through selection of appropriate antigens in combination with specific adjuvants leading to the induction of discrete antigen-specific T cell populations. Adjuvant selection could allow for the generation of classic αβ T cells (e.g. Th1 and CTL CD8 T cells) and/or non-traditional lymphocytes such as γδ T cells, specific for the selected staphylococcal antigens. Upon infection, vaccine induced T cells would produce pro-inflammatory effector cytokines (such as IFNγ and IL-17), the downstream effects of which would promote phagocyte recruitment to the infection site. Upon phagocyte egression to the infection site cytokines derived from vaccine induced T cells could enhance phagocyte bactericidal properties. IL-17 is also capable of inducing AMP production, further contributing to the anti-bacterial response. The appropriate selection of antigens would also allow for the induction of antigen-specific antibody responses, which alone could neutralise virulence factors and toxins, and in the presence of activated phagocytes mediate opsonophagocytic killing of the invading staphylococci. Therefore, induction of both arms of immunity would therefore facilitate more efficient clearance of invading staphylococci.

Chapter 7

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