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Investigation of immuno-regulatory mechanisms during *Staphylococcus aureus* colonisation and infection

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M.Sc. Immunology



A thesis submitted to
Trinity College Dublin
For the degree of
Doctor of Philosophy

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Publications

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2. Interleukin-22 regulates antimicrobial peptide expression and keratinocyte differentiation to control *Staphylococcus aureus* colonization of the nasal mucosa. Mulcahy ME, **Leech JM**, Renauld JC, Mills KH, McLoughlin RM. Mucosal Immunol. 2016 Mar 23.
3. Memory Th1 Cells Are Protective in Invasive *Staphylococcus aureus* Infection. Brown AF, Murphy AG, Lalor SJ, **Leech JM**, O'Keeffe KM, Mac Aogáin M, O'Halloran DP, Lacey KA, Tavakol M, Hearnden CH, Fitzgerald-Hughes D, Humphreys H, Fennell JP, van Wamel WJ, Foster TJ, Geoghegan JA, Lavelle EC, Rogers TR, McLoughlin RM. PLoS Pathog. 2015 Nov 5
4. Manipulation of Autophagy in Phagocytes Facilitates *Staphylococcus aureus* Bloodstream Infection. O'Keeffe KM, Wilk MM, **Leech JM**, Murphy AG, Laabei M, Monk IR, Massey RC, Lindsay JA, Foster TJ, Geoghegan JA, McLoughlin RM. Infect Immun. 2015
5. *Staphylococcus aureus* Colonization: Modulation of Host Immune Response and Impact on Human Vaccine Design. Brown AF, **Leech JM**, Rogers TR, McLoughlin RM. Front Immunol. 2014 Jan 8 (joint first author)

Abbreviations

Agr	Accessory gene regulator
AMP	Antimicrobial Peptide
ANOVA	Analysis of Variance
APC	Antigen Presenting Cell
BMDC	Bone Marrow-derived Dendritic Cell
BMM	Bone Marrow-derived Macrophage
B-reg	Regulatory B-cell
Bcl2	B-cell lymphoma 2
C	Celsius
CA	Community Acquired
CC	Clonal complex
CCL	CC chemokine ligands
CD	Cluster of Differentiation
CFU	Colony Forming Unit
CGD	Chronic Granulomatous Disease
CHIPS	Chemotaxis Inhibitory Protein of <i>Staphylococcus aureus</i>
ClfA	Clumping factor A
ClfB	Clumping factor B
CP	Capsular Polysaccharide
CRAMP	Cathelicidin-related antimicrobial peptide

CSA	Columbia Agar
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
CXCL	C-X-C motif ligand
d	Day
DC	Dendritic Cell
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
<i>E. coli</i>	<i>Escherichia coli</i>
Eap	Extracellular adherence protein
EfB	Extracellular fibrinogen binding protein
ELISA	Enzyme-linked Immunosorbant Assay
FACS	Fluorescence-activated cell sorting
FAS	Apoptosis Stimulating Fragment
FMO	Fluorescence minus one
FoxP3	Forkhead box P3
G-CSF	Granulocyte Colony Stimulating factor
GAS	Group A Streptococcus
GFP	Green Fluorescence Protein
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
h	Hour
H ₂ O ₂	Hydrogen peroxide

HA	Hospital Acquired
Hla	α -toxin (haemolysin α)
HNP	Human Neutrophil Peptide
HOCl	Hypochlorous acid
i.n.	intranasal
i.p.	Intraperitoneal
ICAM-1	Intracellular Adhesion Molecule- 1
IFN- γ	Interferon- γ
Ig	Immunoglobulin
IL	Interleukin
iNOS	inducible Nitric Oxide Synthase
IsdB	Iron regulated surface determinant
Jak	Janus kinase
KC	Keratinocyte chemoattractant
kDa	kilo Daltons
KO	Knockout
LDH	Lactate Dehydrogenase
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide
LTA	Lipotechoic Acid
LukAB	Leukotoxin AB

MCP1	Monocyte chemotactic protein 1
MDSC	Myeloid derived suppressor cells
MEF	Mouse Embryonic Fibroblasts
MFI	Mean Fluorescence Intensity
Mg	milligrams
MHC	Major Histocompatibility Complex
Mins	Minutes
MIP-2	Macrophage Inflammatory Protein 2
ml	Millilitres
MOI	Multiplicity of Infection
MPO	Myeloperoxidase
MRSA	Methicillin Resistant <i>Staphylococcus aureus</i>
Mtb	<i>Mycobacterium tuberculosis</i>
MyD88	Myeloid differentiation primary response gene 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NET	Neutrophil Extracellular Trap
NK	Natural Killer
nm	nanometer
nM	nanoMolar
NO	Nitric Oxide
NOS2	Nitric Oxide Synthase 2

NS	not significant
OD	Optical Density
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PBST	PBS-Tween
PCR	Polymerase Chain Reaction
PD1	Programmed cell death protein 1
PEC	Peritoneal Exudate Cells
PFA	Paraformaldehyde
PGN	Peptidoglycan
PI	Propidium Iodide
PMN	Polymorphonuclear Neutrophil
PRR	Pattern Recognition Receptor
PSM	Phenol soluble modulins
PVL	Panton Valentine Leukocidin
RIP	Receptor-Interacting Protein
RNA	Ribonucleic Acid
Roryt	Retinoid-related orphan receptor γ t
ROS	Reactive Oxygen Species
RPM	Rotation Per Minute
RT	Real Time

<i>S. aureus</i>	<i>Staphylococcus aureus</i>
s.c.	sub-cutaneous
SCID	severe combined immuno-deficient
SCIN	Staphylococcal Complement Inhibitor
Sm	Streptomycin
Sm ^R	Streptomycin-resistant
SPF	Specific Pathogen Free
STAT	Signal transducer and Activator of Transcription
T-bet	T-box Transcription Factor
TCD	Trinity College Dublin
TCR	T-Cell Receptor
TGFβ	Transforming Growth Factor beta
Th1	T helper cell 1
Th17	T helper cell 17
Th2	T helper cell 2
TLR	Toll-like Receptor
TNF	Tumour Necrosis Factor
TRADD	TNF receptor type 1-associated Death Domain protein
T-reg	Regulatory T-cell
TSA	Tryptic Soy broth Agar
TSB	Tryptic Soy Broth

TSST1	Toxic Shock Syndrome Toxin 1
WT	Wild Type
$\alpha\beta$	Alpha Beta
$\gamma\delta$	Gamma Delta
μg	microgram
μl	microliter

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Chapter 1

1. Introduction

1.1 *Staphylococcus aureus* infections and treatment options

Staphylococcus aureus (*S. aureus*) is a Gram-positive coccal bacterium. It is a facultative anaerobe which can be distinguished from other such staphylococci due to its gold pigmentation and ability to ferment mannitol. *S. aureus* is one of the leading causes of a variety of community-acquired and hospital-acquired bacterial infections. It is one of the most prominent causes of bacteraemia, carrying a greater mortality than any other bacterium — currently 20-40% mortality at thirty days despite appropriate treatment (1, 2). It is also a contributing cause for other deep-seated infections including osteomyelitis, septic arthritis, endocarditis, device-related infections and pneumonia. *S. aureus* is unique for its ability to cause primary bacteraemia and serious infections in young, otherwise healthy people, as well as in those with known risk factors (3). Invasive disease is by far the most acute and severe, however, the greatest burden of morbidity is due to chronic and recurrent skin and soft tissue infections (SSTIs), which are extremely common (4). ≤

Methicillin resistant *S. aureus* (MRSA) strains are now a major cause of concern in the healthcare associated (HA) environment, particularly in Western Europe and North America. Lately, there have been reports of *S. aureus* resistance to vancomycin, a glycopeptide antibiotic, considered by many as the last resort of antibiotic treatment to treat infections caused by this bacterium (5). Of further alarm, is the more recent outbreak of community associated (CA)-MRSA among healthy individuals outside the healthcare realm. These CA-MRSA strains can be characterised by distinct genotypes and distinct determinants of virulence, which are often more virulent than HA-MRSA strains (6, 7).

Due to the rapid ability of *S. aureus* to constantly adapt to the pressures of antibiotics, novel alternatives are urgently required to combat this epidemic. Significant efforts have been employed to develop an anti-*S. aureus* vaccine, however to date all candidate vaccines have failed at various stages of clinical trials (8). These vaccines were based upon eliciting a humoral response to *S. aureus*. However recent advances within the field now indicate that an antibody response alone may not be adequate to protect against *S. aureus* infection and that cell-mediated immunity must also be generated (9-

12), consequently, the field now accepts that next generation anti-*S. aureus* vaccines will be required to induce protective effector T-cell responses in addition to humoral immune responses (13). To date however an efficacious anti-*S. aureus* vaccine remains elusive. A significant factor underpinning the lack of success to date in developing and effective anti-*S. aureus* vaccine or other forms of immunomodulatory therapy stems from a paucity of insight into *S. aureus* host: pathogen interactions, particularly in the human host.

1.2. Immune response during *S. aureus* infection

During *S. aureus* infection the innate and adaptive immune responses do not function independently of one another but rather act in synergy in an attempt to ultimately clear *S. aureus* while protecting the host from unwanted immunopathology. Here components of both innate and adaptive immunity that are particularly important in protection against *S. aureus* infection are briefly discussed.

1.2.1 Innate immune response against *S. aureus* infections

1.2.1.1. The role of skin and anti-microbial peptides (AMPs) in protection against *S. aureus*

The initial physical barrier of intact skin is almost certainly the most important host defence element in preventing *S. aureus* infection. Because few organisms have the ability to directly penetrate the skin, they usually gain access by some physical means, such as trauma, surgical incision or intra-venous catheter. Compromised skin integrity is consistently associated with *S. aureus* SSTIs (14-17). In addition, breaches in the skin barrier often precede invasive disease, as evidenced by the increased incidence of *S. aureus* blood stream infections in patients with indwelling catheters, repeated injections or burns (18-20).

In the skin, anti-microbial peptides (AMP) are primarily produced by keratinocytes, but can also be produced by other cell types, such as hair bulb cells and sebocytes (21). Humans express a multitude of AMPs including HNP1-4 (human neutrophil alpha-defensins 1-4), human β -defensin 3 and LL-37 (a cathelicidin) (22-24). A murine

homolog of cathelicidin, cathelin-related antimicrobial peptide (CRAMP) has also been found. Both these human and murine AMPs have been shown to be capable of killing *S. aureus in vitro* (25) and *in vivo*. These AMPs can kill *S. aureus* in a variety of ways. The α -defensins target the cell membrane of *S. aureus* (26) while human β -defensin 3 is not as well characterised, however, it is believed to be mediated through perforation of the bacterial cell wall (23, 24, 27, 28). Unfortunately, *S. aureus* has adapted to this system by producing proteases to degrade AMPs, proteins to bind and inactivate them, and by altering the charge of its cell wall to reduce their affinity to attach (29).

1.2.1.2. The role of complement in protection against *S. aureus*

The complement system plays a crucial role in the innate immune response. It consists of a large number of plasma proteins. These proteins are enzymatically cleaved to their active form as part of the complement cascade. Activation of the three complement cascades: classical, alternative and lectin pathway results in the opsonisation of the pathogen by C3b, release of molecules such as C3a and C5a can increase inflammation and recruit phagocytes, other immune cells and the formation of pore forming, membrane attack complex's. In the context of *S. aureus*, infection, opsonisation by C3b is an important step for the recognition and clearance of the bacteria by monocytes and granulocytes (30, 31). The production of C3a, C4a and C5a - which are induced as a consequence of the activation of C3, C4 and C5 - are generated in response to Protein A on the surface of *S. aureus* and act as a chemotactic signal to granulocytes (30, 32). Furthermore, following an intravenous challenge, C5aR1^{-/-} mice exhibited elevated bacterial loads in systemic organs, along with increased kidney tissue destruction compared with wild type mice (33). However, *S. aureus* has evolved mechanisms including Protein A, chemotaxis inhibitory protein of staphylococci (CHIPS) and staphylococcal complement inhibitor (SCIN), all of which prevent efficient complement killing of *S. aureus* (34).

1.2.1.3. The role of neutrophils in *S. aureus* infection

Neutrophils are considered the first line of cellular defence against *S. aureus*. Neutrophils originate in the bone marrow and circulate in the blood as mature cells (35). They are identified in murine studies by the expression of the surface markers CD11b and Ly6G and in humans by expression of CD14, CD15 and CD16 (36). Morphologically, neutrophils can be identified by their polylobed nucleus and granularity (37). Upon *S. aureus* infection, endothelial cells and resident macrophages in the affected tissue release the chemokine CXCL8 (IL-8) that cause vascular endothelial cells to up regulate their expression of cell adhesion molecules. Neutrophils interact with these adhesion molecules allowing them to migrate from the systemic circulation across the blood vessel endothelium and enter the site of infection. Upon entry into the inflamed tissues, neutrophils migrate along a chemokine gradient secreted by cells at the site of infection (38).

Neutrophils recognise *S. aureus* through the recognition of PAMPs including lipoteichoic acid (LTA) and CpG DNA which interact with Toll-like receptors (TLR) on the neutrophils to promote phagocytosis. Neutrophils also express complement receptors and Fc receptors that bind complement-opsonised bacteria and the Fc region of antibody coated bacteria respectively (38). Upon phagocytosis, the pathogen becomes enclosed within a vesicle, known as a phagosome. This phagosome fuses with a lysosome becoming a phagolysosome. Activation of the NADPH oxidase complex on the phagolysosome membrane leads to the production of reactive oxygen species (ROS) which are important for killing the bacteria within the phagosome. Additionally, fusion of cytoplasmic granules with the bacteria containing phagosome results in the release of anti-microbial peptides with potent microbicidal activity. Post bacterial phagocytosis, neutrophils undergo apoptosis or programmed cell death. The subsequent debris is phagocytosed by macrophages to bring the innate response to its conclusion (38).

Neutrophils also have the ability to kill *S. aureus* extracellularly. This can be achieved through various mechanisms including the secretion of microbicidal AMPs and the formation of neutrophil extracellular traps (NETs). NETs consist of chromatin and granule protein complexes and are released into the extracellular space. They are an

effective mechanism of limiting pathogen dissemination. In addition, this exposes *S. aureus* to high concentrations of antimicrobial molecules. NET formation can be triggered by many factors, including exposure to ROS, activated platelets or bacteria, including *S. aureus* (39, 40).

The importance of neutrophils during *S. aureus* infection has been demonstrated in neutrophil depleted mice which demonstrate an increase in mortality following pulmonary challenge with *S. aureus* compared with immunocompetent mice (41). In addition, patients who suffer from Chédiak-Higashi syndrome have reduced neutrophil migration and phagolysosome activity which results in greater occurrence of *S. aureus* infection (42).

Although neutrophils are clearly important in the clearance of *S. aureus*, some reports however suggest that high numbers of neutrophils may have a negative effect on *S. aureus* clearance, as increased neutrophil influx was shown to increase *S. aureus* burden. Gresham *et al.* has previously demonstrated that administration of MIP-2 into the peritoneal cavity results in an increase in neutrophil infiltration which is subsequently associated with increased bacterial burden. Similarly, McLoughlin *et al.* demonstrated during a surgical wound model of *S. aureus* infection that the inhibition of neutrophil infiltration through administration of an anti-CXCR2 antibody resulted in a decrease in bacterial burden in this model (43). These studies suggest that although neutrophils are vital for the efficient clearance of *S. aureus*, elevated numbers of neutrophils present at the site of infection may be detrimental and may actually impede bacterial clearance. *S. aureus* has been shown to be able to survive within the neutrophil as a mechanism of protecting itself and facilitating disseminating to other tissues (44). Therefore, while neutrophils are an essential part of anti-staphylococcal immunity, it is an 'appropriate' neutrophil response that is needed to control *S. aureus* during infection (45).

1.2.1.4. The role of macrophages in *S. aureus* infection

Macrophages have also been shown to be important in the immune response to *S. aureus* (46). Once activated, macrophages secrete a range of immune-stimulatory molecules, including AMPs, chemokines (such as CXCL8 which recruits neutrophils) and cytokines (such as IL-1 β which is highly pro-inflammatory). Following phagocytosis

of *S. aureus* by macrophages peptide fragments are presented to the adaptive immune system cells via MHC molecules. In the case of Th1 cells, this can promote the recruitment of further macrophages, creating a positive feedback loop.

Macrophages activate CD8⁺ T-cells through MHC I, causing apoptosis of the macrophage, thus eliminating intracellular *S. aureus*. In addition, they perform another important task by the removal of apoptotic cells. This is essential to maintain normal tissue homeostasis by removing dead/dying cells thereby preventing debris build-up. In the case of infections, the neutrophils that are required to clear the infection by phagocytising the bacteria undergo apoptosis, require clearance. This is primarily carried out by macrophages (47).

Upon activation, murine macrophages can become polarised into a range of subsets. The extremes of these subsets include M1 macrophages, which are pro-inflammatory and secrete cytokines such as IL-12, which drives a Th1 type CD4 response (48). They represent the macrophage subset that is responsible for clearance of bacteria (49). In contrast, M2 macrophages have a more regulatory phenotype, secrete IL-10 which is believed to play an important role in resolution of infection and wound healing (50).

The role of macrophages in the direct killing of *S. aureus* is less well defined. Macrophages killing mechanism is similar to that of neutrophils, through phagosome acidification and phagolysosome fusion. ROS and iNOS are generated within the phagosome and have been shown to contribute to intracellular bacterial killing (51, 52). The importance of macrophages in the immune response to *S. aureus* was demonstrated by Brown *et al.*, this study demonstrates that the depletion of macrophages through treatment with clodronate liposomes, resulted in increased bacterial burden both locally and systemically following intraperitoneal infection with *S. aureus* in comparison to mice treated with PBS-filled liposomes, and this was associated with significant mortality in the clodronate treated group (12).

There are however a number of contrasting reports on the role of macrophages during *S. aureus* infection. During *S. aureus* pneumonia, an absence of macrophages was associated with increased survival but no effect on bacterial burden. It was suggested

that this decrease in mortality was linked with a decrease in macrophage mediated inflammatory damage. Therefore, a more important role for macrophages during *S. aureus* infection may be enhancing the differentiation of certain T-cell phenotypes or the clearance of apoptotic neutrophils, as opposed to direct bacterial killing (53).

1.3. Adaptive immune response against *S. aureus* infection

The adaptive immune response provides a more complete defence mechanism against *S. aureus*. It involves both cellular and humoral components which are *S. aureus* specific.

1.3.1. The role of antibodies in *S. aureus* infection

The humoral immune response to *S. aureus* is mediated by antibody producing B-cells. These antibodies are capable of recognising an infinite number of foreign antigens and can provide the host with both short and long term protection against the pathogen. Antibodies are critical in the immune response against *S. aureus*. *S. aureus* infection has been shown to result in elevated concentrations of *S. aureus* specific antibody titres in the blood (54, 55). These antibodies are important for neutralising toxins (54), inhibiting bacterial adherence to host tissues (56) and facilitating opsonisation of the bacteria to aid in opsonophagocytic clearance (57).

The majority of vaccination strategies to date have focused on the induction of humoral responses. B-cell deficiencies in patients or mice however are not associated with increased susceptibility to *S. aureus* infection (58). In addition, numerous studies have shown that *S. aureus*-specific antibodies are produced upon exposure to the bacterium during infection (54, 55, 59), however these antibodies do not necessarily protect against *S. aureus* infection (55, 60) and recurrence of infection is often observed in the clinic (59, 61). Mice that received sera containing anti-staphylococcal antibodies or B-cells isolated from mice vaccinated with a model anti- *S. aureus* vaccine were not protected against subsequent bacteraemia (58). These studies suggest that induction of an antibody response alone is incapable of mediating full protection against *S. aureus* infections.

1.3.2. The role of cellular immunity in *S. aureus* infection

T-cells have been shown to play a crucial role in protection against *S. aureus* infection. Both patients and mice with T-cell deficiencies demonstrate increased susceptibility to *S. aureus* infection (13). T lymphocyte deficient mice were shown to demonstrate increased susceptibility to *S. aureus* infection compared with WT mice (58). Mice lacking $\gamma\delta$ cells had increased morbidity in response to *S. aureus* than their wild type littermates in a model of subcutaneous abscess infection (62), as well as increased bacterial burden at the site of a *S. aureus* surgical wound infection (63). In all of these models, T-cells were shown to mediate their protective effects through the production of cytokines that control downstream phagocyte responses. IFN- γ , IL-17 and to a lesser extent IL-22 are the primary T-cell cytokines that are known to regulate phagocyte responses and have been shown to be instrumental in protecting against *S. aureus* infections at various sites.

1.3.2.1. Interferon- γ

Th1 cells and NK cells are primary producers of IFN- γ , a cytokine which is classically associated with the activation of macrophages. Macrophages themselves can also produce IFN- γ , inducing a positive autocrine effect. IFN- γ upregulates MHC expression on macrophages, enhances phagocytosis and primes intracellular bactericidal components such as NO and lysosomal enzymes (64). Th1 cells are expanded during *S. aureus* infection (12). This cell phenotype produces mainly IFN- γ , which is known to drive macrophage and neutrophil recruitment and activation (65) (66).

However, the role of IFN- γ during *S. aureus* infection is not fully understood. *In vivo* studies examining the role of Th1 cells in varied *S. aureus* infection models have generated conflicting results. Some studies have demonstrated that IFN- γ signalling is not required for protection against *S. aureus*, with IFN- γ R^{-/-} and WT mice displaying similar pathologies upon cutaneous staphylococcal infection (11). While other studies have shown a detrimental effect for IFN- γ , as deficiency in IFN- γ was associated with protection in a model of surgical site infection (65). It appears that the role of IFN- γ is dependent on the site of infection, as significant evidence suggests that during a systemic infection, IFN- γ plays a protective role. Studies have shown that administration

of recombinant IFN- γ leads to an increase in phagocyte recruitment and bacterial clearance during intravenous infection, suggesting a protective function for IFN- γ in this model (67). In addition, Brown *et al* demonstrate in both mice and humans that Th1 cells were elevated during *S. aureus* blood stream infection. Furthermore, adoptive transfer of *S. aureus* antigen-specific Th1 cells was protective in naïve mice, by promoting macrophage activation during *S. aureus* peritonitis (12), thereby providing concrete evidence that Th1 cells are involved in a systemic infection.

1.3.2.2. Interleukin-17

IL-17 is a pro-inflammatory cytokine which has come to the forefront of host immunity against *S. aureus*. IL-17 has been shown to be produced by innate-like $\gamma\delta^+$ T-cells and by Th17 cells in response to *S. aureus* infections (68, 69). In the context of infection IL-17 plays an important role in directing neutrophil recruitment through its ability to induce CXC chemokines (70) and also plays an important role in neutrophil survival via induction of Granulocyte-colony stimulating factor (G-CSF) (71). It has also been shown to play a role in improving bacterial killing by neutrophils (72).

$\gamma\delta^+$ T-cells have been identified as a potent source of innate IL-17. In a cutaneous infection model, $\gamma\delta^+$ T-cell deficient mice were found to have reduced neutrophil recruitment to the infection site and impaired bacterial clearance, compared with WT controls (11). $\gamma\delta^+$ T-cells were also shown to have a protective role during surgical site infection (63) through their capacity to produce IL-17. Most recently, IL-17 produced by these cells has been shown to play a protective role against re-exposure to *S. aureus* by promoting neutrophil recruitment and activation (69).

Th17 cells are characterised by their ability to produce IL-17 as well as IL-22 (11). Compelling evidence demonstrating a protective role for Th17 cells in response to *S. aureus* comes from studies in Hyper IgE syndrome patients. These patients exhibit defects in STAT 3 function, resulting in a failure to induce functional Th17 cells and as a consequence have increased rates of *S. aureus* infections, particularly recurrent skin abscesses (73). Ishigame *et al* demonstrated that IL-17A^{-/-}IL-17F^{-/-} mice get spontaneous skin abscesses, however these mice were not more susceptible to

systemic bacterial burden following intravenous infection (74). Therefore, in contrast to IFN- γ , IL-17 may play a more protective role at mucosal sites of *S. aureus* infection.

1.3.2.3. Interleukin-22

IL-22 has been shown to be produced primarily by conventional $\alpha\beta$ T-cells, $\gamma\delta^+$ T-cells, and innate lymphoid cells (ILCs). Studies using an IL-22 fate reporter mouse have indicated that $\gamma\delta^+$ T-cells are the predominant source of IL-22 in the mouse skin and lungs (75). Recently, IL-22 produced by both conventional CD3⁺ T-cells and also a population of CD3⁻ cells, potentially ILCs, were shown to be protective in *S. aureus* pneumonia infection (76). During *S. aureus* pneumonia neutralisation of IL-22 with an anti-IL-22 antibody prior to challenge with *S. aureus* led to increased susceptibility while, conversely, intra-nasal administration of IL-22 at the time of infection with *S. aureus* renders mice more resistant to infection, suggesting that IL-22 is protective against *S. aureus* pneumonia (76). During *S. aureus* sub-cutaneous infection IL-22 was shown to play a non-redundant in host protection by promotion of AMP expression (77). Most recently, using WT and IL-22^{-/-} mice, IL-22 was found to be important for limiting the growth of *S. aureus* on mechanically injured skin (78). Therefore it appears that IL-22 plays a valuable protective role via the promotion of AMPs. Similar to the role of IL-17, IL-22 appears to have its most profound affect at mucosal/ barrier sites.

1.4. *S. aureus* colonisation

In contrast to its invasive infectious potential, *S. aureus* is a common coloniser of most of the population. The primary site of *S. aureus* colonisation is identified as the anterior nares, clinical reports support the presence of multiple sites of colonisation, deeper within the nasal cavity, including the throat (79, 80). Autopsy studies have shown *S. aureus* residing within the squamous epithelial layer, associated keratin and mucous debris, and even within hair follicle shafts (81). Extra-nasal colonisation sites include skin, throat, perineum, vagina and gastrointestinal tract (82-84). However, it appears that the nasal site is often the source of inoculation of other sites via hand transfer. The greater the bacterial load in the nares, the greater the likelihood that other body sites are colonised (85). *S. aureus* nasal carriers fall into two categories – persistent carriers

and intermittent or non-carriers (86). Approximately 20% of individuals are persistently colonised with a relatively high bacterial load, and the remainder are either never colonised or only intermittently colonised (87).

S. aureus appears to maintain a unique relationship with its host during colonisation. Persons who first underwent *S. aureus* eradication, followed by artificial inoculation with a mixture of *S. aureus* including the original carrier strain were found to re-acquire with their autologous strain, proving that there is an intrinsic relationship between host and bacteria. Accurate assessments of factors that promote persistent carriage are difficult to determine, but among healthy adults chronic skin disease, recent skin infection, male sex and being a non-smoker are associated with increased nasal colonisation rates (88, 89).

S. aureus nasal colonisation has been defined as a risk factor for subsequent invasive disease. Approximately 80% of *S. aureus* bacteraemias are endogenous, as it has been demonstrated that the infecting bacteria have been shown by genotypic analysis to be identical to that found in the nasal mucosa (90, 91).

To-date the majority of research into understanding the factors that control *S. aureus* nasal colonisation have focused on bacterial factors. During successful human colonisation *S. aureus* preferentially expresses tissue-adherence and immune evasion molecules and down-regulates expression of virulence factors and toxins (92, 93). Specific interactions between *S. aureus* and the nasal epithelium during colonisation have been defined for two distinct sites within the nasal cavity: the anterior nares and epithelial cells lining the inner nasal cavity (94, 95). Bacterial adherence to the anterior nares is enabled through clumping factor B (ClfB), which is the most well characterised cell surface protein on *S. aureus* to promote adhesion to desquamated nasal epithelial cells (95). ClfB targets host ligands include loricrin and cytokeratin 10. Cytokeratin 10 is a major component of squamous epithelial cells while loricrin is the most abundant protein (~85%) in the keratinised epithelial layer of the nares. Binding of *S. aureus* to these cells has recently been shown to be ClfB-dependent (96), and the absence of either loricrin or ClfB significantly impairs *S. aureus* nasal colonisation in a murine

model (95, 97). Studies in loricrin deficient mice have defined for the first time, a host ligand for ClfB that facilitates colonisation *in vivo*. A *S. aureus* strain deficient in ClfB, was shown to colonise both wild-type and loricrin-deficient mice to similar extents, thereby demonstrating that the interaction is ClfB-dependent (95).

Colonisation of the inner nasal cavity has been shown to be facilitated by the cell wall glycopolymer wall teichoic acid. At this site, Wall teichoic acid (WTA) adheres to an F-type scavenger receptor, known as scavenger receptor expressed by endothelial cell-1 (SREC-1), which is expressed on the surface of epithelial cells in the inner nasal cavity (94). WTA glycosylation has recently been shown to be a vital step in its adherence to both human epithelium and for *in vivo* nasal colonisation in a cotton rat model (98).

IsdA is another surface protein of *S. aureus* which has the ability to bind to cytokeratin 10, loricrin and involucrin proteins in the matrix surrounding the upper layers of the epithelium, contributing to bacterial adhesion to squamous cells (99), in addition to promoting bacterial colonisation of cotton rats (100)

Although the majority of investigations into host: pathogen interactions during *S. aureus* nasal colonisation to-date have primarily focused on the characterisation of how bacterial factors interact with the nasal tissue environment, polymorphisms in immune genes such as mannose binding lectin, TLR2, C-reactive protein, IL-4 and HLA-DR3 have all been shown to be associated with increased risk for *S. aureus* nasal carriage (101-104). The identified IL-4 polymorphism causes lower concentrations of IL-4, resulting in reduced mucin production and dampening of the Th2 response (105, 106) which was associated with increased colonisation. In a sample of elderly Dutch patients, the glucocorticoid receptor polymorphism found in persistent carriage was associated with a phenotype of putative high cortisol concentrations causing immunosuppression, and the non-carriage haplotype was thought to reflect reduced immunosuppression (107). Instead of interrogating single genes, some studies have looked for individuals likely to share multiple genetic similarities to ascertain the role of the host genome in colonisation. Persistent carriage patterns were not concordant among same-sex siblings or even among twin pairs, regardless of gender or zygosity (108, 109). The fact that no

strongly convincing genetic trait has accounted for successful or unsuccessful colonisation, this indicates that it must be determined by multiple factors that may even differ from carrier to carrier.

1.5. The immune response during *S. aureus* colonisation

1.5.1. Innate Immunity during *S. aureus* colonisation

In general there is very limited knowledge on the specific mechanisms of immunity that control or determine *S. aureus* nasal carriage; however a few studies have begun to decipher these interactions. Nasal secretions from *S. aureus* carriers contain higher concentrations of α -defensins (human neutrophil peptides 1-3) and human β -defensin 2, possibly as a consequence of the organism's presence (101). However, in comparison to nasal fluids from non-carriers which were bactericidal in nature, the secretions from carriers seem to be less damaging to *S. aureus in vitro*, and create a permissive environment for successful colonisation due to ineffective defensins (79). The multifunctional cutaneous cathelicidin LL-37 is impressively effective at *in vitro* killing of both extra- and intracellular *S. aureus* (110, 111). Despite this, in a single small study which included Granulomatosis with polyangiitis (GPA/Wegener's) patients, the nasal secretions of those colonised with *S. aureus* contained higher concentrations of LL-37 than non-colonised participants, and its production by nasal epithelial cells was induced by stimulation with *S. aureus* (112). The significance of this for the healthy population is unclear. The cathelicidin gene carries a vitamin D response element, and vitamin D increases expression and function of many antimicrobial peptides (113, 114). However, vitamin D supplementation does not reduce persistent carriage in healthy adults (115).

It was also found that a carrier strain suppressed production of IL-1 compared with a non-carrier strain in nasal epithelial cells; however, only the carrier strain exhibited sensitivity to human epithelial cells pre-incubated with IL-1 (116, 117). In agreement with this data, during a human colonisation study, upregulation of certain pro-inflammatory mediators was observed in volunteers who could not be artificially persistently colonised [76]. Following intra nasal inoculation, individuals who cleared *S.*

aureus from their nose had greater concentrations of the pro inflammatory cytokines including IL-1 β , IL-6, IL-8, G-CSF, TNF, and IFN- γ , compared with baseline concentrations, than individuals who were unable to clear *S. aureus* (118). These results suggest that a robust pro-inflammatory response maybe beneficial to the host in preventing *S. aureus* colonisation and specifically highlight that dysregulation of IL-1 β -signalling maybe a factor facilitating *S. aureus* persistence in the nose.

1.5.2. Adaptive Immunity against *S. aureus* colonisation

It is well established that *S. aureus* nasal colonisation imprints an adaptive immune response with anti-staphylococcal antibody concentrations previously been shown to be elevated in persistent carriers when compared with non-carriers (119). Levels of IgG and IgA antibodies against chemotaxis inhibitory protein of *S. aureus* (CHIPS), extracellular fibrinogen binding protein (Efbp), iron-responsive surface determinant A (IsdA) and iron-responsive surface determinant H (IsdH) have all been shown to be significantly higher in colonised children than in non-colonised children. Despite the evidence showing that colonisation with *S. aureus* promotes antibody production, there is no evidence that antibody production impacts carriage rate, as children are not resistant to nasal carriage having inherited maternal anti-staphylococcal IgG (120).

Immunocompromised individuals such as those infected with HIV, were shown to have a higher prevalence and bacterial burden of persistent colonisation compared with the healthy population (121, 122), suggesting a connection between *S. aureus* nasal carriage and host responses involving adaptive cellular immunity. Very recently studies have begun to decipher the role played by T-cells during murine *S. aureus* nasal carriage. In particular, a paper by Archer *et al.*, demonstrated using lymphocyte-deficient SCID mice, that T-cells were essential for clearance of *S. aureus* from the nose. Mice deficient in T-cells retained higher levels of bacteria in their noses following *S. aureus* nasal colonisation compared with B-cell-deficient mice and WT controls. Furthermore, IL-17 was identified as a key driver of *S. aureus* decolonisation through this cytokines ability to recruitment of neutrophils to the site. Mice deficient in IL-17

displayed increased nasal bacterial burden compared with WT mice, coupled with an impaired neutrophil influx to the nose (123).

1.6. *S. aureus* immune evasion

S. aureus can be described as a consummate immune evader, the multiplicity of mechanisms utilised by *S. aureus* to evade the innate immune system are staggering and have been extensively reviewed (124-127). Its means of evading the adaptive immune system are less well appreciated but its ability to do so, potentially explains the lack of an protective immunological memory seen in patients who develop recurrent *S. aureus* infections (4). Invasive *S. aureus* infections are associated with decreased transcription of genes relating to adaptive immunity (128, 129). Several microbial factors are known to directly interact with and inhibit lymphocytes.

Secreted or wall-anchored staphylococcal protein A binds IgG in the incorrect orientation for neutrophil recognition and thus inhibits opsonophagocytosis. It also binds to the V_H3 region of IgM on the surface of B lymphocytes, initiating a B-cell receptor-mediated programmed cell death that results in significant depletion of the reservoir of potential antibody-producing cells (130). The production of an array of superantigenic toxins by *S. aureus* directly activates vast numbers of T-cells, but this is followed by the loss of the T-cells ability to respond to these antigens (anergy). This prevents normal development – via MHC class II/TCR presentation of processed antigens – of true microbial antigen-specific effector and memory T-cells (131). At least one of these superantigens is expressed by most circulating clinical isolates of *S. aureus* (132, 133). The MHC class II analogue protein (Map) is a secreted *S. aureus* protein with similarity to the MHC peptide-binding groove. Its binding reduces lymphocyte proliferation and shifts this response in a Th2 direction, thus suppressing the Th1 responses shown to be important for bacterial clearance in animal systemic infection (134). Therefore, it is clear that *S. aureus* has the ability to evade the hosts immune response by directly impeding aspects of both adaptive humoral and cellular response, thereby creating a setting in which it can persist. There have been numerous studies demonstrating how these

immune evasion strategies promote persistence of *S. aureus* during infection (Table 1.1).

Table 1.1. Staphylococcal factors implicated in directly modulating the host adaptive immune response.

Immunomodulatory Factor	Human Target	Effect	Role in colonisation
Protein A (Spa)	(i) Fc region free IgG (ii) B-cell-surface IgM V _H 3 region	(i) inhibits opsonophagocytosis (ii) programmed B-cell death	Mostly transcribed in persistent carriers (92, 135)
Superantigens (staphylococcal enterotoxins and toxic shock syndrome toxins)	MHC Class II	Binds MHC Class II to the T-cell receptor to cause initial activation followed by anergic unresponsiveness	Variably transcribed during carriage (136)
MHC Class II analogue protein (Map)	MHC peptide binding groove	(i) Reduced lymphocyte proliferation (ii) Th2-predominant response	Unknown
Leukotoxin ED	CCR5 (T lymphocytes and macrophages)	Cell membrane pore formation causing cytotoxicity	Unknown

1.7. Immune regulation

Immune regulation plays a vital role in maintaining the delicate balance between facilitating adequate inflammation to clear the infection and by controlling excessive inflammation which can cause host tissue damage and potential mortality (137). Immune regulation is multifaceted, and comprises a multitude of regulatory immune cells within both the innate and adaptive arms of the immune response. These cells can exert their suppression either by cell: cell contact or by the production of immuno-regulatory cytokines.

1.7.1. Interleukin-10

Interleukin-10 (IL-10) is a potent anti-inflammatory cytokine that circulates as a homodimer consisting of two tightly packed 160-amino-acid proteins (138). In 1989, IL-10 was first described as cytokine synthesis inhibiting factor (CSIF) due to its ability to suppress cytokine synthesis in certain T-cells (139). IL-10 has been shown to bind to two receptor chains, IL-10R1 and IL-10R2 (140). It belongs to a family that also includes IL-19, IL-20, IL-22, IL-24 and IL-26 (138).

IL-10 family cytokines activate the Jak-STAT signalling pathway. STAT3 in particular is a key downstream transcription factor used by IL-10 although it can sometimes activate STAT1 and STAT5 (141, 142). Jak1 and Tyk2 are associated with IL-10R1 and IL-10R2, respectively (143, 144). The suppressive effects of IL-10 on the production of pro-inflammatory cytokines are completely abolished in macrophages and neutrophils lacking STAT3 (142).

IL-10 has been shown to be produced by cells of both the innate and the adaptive immune system, including dendritic cells (DCs), macrophages, mast cells, natural killer (NK) cells, eosinophils, neutrophils, CD4⁺ and CD8⁺ T-cells, and B-cells (145). IL-10 production from dendritic cells can promote the differentiation of CD4⁺ T-cells into Tr1 T-regs which express IL-10 (146). Initially, only T helper (Th) 2 cells were known to produce IL-10 but recently Th1 and Th17 cells were both reported to produce IL-10 (138).

IL-10 plays an important role in maintaining immune homeostasis by inhibiting pro-inflammatory cytokine and chemokine production. IL-10 impairs the antigen-presenting ability of macrophages and dendritic cells via the inhibition of MHCII expression and the down-regulation of CD80 and CD86 (147). IL-10 inhibits the synthesis of IL-12 (limiting T-cell activation), IL-2 (limiting T-cell expansion) and IL-5 (limiting mast cell proliferation). Autocrine IL-10 signalling in DC inhibits chemokine production, thereby preventing their trafficking to lymph nodes as shown in mycobacterial infection, leading to the failure to recruit and induce Th1 differentiation of naive T-cells (148). Similarly, the secretion of IL-10 by Th1 cells represents a potent auto-regulatory feedback loop that protects against excessive inflammation and potential tissue destruction during pro-inflammatory Th1-driven immune responses in infections (149).

In contrast to its mainly immunosuppressive role, IL-10 has been shown to be immune activating under certain conditions, it has been shown to promote B-cell proliferation and differentiation (147), however it is widely accepted that this cytokines role is primarily immunosuppressive.

Toll-like receptor 2 (TLR2) signalling has been shown to be critical for IL-10 production upon exposure to *S. aureus*. Following systemic challenge with *S. aureus*, there is greater mortality in TLR2 knockout mice (TLR2^{-/-}) as a result of excessive pro-inflammatory cytokines and reduced production of the anti-inflammatory cytokine IL-10 (150). *S. aureus* induced activation of TLR2 signalling on monocytes and macrophages leads to a primarily anti-inflammatory response characterised by IL-10 production while TLR2 signalling on dendritic cells (DCs) leads to a Th1/Th17 pro-inflammatory response through the production of IL-12 and IL-23 (151). Differential signalling downstream of TLR2 have been shown to activate either an anti-inflammatory response through activation of phosphoinositol 3-kinase (PI3K)-Akt-mTOR and extracellular signal-regulated kinase (ERK) or a pro-inflammatory response through MAPK p38 *in vitro* (152). These pro- or anti-inflammatory responses were shown to be independent of each other as *S. aureus* nasal isolates from community carriers were shown to engage both simultaneously. However, the *S. aureus* ligands responsible for engaging these two signalling pathways are now yet known (152).

1.7.2. Transforming growth factor β

Transforming growth factor- β (TGF- β) includes the three different TGF- β isoforms (TGF- β 1, TGF- β 2, TGF- β 3). It is secreted into the extracellular matrix (ECM) in a heterotrimeric complex with latency-associated protein (LAP) and the latent TGF- β binding protein (LTBP). It remains inactive until it is activated and released from the latent complex in a mechanism involving thrombospondin 1 (TSP1), enzymatic activation by matrix metalloproteinases (MMPs) and integrins (153).

TGF- β binds to a tetrameric structure, which is composed of two type I TGF- β receptors (TGF- β RI) and two type II TGF- β receptors (TGF- β RII). Ligation of TGF- β to the receptor complex recruits the intracellular receptor SMAD. Different isoforms of TGF- β are thought to activate different SMAD signaling pathways (154). In addition to SMAD there are also a number of other signaling pathways activated independently of SMAD including MAPK and PI3K (153).

TGF- β is a pleiotropic cytokine whose role can vary substantially depending on the environment in which it is released. TGF- β controls a multitude of cellular processes such as proliferation, differentiation, migration and apoptosis, embryonal development, tissue repair and immune system modulation. TGF- β plays fundamental roles in regulating differentiation of T-cells into effector and regulatory subsets. The effects of TGF- β on T-cell differentiation are highly context specific: TGF- β has been shown to potently inhibit Th1 cell differentiation by inhibiting the induction of T-bet (155). In addition, TGF- β is capable of downregulating the expression of IL-12R on T-cells, resulting in a reduction in their responsiveness to IL-12 (156). TGF- β can also act indirectly to inhibit Th1 cell differentiation by inhibiting IFN- γ production by natural killer cells (157). TGF- β has also been shown to potently downregulate differentiation of Th2 cells. Inhibition of Th2 differentiation is due to modulation of the transcription factor GATA3, a key transcription factor in Th2 cell induction (153, 158).

In addition to limiting Th1 and Th2 differentiation and proliferation, TGF- β also plays important but distinct roles in the regulation of both natural T-reg and inducible T-reg populations. TGF- β signaling in T-cells protects nT-regs from apoptosis during thymic development by suppression of pro-apoptotic proteins and upregulation of the anti-

apoptotic protein Bcl2 (159). TGF- β plays a prominent role in promoting iT-reg development. In combination with IL-2 and retinoic acid, TGF- β promotes the conversion of naive CD4⁺ T-cells to iT-regs by upregulating expression of Foxp3 (160, 161). In addition to its suppressive role, TGF- β also has a fundamental role in driving the expansion of Th17 cells, this expansion of Th17 cells requires the presence also of pro-inflammatory cytokines such as IL-6, IL-1 β and IL-21 (162).

1.7.3. T-regulatory cells

1.7.3.1. Classification and function

It was the research work of Sakaguchi *et al.* that brought the therapeutic suppressive potential of T-regs into focus by the observation that the deletion of the CD4⁺CD25⁺ subset from a population of normal adult mice CD4⁺ T-cells led to the development of a spectrum of autoimmune diseases (163). This CD4⁺CD25⁺ population is now termed T-regulatory cells (T-reg) (163). T-regs make up approximately 8-10% of total CD4⁺ T-cells. The main functions of T-regs are to maintain immune homeostasis, prevent autoimmunity and control inflammation, all of which now comes under the umbrella term peripheral tolerance, which is an extra tolerance mechanism (in addition to central tolerance) that prevents the activation of autoreactive T or B-cells against harmless antigen or self-tissue (164).

Forkhead box P3 (FoxP3) has been shown to be the key transcriptional factor required for the development, maintenance and function of T-regs. The use of FoxP3 knockout mice demonstrate the importance of T-regs in controlling autoimmunity, these mice develop severe autoimmune like hyper-proliferative diseases (165). FoxP3 is an invaluable T-reg marker in mice, however in human T-regs it is not a sufficient marker as many activated T-cells express FoxP3 but are not suppressive (164, 166). Other phenotypic markers on murine T-reg cells include high levels of CD25, CTLA-4, CD103, CD73 and CD39 (164, 167) and in humans CD4, CD25, Foxp3 and low levels of CD127 is used to characterise these cells (168).

T-regs can be further divided into natural T-regs (nT-regs) and inducible T-regs (iT-regs). nT-reg and iT-reg cells express similar phenotype markers and have similar general functions. nT-regs are selected by autoantigen presentation in the thymus. These nT-regs possess an autoreactive T-cell receptor (TCR) repertoire when they migrate to the periphery (169). nT-reg cells main function is to control overall immune homeostasis of the host by preventing undesirable inflammation.

iT-reg cells are derived from naive T-effector cells in the periphery in the absence of optimal CD3 and CD28 stimulation. They have the same antigen specificity as the auto-

reactive T-effector cell. However, their activation requires low TCR engagement, low CD28 signalling and cytokines especially TGF- β . Another factor which can influences iT-reg development is retinoic acid (RA). RA is highly expressed in the gut and in synergy with TGF- β , enhances iT-reg development (170). Well characterised iT-reg include Tr1 and TH3 iT-reg which produce predominantly IL-10 and TGF- β respectively. Research suggests that the generation of Tr1 cells is dependent on IL-27 and/or IL-10 from APC's, while TGF- β derived from APCs drives a TH3 phenotype (171). To date many other different types of T-cells with regulatory functions outside of the traditional nT-reg and iT-reg phenotypes have been identified, these include CD8⁺, CD4⁻CD8⁺TCR⁺, Natural Killer (NKT) regulatory cells (172, 173) however they are less well studied compared with CD4⁺ regulatory cells (174).

T-reg cells can directly suppress the function of effector CD4⁺ and CD8⁺ T-cell by preventing their differentiation, activation and proliferation. They can achieve this via a number of mechanisms (**Figure 1.2**) as summarised in **Table 1.2**. Cell-cell contact as well as contact independent routes including; secretion of inhibitory cytokines, suppression via cytotoxicity, metabolic disruption or by the interference of dendritic cell (DC) maturation and function (164, 166, 175, 176).

Table 1.2. T-reg mechanisms of suppression

Suppression of mechanism	Result	References
IL-10	Th1 suppression Down-regulation MHCII	(147)
TGF- β	Th1 suppression Induce T-reg	(155)
IL-35	Increased immune suppression	(177, 178)
CD25 (IL-2R)	T-effector cells undergo apoptosis.	(179)
Perforin and granzymes	Induce Apoptosis	(180)
Galectin	Induce T-cell apoptosis Inhibit T-cell pro-inflammatory cytokine release.	(181)
CTLA-4	Internalising and degrading CD28 on the surface of T-cells	(182)
CD39/ CD73	Adenosine production	(183)
Cyclic adenosine monophosphate (cAMP)	Down regulation of the co-stimulatory molecules CD80/CD86	(184)

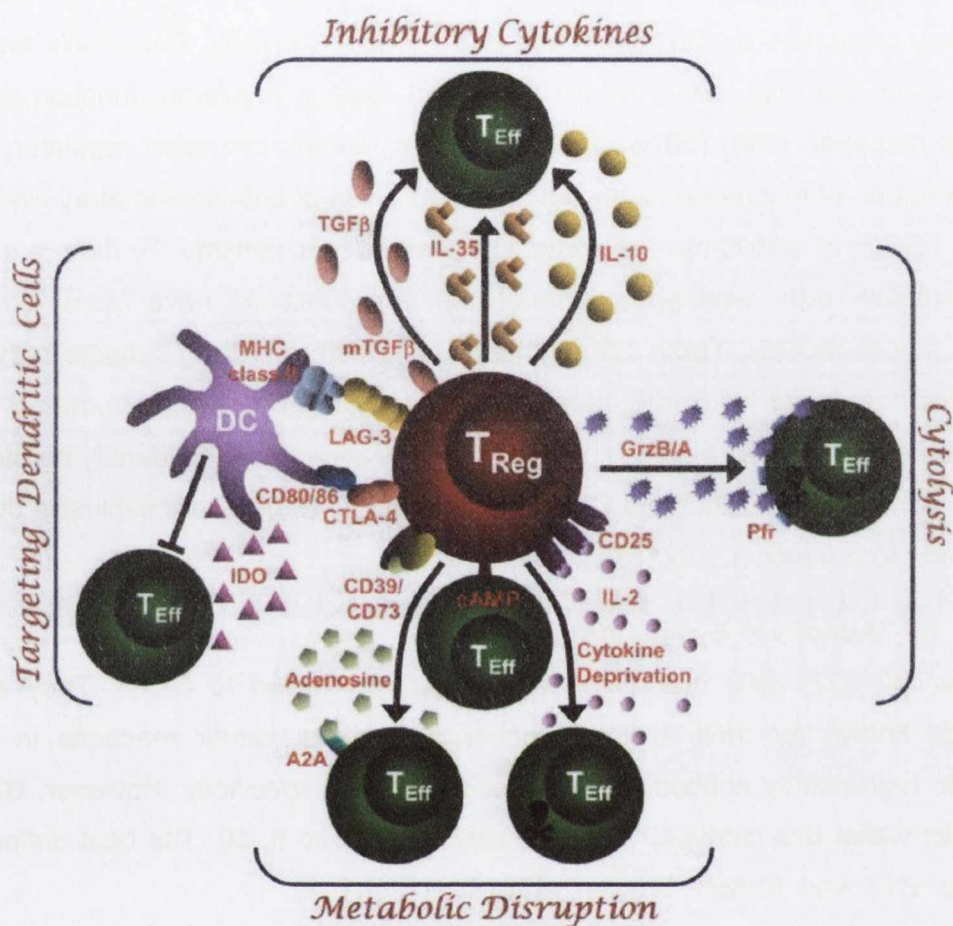


Figure 1.1 T-reg cells modes of suppressive

T-regs may target T-effector cells by several mechanisms including (i) Metabolic disruption via IL-2 deprivation, cleaving of AMP by CD39 and CD73 to reduce pro-inflammatory effects (ii) Cytolysis by perforin and granzyme (iii) Inhibitory cytokines such as IL-10, TGF, IL-35 (iv) Targeting Dendritic cells by degradation of co-stimulatory molecules or expressing CTLA-4 which plays a role in up regulating indoleamine 2, 3-dioxygenase (IDO) which in turn down regulates the immune response. Adapted from [Vignali *et al*, (2008)] (164)

1.7.4. B-regulatory cells

The term “regulatory B-cells” (B-reg) was first introduced to define B-cell subsets with regulatory properties by Mizoguchi and Bhan (185). Currently, B-reg cells are defined on the basis that they can produce IL-10 and exert a regulatory function during the immune response (186) (187). Similar to T-regs, B-regs can exert regulatory function via a multitude of mechanisms, including the secretion of anti-inflammatory cytokines IL-10 and TGF- β , in addition to cell contact dependent mechanisms. To date, a number of B-reg subsets with overlapping phenotypes and functions have been identified in various mouse models. **Table 1.3** summarises the different B-reg subsets in the mouse that have been reported in the literature. Similar to murine B-regs, human B-regs are mainly identified by their ability to produce IL-10. Markers used to identify human B-regs include CD19, CD24, CD27 and CD38, however they are generally exclusive defined by their ability to produce IL-10 (187).

1.7.4.1. B2 “Adaptive” B-regulatory cells

Follicular B2 (B2) cells are the most commonly referred to B-cell. They are most generally known for their ability to undergo germinal center reactions in order to generate high-affinity antibodies with precise antigen specificity. However, B2 B-regs also exist within this subtype, with an ability to produce IL-10. The best defined B-reg cells are “B10” and T2-MZP B-regs (187).

“B10” cells that are characterised by the co-expression of CD1d^{hi}CD5⁺ are generally found in the spleen. These cells have been shown to uniquely secrete IL-10 upon LPS stimulation (188). Adoptive transfer of these B10 cells has been shown to ameliorate disease progression in a number of animal models of autoimmune and inflammatory diseases including EAE (189), arthritis (190) and intestinal inflammation (191).

Another well-defined B-reg subtype is Transitional 2-marginal zone precursor “T2-MZP” cells. These cells are characterised by the cell surface expression of CD21^{hi}CD23⁺ and an ability to secrete IL-10 in response to anti-CD40 stimulation (192). IL-10-producing T2-MZP B-cells have been shown to have a suppressive function in a range of scenarios including allergic airway inflammation (193), gastric immunopathology caused by *Helicobacter* and transplant tolerance (187, 194).

1.7.4.2. B1 “innate” B-regulatory cells

Unlike the above mentioned B10 and T2-MZP B-reg cells, which are derived from the conventional B2 or conventional cell lineage. B1 cells represent a unique innate-like population of B-cells capable of IL-10 production (195). B1 B-cells are highly enriched in peritoneal and pleural cavities. B1 cells can be subdivided based on expression of the surface marker CD5 into CD5⁺ B1a and CD5⁻ B1b B1 cells. Alongside the expression of CD5, B1a cells express CD19 and CD11b. B1 cells express a range of TLRs including TLRs 1, 2, 3, 4, 7, 8, 9. These TLRs are the primary mechanism by which B1 cells are activated. TLR2, TLR4 and TLR9 in particular have been shown to be important for IL-10 production by B1a cells (196). IL-10 producing B-1a cells have been shown to play a crucial role in protecting against TLR-mediated lethal inflammation in neonatal mice by preventing excessive inflammation (196).

In addition to producing IL-10, B1a B-cells spontaneously secrete IgM, providing the initial defence against encapsulated bacteria and are thought to provide a more first line defence function compared with their B2 counterparts (197, 198). Therefore, B-1a B-cells play a vital role in immune regulation during infection as they can neutralise invading pathogens with antibody production and also suppress the inflammatory responses with the production of IL-10.

Table 1.3. Different sub-types of B-reg cells

Murine B-reg Subtype	Phenotype	Mechanism of suppression	Reference
B-1a	CD5	IL-10	(199)
B10	CD19 ^{hi} CD1d ^{hi} CD5 ⁺	IL-10	(188)
T2-MZP B-cells	CD19 ⁺ CD21 ^{hi} CD23 ^{hi} CD24 ^{hi} IgM ^{hi} IgD ^{hi} CD1d ^{hi}	IL-10	(192)
MZ B-cells	CD19 ⁺ CD21 ^{hi} CD23 ⁻ CD24 ^{hi} IgM ^{hi} IgD ^{lo} CD1d ^{hi}	IL-10	(200)
Killer B-cells	CD5 ⁺ CD178 ⁺	FasL	(201)
GIFT-15 B-cells	B220 ⁺ CD21 ⁺ CD22 ⁺ CD23 ⁺ CD24 ⁺ CD1d ⁺ CD138 ⁺ IgD ⁺ IgM ⁺	IL-10	(202)
Plasma cells	CD138 ^{hi} IgM ⁺ TACI ⁺ CXCR4 ⁺ CD1d ^{hi} Ti m1 ^{int}	IL-10, IL-35	(203)
Plasmablasts	CD138 ⁺ CD44 ^{hi}	IL-10	(204)
TIM-1 ⁺ B-cells	-	IL-10	(205)
PD-L1 ^{hi} B-cells	CD19 ⁺ PD-L1 ^{hi}	PD-L1	(206)

1.7.4.2. B-reg mechanisms of suppression

Even though the production of IL-10 is regarded as vital to B-reg function, recent identification of other diverse suppressive mechanisms, similar to T-regs, are being discovered. This section provides an overview of the mechanisms of B-reg suppression in general (**Figure 1.2**). As stated IL-10 producing B-regs are the most common and well defined B-reg cell population. IL-10-producing B-regs have been shown to successfully limit T-cell proliferation and the production of pro-inflammatory cytokines (IFN- γ and TNF- α) by Th1 cells (188, 192, 207, 208). B-regs have not only been shown to suppress Th1-mediated immune responses but also have the ability to convert effector T-cells into regulatory IL-10 producing Tr1 cells (208, 209). It was observed that longer contact time between IL-10-producing B-cells as opposed to IL-10-deficient B-cells with CD4⁺CD25⁻ effector T-cells, resulted in IL-10⁺ B-cells being able to convert T-effector cells into Tr1 cells (209). B-regs were also shown to be capable of regulating DCs, by forcing them to downregulate their IL-12 production, which subsequently influences the Th1/Th2 balance (210).

In addition to IL-10-producing B-reg cells, TGF- β -producing B-reg cells have also been identified following LPS stimulation *in vitro* (211). These TGF- β producing B-regs have been compared with Th3 T-regs. TGF- β producing B-regs generate a negative autocrine loop, as TGF- β production has the ability to inhibit CD5⁺ B-cells, forcing them into apoptosis. On the other-hand, TGF- β producing B-regs can also work in tandem with T-cells by promoting the emergence of iT-regs.

B-reg cells can also express FasL and other death-inducing ligands under many circumstances. The expression of both FasL and IL-10 is the highest in the CD5⁺ B-cell population, which indicates that CD5⁺ B-cells may exert regulatory function by direct killing of target cells (212). Other suppressive markers on B-regs include CD73 (213) and PDL1 (214).

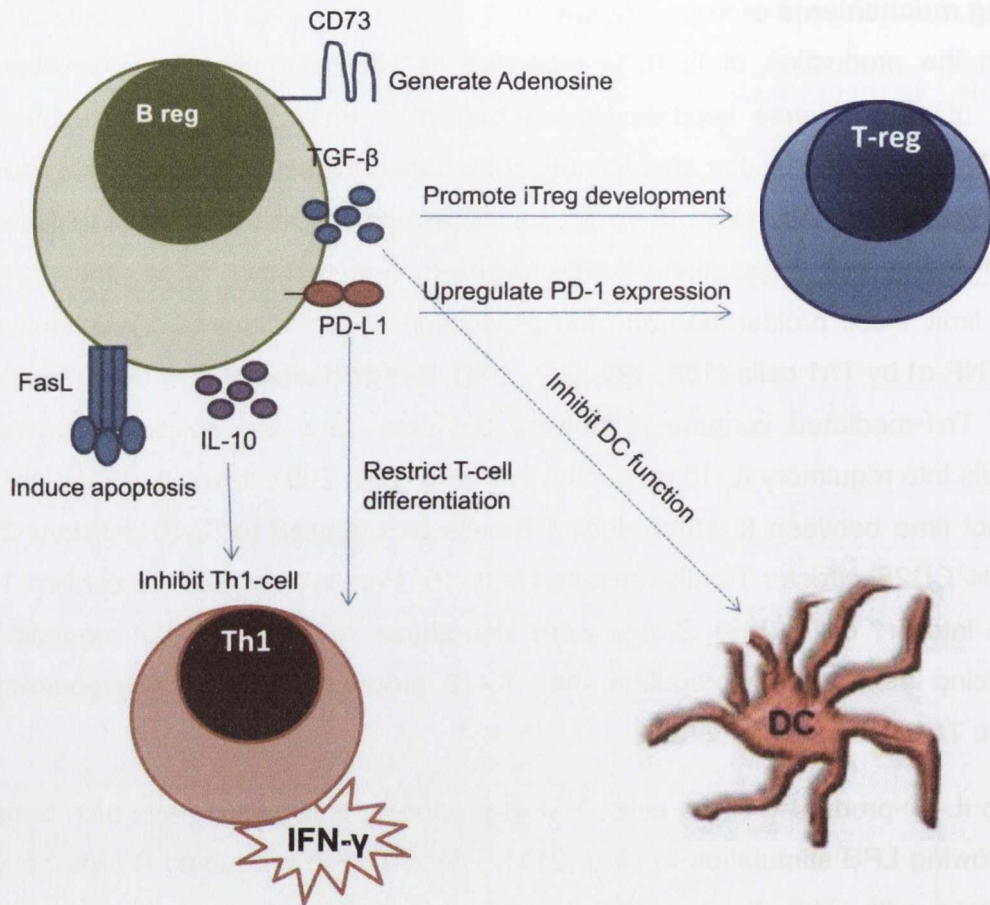


Figure 1.2 Mechanisms of suppression of regulatory B-cells identified in human and animal models

In mice, regulatory B-cell suppression is fulfilled by IL-10 secretion, activation of the CD40 pathway, TLRs and possibly via contact with T lymphocytes. It has numerous effects: (1) inhibition of Th1 and Th17 differentiation, (2) inhibition of antigen presentation by DCs, and (3) induction of natural regulatory T-cells. (4) induce apoptosis.

1.8. Myeloid derived suppressor cells

Myeloid derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells. Populations of MDSCs have been identified in the blood, spleen, liver, bone marrow and lymph nodes of mice. MDSCs are generally identified in murine studies as CD11b⁺Gr1⁺ cells. Human MDSC can be characterised by their expression of CD11b⁺ and CD33⁺, but are usually negative for HLA-DR, CD3, CD19 and CD57 (215). Difficulties arise as the markers Gr-1 and CD11b identify a multitude of populations of immune cells including neutrophils, eosinophils and monocytes (216, 217). The Gr-1 antibody recognises two epitopes, Ly6G and Ly6C, making conclusions about the type of cell responsible for suppressive function among the Gr-1⁺CD11b⁺ cell phenotype very difficult. MDSC have recently been subdivided into either monocyte or granulocyte like cells based on their Ly6C and Ly6G expression (218, 219). CD11b⁺ cells expressing Ly6G and low Ly6C levels are classified as granulocytic MDSC and express arginase 1 (Arg1). CD11b⁺ cells expressing high levels of Ly6C but not Ly6G are identified as monocytic MDSC expressing both Arg1 and inducible nitric oxide synthase (iNOS). Both subpopulations of MDSC have been shown to have suppressive function; however, whether granulocytic or monocytic MDSC are preferentially expanded seems to be independently associated with inflammatory stimuli present in a particular disease (219, 220).

Indeed, in certain models F4/80, CD40, IL-4R, and CD49d have all been found to be expressed on MDSC with enhanced regulatory function (221-224). This represents a major challenge as to date there is no specific marker or phenotype that can consistently identify non-regulatory Gr-1⁺CD11b⁺ cells from functional MDSC with suppressive capabilities (220). Because of this heterogeneity in Gr-1⁺CD11b⁺ subsets, it is now considered important to demonstrate the regulatory capacity or suppressive function of these cells to confirm that they are regulatory MDSCs. MDSCs are effective suppressors of both the innate and adaptive immune responses and **Table 1.4** provides a summary of MDSC suppression mechanisms.

Table 1.4. MDSC mechanisms of suppression

Mechanism of Suppression	Effect	Result	Reference
Arginase 1	Arginase deprivation	CD3 down modulation nT-reg expansion	(225, 226)
Inducible nitric oxide		T-cell apoptosis Inhibition of T-cell proliferation	(227, 228)
NADPH oxidase 2 (NOX2)	Reactive oxygen species	Inhibition of T-cell proliferation	(229)
Heme oxygenase 1 (HO1)		Inhibition of T-cell proliferation	(230)
Compete with DC for cysteine		Inhibition of T-cell proliferation	(231)
ADAM17	cleavage of L-selectin (CD62L)	Inhibition of the homing	(232)
TGF- β		inhibition of cytotoxic T lymphocytes	(233, 234)
IL-10		expansion of induced-T-reg Macrophage polarisation	(234)

1.9. The contrasting effects of immune regulation during bacterial colonisation and infection

1.9.1. The protective role of immune regulation during bacterial infections

Immune regulation plays a pivotal function in protecting the host from self harm, by controlling inflammation and immune pathology during infections (137). Systemic infection with *Escherichia coli*, *Toxoplasma gondii*, *Trypanosoma cruzi* and *Plasmodium chabaudi* in IL-10-deficient mice, resulted in extreme production of pro-inflammatory cytokines including TNF- α and IFN- γ which resulted in increased host immunopathology, morbidity and mortality. These examples demonstrate the crucial function played by immune regulation in order to protect the host (235).

In a pneumococcal pneumonia model of infection, it was shown that higher concentrations of TGF- β in the lung correlated with a rapid increase in Foxp3⁺Helios⁺ T-regs in the lungs. This increase in T-regs was responsible for protecting the host against increased bacterial dissemination from the lung to the blood which was hypothesised to be IFN- γ driven (236). This confirms the importance of TGF- β in driving the recruitment of T-regs to the lung and the importance of these regulatory cells in controlling IFN- γ producing NK cells and also the potentially inappropriate neutrophil response which results in bacterial dissemination to the blood (236).

Research has recently addressed the potential role of MDSCs in bacterial infections (237). Although, it is difficult to have a definite conclusion on the role of MDSCs during bacterial infection, they have been reported to play a beneficial role in models of polymicrobial sepsis. Polymicrobial sepsis resulted in an accumulation of MDSCs in spleens. Adoptive transfer of day 10 MDSCs to septic mice attenuated peritoneal cytokine production, increased bacterial clearance and dramatically improved survival rate (238). Studies examining the protective role of B-regs during bacterial infections are limited, however it was previously demonstrated that peritoneal B1 cells were capable of immunosuppression and host protection by containing bacterial burden in response to systemic *Coxiella burnetii* infection (239).

Very few studies have examined the protective role of immune regulation during *S. aureus* infections. One such study found that following intravenous challenge with *S. aureus*, IL-10^{-/-} mice developed more frequent and destructive arthritis as compared with WT mice. Furthermore, these IL-10^{-/-} mice resulted in higher bacterial load in blood and kidneys compared with WT mice. This study indicates that IL-10 is critical for controlling excessive pathological production of IFN-γ which was responsible for the impaired bacterial clearance/bacterial dissemination/pathology and more frequent and destructive arthritis (240). During a mouse model of biofilm-mediated prosthetic implant infection, C57/BL6 mice developed chronic implant infection, while Balb/c mice cleared the infection. This clearance was associated with a more robust Th2/T-reg response in the Balb/c mice indicating that a controlled response, where the bacteria is contained, is more efficient than an overly pro-inflammatory immune response which could cause tissue damage, thereby facilitating bacterial dissemination to other sites (241).

To summarise, it is clear that in certain circumstances/environments, immune regulation which involves diverse mechanisms depending on nature of infection or site of infection, including IL-10, T-regs and maybe B-regs or MDSCs play a critical role in protecting the host from an excessive pro-inflammatory setting that could cause tissue damage and potentially host morbidity and mortality.

1.9.2. The role of immune regulation in bacterial persistence during infection

Immune regulation plays an essential role in preventing excessive inflammation during the immune response to infection. In addition, it is essential for the establishment of immune tolerance, which allows the host to distinguish innocuous ingested or inhaled antigens and prevent the activation of an undesirable immune response to these antigens. Some pathogens however have evolved highly refined mechanisms which allow them to modulate these immune tolerance mechanisms and manipulate the host immuno-regulatory machinery for their own advantage in order to promote their own survival and transmission.

Bordetella pertussis has been shown to achieve persistence by targeting TLR4 on DCs to induce IL-10 production, which in turn promotes the expansion of IL-10 producing T-

regs. This induction of IL-10, functions to suppress antigen specific Th1 cells, which enhances the bacterium's chances of transmission to other hosts (242), ultimately preventing its efficient clearance (243, 244). *B. pertussis* achieves this IL-10 production by both macrophages and dendritic cells through expression of the virulence factors filamentous hemagglutinin (FHA) and adenylate cyclase toxin (ACT). This IL-10 then inhibits IL-12p70 production by DCs, and these DCs direct naive T-cells towards a regulatory phenotype (245-247).

In the case of Mtb, a common finding in patients with symptomatic tuberculosis, is a high concentration of IL-10 in the blood. Studies using PBMCs from Mtb infected patients have shown that antigen specific IFN- γ secretion is increased *in vitro* via the addition of anti-IL-10 antibodies, suggesting that IL-10 plays a role in controlling IFN- γ production by PBMCs. In murine studies it has been shown that IL-10 can directly inhibit macrophage activation, which therefore impairs the development of an effective Th1 response (248). *In vitro* experiments have also demonstrated that blocking IL-10, enhanced phagosome maturation in Mtb infected human macrophages after 3 h, while the addition of recombinant IL-10 significantly inhibited this process (249, 250).

Helicobacter pylori, a Gram negative bacteria which causes more the half of worlds cases of chronic gastritis, peptic ulcers and gastric adenocarcinoma, has been shown to manipulate the expansion of T-regs in both humans and mice. Individuals infected with *H. pylori* were found to have increased frequencies of CD4⁺CD25⁺Foxp3⁺CTLA4⁺ T-reg cells both the stomach and duodenal mucosa compared with uninfected controls.(251). While mice infected with *H. pylori* resulted in a substantial induction of a gastric Foxp3⁺ T-cell response. T-reg depletion resulted in increased gastric inflammation and reduced bacterial colonisation (252). The expansion and activation of these T-regs down regulate the Th1 adaptive immune response and thereby facilitate the persistence of the *H. pylori* infection (253).

S. aureus has been shown to manipulate host immuno-regulatory mechanisms to facilitate persistence during both a biofilm and chronic systemic infections. IL-10 production played a major role in this evasion strategy (254). During a chronic *S. aureus* bloodstream infection, it was observed that kidney abscesses were still present on day

56 post-infection. Reason for this was due to impaired T-cell responses (255). In a later study, this impaired T-cell response was attributed to the expansion of MDSCs and to a lesser extent T-regs in the spleens of infected mice compared with uninfected controls (256). Although both IL-10 and TGF- β were produced by an unidentified source, immunosuppression was primarily dependent upon cell-cell contact which inhibited effector T-cell responses (256). Adoptive transfer of MDSCs exacerbated systemic infection in recipient mice following *S. aureus* intra-venous challenge (256). Similarly, during chronic *S. aureus* orthopaedic biofilm infection, Heim *et al.* observed an expansion of IL-10-producing MDSCs at the site of infection, which was associated with bacterial persistence (257, 258). Targeted depletion of these cells resulted in an enhanced pro-inflammatory response that resulted in increased bacterial clearance (258, 259).

Not much is known regarding the role of B-regs during *S. aureus* infections. During a chronic systemic model of infection it was shown that B220⁺ B-regs did not play a role in immune suppression (256). However given that a variety of B-reg subsets express low levels of the marker B220 (260), this does not definitely rule out B-regs from this model of infection.

1.9.3. The role of immune regulation during bacterial colonisation

Unlike pathogenic bacteria, commensal bacteria do not represent a significant threat to the host under normal circumstances and in fact commensal species have been shown to actually provide benefits to the host in certain circumstances. In order to survive within the host, commensal organisms have evolved mechanisms to suppress inflammation and actively induce immune tolerance, which facilitates their survival. There is increasing evidence that the mechanisms regulating tolerance to the commensal flora are actually critically involved in maintaining systemic immune tolerance, which is instrumental in protecting against allergic and autoimmune diseases (261).

Commensal organisms, especially in the gut, have the ability to switch on the production of IL-10 from multiple cell populations to facilitate their survival. Studies in germ-free (GF) mice have identified an important link between the intestinal microbiota and the development of T-reg cells both locally in the gut and systemically. *Bacteroides fragilis* is an important component of the human intestinal microflora, maintaining a complex and generally beneficial relationship with the host. Through the expression of the carbohydrate molecule Polysaccharide A (PSA), *B. fragilis* can promote the expansion of IL-10 producing inducible CD4⁺CD25⁺FoxP3⁺ cells in a TLR2 dependent manner (262, 263). Similarly, *Bifidobacterium breve* has been shown to activate intestinal CD103⁺ DCs to produce IL-10 via the TLR2/MyD88 pathway thereby inducing IL-10-producing Tr1 T-reg cells in the large intestine (264).

In contrast to the gut micro-environment, little is known about how commensal species within other microbiomes e.g. respiratory tract, interact with the hosts immune-regulatory system. *S. pneumoniae* is a common coloniser of the upper-respiratory tract. In a murine model of *S. pneumoniae*, there was an expansion of T-reg cells in the nasopharynx at 3, 7 and 14 days following low density colonisation (265). This expansion was TGF- β dependent, as blocking of TGF- β led to a failure to induce T-reg cells but interestingly had no effect on IL-10 production, suggesting that in this model T-reg cells are not the main source of IL-10. In summary, this study found that TGF- β and T-reg cells were important in facilitating *S. pneumoniae* persistence during nasal colonisation, however they also played a protective role in preventing bacterial dissemination to the lung (265).

Zhang et al (2011) demonstrated that in children, the immune tissues called adenoids (at the back of nose) contain regulatory cells, CD4⁺CD25^{hi}Foxp3^{hi}CD39CTLA4 with the ability to inhibit the development of a Th17 response. While the presence and action of these T-reg cells is important in preventing self-tissue damage during infection (due to an excessive immune response), the study demonstrated a correlation between T-reg cell numbers and persistence of pneumococcal carriage (266).

There is currently no information regarding the capacity of *S. aureus* for immune regulation in the context of nasal colonisation. However, this thesis hypothesises that

during commensal colonisation of the host, *S. aureus* has the potential to modulate T-effector cells and the subsequent activation of downstream phagocytes in order to facilitate its own survival and persistence within its niche.

In conclusion it is clear that immune regulation can act as a “double edged sword” during exposure to bacteria. During an infection, it is imperative that the hosts’ pro-inflammatory responses are tightly controlled in order to prevent an excessive response capable of causing host morbidity in response to either pathogens or harmless antigens. In contrast to that, a sufficient immune response must be activated in order to clear the pathogen and not allow its persistence.

1.10. Project Aims

The fact that *S. aureus* is a lethal pathogen in addition to a harmless commensal coloniser, poses a unique set of questions when it comes to understanding the mechanisms of host: pathogen interactions with this organism. In the first instance, research is required to significantly advance the understanding of how colonisation impacts upon host immune response, as it is possible that prior exposure to *S. aureus*, a bacterium known for its capacity to induce immuno-suppression could impede the immune response to subsequent vaccination.

This project focuses specifically on understanding how *S. aureus* induces immune regulatory mechanisms including IL-10 and T-regs in the context of either localised or systemic infection and during nasal colonisation. Here this thesis attempts to decipher the mechanisms by which *S. aureus* activates these regulatory processes and the subsequent downstream effects of this on the activation of T-cells and phagocytes.

Recently, *S. aureus* has been shown to be very effective at manipulating the hosts' immune regulatory machinery during both biofilm and systemic models of chronic infection in order to generate a niche in which it can survive and persist. IL-10 was identified as key player in this immune evasion. Consequently, this work focuses particularly on IL-10, as recent research suggests it is at the forefront of *S. aureus* immune evasion strategy. Specifically, this project will address the impact of *S. aureus* induced IL-10 on different disease outcomes. The role of IL-10 will be examined during contrasting types of infection (colonisation v infection) and also at contrasting sites of infection (local v systemic). The role and consequences of IL-10 in regulating effector T-cell responses during three contrasting models i.e. colonisation, skin and peritonitis will be investigated. *S. aureus* might also engage other regulatory cytokines or cells including TGF- β and/or T-regs during colonisation or infection. The impact of these immune-regulatory mechanisms in terms of host protection will also be examined.

To achieve this, this project will address the following specific aims:

Aim 1: Characterise the immune response to *S. aureus* during nasal colonisation

1. Optimise a murine model of *S. aureus* nasal colonisation

2. Characterise the immune response occurring locally within the nasal cavity during *S. aureus* colonisation. Specifically exploring a role for Th17 cell cytokines IL-17 and IL-22 in controlling *S. aureus* nasal colonisation
3. Determine if IL-10 is induced during *S. aureus* nasal colonisation and if so investigate its role in regulating pro-inflammatory effector T-cell responses and then subsequent impact on phagocytic cells
4. Investigate if *S. aureus* expands T-reg cells locally in the nasal tissue during nasal colonisation and examine the role of these regulatory cells in facilitating *S. aureus* persistence.
5. Determine if IL-10 acts directly to inhibit *S. aureus* killing by phagocytes
6. Establish if *S. aureus* from persistent human carriers induces simultaneous production of pro- and anti-inflammatory cytokines from phagocytic cells, and examine if these colonising strains compare with invasive strains in terms of phagocyte cytokine production

Aim 2: Investigate the role of IL-10 during acute local vs systemic *S. aureus* infections

1. Establish the source of IL-10 during acute systemic peritoneal infection and examine if it controls effector T-cell responses to *S. aureus*.
2. Investigate the mechanism of activation of IL-10 producing cells
3. Compare the role of IL-10 during a systemic infection compared with a localised sub-cutaneous infection

Chapter 2

2. Materials and Methods

2.1. Materials

Table 2.1 General Reagents

Name	Recipe
Tryptic Soy Agar/ Broth (TSA)/(TSB)	30g Tryptic Soy (TS) Broth (Sigma Aldrich) 15g Agar (Sigma Aldrich) 1L dH ₂ O
Columbia Soy Agar (CSA)	35g Columbia Soy (CS) Broth (Sigma Aldrich) 15g Agar (Sigma Aldrich) 1L dH ₂ O
5% Blood Agar	25ml Horse Blood Defibrinated (Fischer) 1L TSA
cRPMI Media	500 ml RPMI (Sigma Aldrich) 10% (v/v) Heat-inactivated Fetal Calf Serum (FCS; Biosera) 100mM L-Glutamine (Gibco) 100µg/ml penicillin/streptomycin (Gibco)
Ammonium Chloride Lysis Buffer	0.77g NaH ₄ Cl (Sigma Aldrich) 1L dH ₂ O
10X PBS	80g NaCl (Sigma Aldrich) 11.6g Na ₂ HPO ₄ (Sigma Aldrich) 2g KH ₂ PO 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 ddH2O

Table 2.2 Additional Reagents

Reagent	Supplier
PBS	Sigma Aldrich
TRizol	Invitrogen
Brefeldin A	Sigma Aldrich
123-Dihydrorhodamine (DHR)	Sigma Aldrich

Table 2.3 ELISA Reagents

ELISA Reagents		
Wash Buffer		1L 10X PBS 9L dH ₂ O 5ml Tween 20 (Sigma Aldrich)
Reagent Solutions	Diluent	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 ₂ HPO ₄ (Sigma Aldrich) 10.1g Anhydrous Citric Acid (Sigma Aldrich) 1L 1X PBS pH 5.0
Substrate Solution		25ml Phosphate Citrate Buffer 10mg o-Phenylenediamine dihydrochloride Tablet (Sigma Aldrich) 7µl 30% H ₂ O ₂ (Sigma Aldrich)
Stop Reagent		1M H ₂ SO ₄ (Sigma Aldrich)

Table 2.4 ELISA dilutions

Cytokine	Supplier
IL-1 β	R&D
IL-10	R&D
IL-17A	R&D
IL-22	R&D
CXCL1	R&D
MPO	R&D
TNF- α	R&D
TGF- β	R&D
IFN- γ	R&D
IL-12	R&D
IL-23	R&D

Table 2.5 FACS Antibodies

Location	Antigen	Clone	Supplier
Extracellular	CD3	clone 500A2	BD Biosciences
	CD4	clone RM4-5	BD Biosciences
	CD8	clone 53-6.7	ebioscience
	$\Gamma\delta$	clone GL3	ebioscience
	CD11b	clone M1/70	ebioscience
	CD19	clone 1D3),	ebioscience
	F480	clone BM8	ebioscience
	Ly6G	clone IA8),	BD Biosciences
	Ly6C	clone HK1.4	ebioscience
	CD1d	clone 1B1	BD Biosciences
	CD5	clone 53-7.3	ebioscience
	CD25	clone PC61.5	ebioscience
	hCD11b	clone ICRF44	eBioscience
	hDC SIGN	clone eB-h209	eBioscience
	h CD11c	clone 3.9	eBioscience
	hCD14	clone TÜK4	Miltenyi Biotec
Intracellular	IL-17	clone 17B7	ebioscience
	IFN- γ	clone XMG1.2	ebioscience
	IL-22	clone 1H8PWSR	ebioscience
	NOS2	clone CXNFT	ebioscience
	FoxP3	clone FJK-16s	ebioscience
Blocking	IL-10 (human)	clone JES3-9D7	ebioscience
	IL-10 (mouse)	clone JESS-16E3	Ebioscience

2.2 Mice

Age- and sex-matched wild-type (WT) C57BL/6, BALB/c, B6.129S6-*Il10*^{tm1Flv}/J (267) and IL-10 knockout (IL-10^{-/-}) mice were housed under specific pathogen-free conditions at the Trinity College Dublin Comparative Medicines (CM) unit. All experiments were conducted in accordance with the recommendations and guidelines of the Health Product Regulatory Authority (HPRA), the competent authority in Ireland, and in accordance with protocols approved by Trinity College Dublin Animal Research Ethics Committee

2.3 Bacteria

2.3.1 Bacterial Strains

Streptomycin-resistant (Sm^R) mutant strains of *S. aureus* Newman (Newman Sm^R) (95), *S. aureus* PS80 (268) and USA 300 LAC::*lux* (269) have been previously described. All bacteria were cultivated on TSA from frozen stocks for 24 h at 37°C. All bacterial work was carried out in a Class II biological safety hood.

2.3.2 Streptomycin resistant Strain construction (Human clinical isolates)

S. aureus clinical isolates were grown overnight in TSB, and then plated onto TSA plates containing streptomycin (Sm) (500 µg/ml). Sm^R colonises were plated again on Sm-supplement TSA plates. Sm^R human clinical isolates were phenotypically identical to their parental strains in terms of growth rate, toxin production and adherence to loricrin.

2.3.3 *In vitro* loricrin adherence assay

Medium-binding microtiter plates (Nunc) were coated with recombinant loricrin (1 µM) in carbonate buffer and incubated overnight at 4°C. Wells were blocked with 5% (w/v) bovine serum albumin (BSA) for 2 h at 37°C. The plates were washed three times with PBS. A bacterial cell suspension (OD₆₀₀ = 1.0 in PBS) was added (100 µl per well), and the plates were incubated for 2 h at 37°C, washed three times with PBS, and bound cells were fixed with formaldehyde (25% v/v) for 20 min and stained with crystal violet (0.5% v/v, 100 µl per well) for 1 min, before washing with PBS and acetic acid (5% v/v). The absorbance was measured at 570 nm.

2.3.4 Preparation of bacterial inoculum for *in vivo* challenge studies

Following overnight growth on TSA agar from frozen stocks, a turbid solution of bacteria (1ml) was prepared by scraping bacterial colonies using a sterile loop and thorough mixing in sterile PBS. The optical density (OD) of the bacterial solution was determined at 600nm using a spectrophotometer and the bacterial suspension adjusted to 1×10^9 CFU/ml. The inoculum concentration was confirmed by plating serial dilutions of the bacterial suspension on TSA plates for 24 h at 37°C, after which the number of colonies per ml was determined.

2.4. *In vivo* models of *S. aureus* infection

2.4.1. *In vivo* model of *S. aureus* peritonitis

Mice were infected with *S. aureus* by (intra-peritoneal (i.p.) injection of 1×10^8 CFU suspended in sterile PBS. *S. aureus* Newman was used in all experiment unless otherwise stated. At specific time points post-infection, mice were sacrificed to assess local and systemic infection and associated immune responses.

2.4.2. *S. aureus* –induced acute sub-cutaneous infection.

Mice were shaved on the back and inoculated sub-cutaneously (s.c.) with a fluorescently tagged strain of *S. aureus* USA300 LAC::*lux* strain (2×10^7 CFU) in 100µl sterile PBS using a 27-gauge syringe (BD). At specific time points, mice were anaesthetised via inhalation of isoflurane (Iso-vet), bioluminescence imaging was performed using the Biospace Lab Photon Imager system. Bioluminescent activity is presented as ph/s/sr. Measurements of total lesion size (cm²) were made by analysing digital images using the software program (Biospace Lab Photon Imager) and a millimetre ruler as a reference. To confirm that the *in vivo* bioluminescent signals reflected bacterial burden, 8mm punch biopsies of lesional skin were taken at specific time points, homogenised in sterile PBS and total bacterial burden determined by plating out serial dilutions on TSA.

2.4.3. *S. aureus* nasal colonisation model.

Groups of mice (8-12 weeks) were given sterile water containing 0.5 mg/ml streptomycin for 24 h prior to nasal inoculation and throughout the duration of the

experiment. Mice were inoculated intra-nasally (i.n.) with *S. aureus* strain Newman Sm^R, 2x10⁸CFU in 10µl per nostril. At specific time points post inoculation, mice were euthanised and the nose excised, homogenised in 500µl PBS and plated onto TSA containing 0.5 mg/ml streptomycin to quantify *S. aureus* CFU per nose

2.4.4. Harvesting of tissues

To harvest the nose, the area surrounding the nose was wiped with 70% ethanol and the nose was excised (1 cm deep approximately) in 500 µl PBS. In order to isolate the nasal associated lymphoid tissue (NALT), which is localized bilaterally on the posterior side of the palate, the lower jaw was removed. The NALT was then separated from the rest of the nasal tissue by peeling away the palate, as previously described (270) (Figure 2.1).

Peritoneal exudates cells (PECs) were recovered from the peritoneal cavity following lavage with sterile PBS (2 ml i.p.). Livers, Spleens, kidneys and blood were collected for analysis of bacterial burden. Lavage samples were centrifuged at 300 x g for 10 min. Cell-free supernatants were then removed and stored at -20°C for cytokine analysis by Enzyme Linked Immunosorbent Assay (ELISA). Red blood cells were lysed using lysis buffer and PEC re-suspended in cRPMI for assessment of local immune response. A proportion of recovered blood was allowed to settle at room temperature (RT) for 1 h, centrifuged at 750 x g for 5 min, and sera contained in the supernatant transferred to a fresh 1.5ml tube and frozen at -20°C until further analysis.

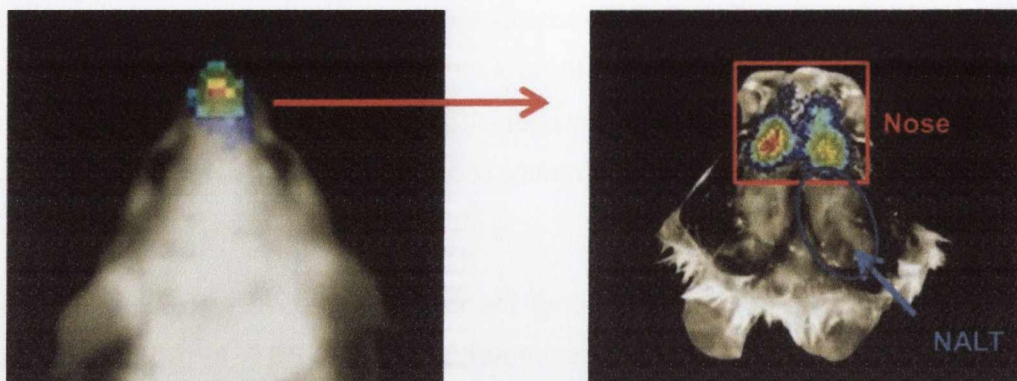


Figure 2.1. Sites of *S. aureus* nasal colonization.

Bioluminescence from mice inoculated intra-nasally with *S. aureus* (MRSA Xen31), demonstrates the anatomical location of *S. aureus* nasal colonization. The entire bioluminescent signal was emitted from the nose (Red), while no signal could be detected from the Nasal Associated Lymphoid tissue (NALT). Figure adapted from (271).

2.4.5. Isolation of cells from blood

To isolate blood leukocytes, blood was layered onto Histopaque-1083 (Sigma) for density gradient centrifugation at 400 x g for 30 min. Leukocytes were collected between the plasma layer and the pellet containing red blood cells (RBCs) and extracellular bacteria. As density gradient centrifugation separates cells based on their densities, the extracellular bacteria collect in the pellet with the RBCs. Isolated leukocytes were washed and re-suspended in Fcy block for flow cytometric analysis.

2.4.6. Quantification of Bacterial Burden

Serial dilutions of the peritoneal lavage contents (cells and fluid) and organs (liver, kidneys and spleen) were homogenised in 3 ml of sterile PBS using a homogeniser (Kinematica Polytron PT 2500E) 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, and CFU/ml determined. The homogeniser blade was cleaned between samples using 1M NaOH, sterile H₂O, 70% (v/v) EtOH and sterile PBS. All plates were kept at RT for a further 48 h to confirm a lack of contaminating bacterial species.

2.5 Eukaryotic Cell Counting

Cell counting was performed using a Trypan Blue exclusion assay. Briefly, 10 µl of cells were diluted in 90 µl of Trypan blue and 10 µl of the cell suspension loaded onto a disposable haemocytometer. Viable cells were counted using a light microscope and the number of cells was calculated according to the manufacturer's protocol.

2.6. B1 regulatory cell preparations

2.6.1. B1a regulatory cells isolation and adoptive transfer

Purified CD19⁺ CD11b⁺ CD5⁺ B1a regulatory B-cells were isolated from the peritoneal cavity of naïve WT and IL-10^{-/-} mice by FACS sorting using antibodies specific to CD19 (PerCp-Cy5.5), CD11b (Pe-Cy) and CD5 (APC) (Beckman Coulter [DakoCytomation] MoFlo Cell Sorter). The level of purity of purified B1a cells was routinely >95%. WT B1a cells or PBS were administered by i.p. injection to naïve mice (2 x 10⁵ cells/ mouse). At 12 h post transfer, mice were challenged by i.p. injection of *S. aureus* Newman (1 x 10⁸

CFU). On day 5 post-infection, the peritoneum was lavaged and the kidney, spleen, and liver isolated to determine local and systemic bacterial burden as previously described.

2.6.2. *In vitro* culture of B1a cells

B1a cells (1×10^5) were plated in cRPMI media– without Pen/ Strep prior to infection with *S. aureus* MOI 100. For cytokine blocking experiments, cells were incubated with monoclonal antibody for 1 h prior to infection. Supernatants were collected 15 h post-infection and assessed for IL-10 by ELISA.

2.7. Determining IgM antibody concentration

Total IgM in peritoneal fluid was determined by ELISA. Briefly, medium-binding 96-well plates were coated with purified Rat Anti-mouse IgM (1µg/ml) (Clone II/41) (BD Pharminger). Biotinylated Rat anti-mouse IgM (0.5µg/ml) (Clone R6-60.2) (BD Pharminger) antibody was used as a secondary antibody. Colour development was achieved using o-phenylenediamine dihydrochloride (OPD) (Sigma-Aldrich) in substrate buffer. The o-phenylenediamine dihydrochloride reaction was stopped using 1M sulphuric acid, and OD450 was measured using a micro-plate reader and Softmax Pro software.

2.8. Cytokine ELISAs

ELISA assays were carried out on cell supernatants to determine various cytokine concentrations (Table 2.4). A high binding 96-well flat bottomed plate was coated with capture antibody and incubated at 4°C overnight. Wells were washed in ELISA wash buffer 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 a serial dilution of cytokine standards, made up in reagent diluent (RD), and samples were added to the appropriate wells for 2 hr at RT or overnight at 4°C. Following incubation, wells were washed and detection antibody solution was added for 2 h, at RT. After further washing, streptavidin-horse radish peroxidase (HRP) solution, diluted to a working concentration in RD, was added to the wells for 20 min at RT in the dark. After washing, substrate solution was added to the wells for 20 min at RT in the dark. The reaction was stopped using 1M H₂SO₄. Absorbance was measured at 450nm using a micro-plate reader and Softmax Pro software.

2.9. Flow Cytometry

Single cell suspensions, prepared from various tissues were incubated at 37°C and 5% CO₂ for 4 h with Brefeldin A (BFA, 5µg/ml) in the presence or absence of PMA (10ng/ml) and Ionomycin (1µg/ml). Cells were then washed in PBS and centrifuged at 300 x g for 5 min. Cells were re-suspended in Fcy block (1 µg/ml) for 10 min at 4°C, to block non-specific antibody binding before extracellular staining with antibodies against specific cell surface antibodies. Cells were suspended in a final concentration of (2µg/ml) of surface antibody solution diluted in FACS buffer and incubated for 20 min at 4°C before washing in FACS buffer and centrifugation at 300 x g for 5 min. For intracellular staining, 60µl of Intrastain Reagent A (Dakocytomation Intrastain Kit (Alere), a fixative reagent (contains 5-10% formaldehyde) was added to cells following incubation with surface antibodies and incubated at RT for 15 min. Samples were then washed in FACS buffer and centrifuged at 300 x g for 5 min. Next, 60µl of Intrastain Reagent B, a permeabilisation reagent containing fluorescence-conjugated antibodies specific for intracellular cytokines was added to each sample, at final concentration of (2µg/ml) and incubated for 15 min at RT in the dark. Samples were washed again and re-suspended in FACS Buffer. Flow cytometry data acquired using BD FACSCanto II and FACS DIVA software (Treestar, Inc). Flow cytometry data was analysed using FloJo software with gates set on respective Fluorescence Minus One (FMO) samples. To investigate reactive oxygen species (ROS) activity within phagocytes, 123-dihydrorhodamine assays were carried out. ROS breaks down 123-DHR to rhodamine within the cell. Rhodamine fluoresces on the FL1 channel allowing rhodamine positive cells to be identified by flow cytometry. After surface staining, as above, 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, centrifuged at 300 x g and re-suspended in 1% paraformaldehyde. Flow cytometric analysis of rhodamine was performed immediately. For full gating strategy see Appendix.

2.10. *In vitro* cell culture

2.10.1. Generation and infection of murine Bone marrow derived Macrophages

WT C57/BL/6 and IL-10^{-/-} mice were euthanised and their hind legs dissected from the muscle. The femur and the tibia of each leg were isolated and the ends of the bones removed. The bone marrow was flushed out with cRPMI using a 27 gauge needle and 5ml syringe. The cells were centrifuged at 1200 rpm for 5 min and re-suspended in 3ml ammonium chloride at room temperature for 2 min to lyse red blood cells. Cells were centrifuged at 300 x g for 5 min, re-suspended in 5ml of cRPMI and counted by Trypan blue exclusion assay. Cells (1x10⁶/ml) (x10ml) were seeded in petri dishes in cRPMI containing 20% Macrophage Colony Stimulating Factor (M-CSF), in the form of supernatant from the M-CSF producing cell line L929. After day 3, 15 ml of cRPMI supplemented with 20ng/ml M-CSF was added to each petri dish. On day 6, supernatant was removed, adherent cells (BM-Macrophages) were collected by gently scraping of the petri dish. Cells were centrifuged at 300 x g for 5 min. BM-Macrophages were counted using Trypan Blue exclusion assay, plated at 1 x 10⁶ cells/ml in RPMI supplemented with 10% M-CSF. Cells were rested overnight before infection with bacteria of various MOI.

2.10.2 PBMC isolation

Human peripheral blood mononuclear cells (PMBCs) were isolated from buffy coats (provided by the Irish Blood Transfusion Service (IBTS)) by density gradient centrifugation of PBS-diluted fresh heparinised blood. Briefly, blood was diluted 1:1 with sterile PBS and slowly layered over Lymphoprep (Axis-Shield) in a 5:4 ratio to create a density gradient. The solution was centrifuged at 300 x g for 20 min with the brake off. The top plasma layer was discarded and the buffy coat layer was removed and added to ~10 ml of PBS. The solution was centrifuged at 650 x g for 10 min to remove any additional plasma. The pellet was washed in 50 ml of PBS and centrifuged at 400 x g for 7 min to remove platelets. The remaining pellet was resuspended in 10 ml of cRPMI and cell viability assessed by Trypan Blue exclusion assay.

2.10.3. Monocyte-derived macrophages

CD14⁺ monocytes were prepared from PBMCs by magnetic separation using Miltenyi Biotec CD14⁺ beads according to manufacturers' instructions. Purified CD14⁺ monocytes were cultured at 1x10⁶cells/ml in cRPMI containing 20 ng/ml M-CSF in a 6-well plate. After a day 3 incubation at 37°C and 5% CO₂, 1 ml of fresh cRPMI containing 80 ng/ml M-CSF (4x) was added to each well.

2.10.4. Monocyte-derived dendritic cells

Purified CD14⁺ monocytes were cultured at 1x10⁶cells/ml in cRPMI containing 50ng/ml GM-CSF in a 6-well plate. After a day 3 incubation at 37°C and 5% CO₂, 1 ml of fresh cRPMI containing 200 ng/ml GM-CSF (4x) and 200 ng/ml IL-4 (4x) was added to each well.

On day 6, both cell types were collected by repeat pipetting, viability assessed using Trypan blue exclusion assay, and re-suspended at a final concentration of 1x10⁶cells/ml in penicillin/streptomycin free media. The cells were plated at 2x10⁵cells/well in a 96-well flat-bottomed plate.

2.11. Infection of murine and human phagocytes

2.11.1. Assessment of bacterial killing

Cells were infected with live *S. aureus* at multiplicities of infection (MOI) of 1, 10 or 100 for the indicated times. At the indicated time point, infected cells were centrifuged at 300 x g, the supernatant removed and cells lysed by the addition of 20 µl 0.1% Triton-X 100. The supernatant was then re-introduced into the well and mixed with the cell lysate. Serial dilutions of the suspension were prepared in PBS and plated on TSA to determine the CFU/ml. Bacterial killing was determined as the % reduction of CFU in wells containing bacteria and phagocytes as compared with wells containing bacteria only.

2.12. Analysis of viability

To assess *S. aureus* induced cytotoxicity, B1a cells, BMMs or human macrophages or DCs were infected as described above. At specified timepoints, the plates were centrifuged (300 x g, 5 min) and the cell-free supernatant collected. LDH release into

the supernatant was measured using the Pierce LDH Cytotoxicity Assay kit according to the manufacturer's instructions.

2.13. Ethics Statement

Anonymised buffy coats were obtained from healthy blood donors at the Irish Blood Transfusion Service, Dublin. Donors have given written approval for the use of cells for scientific purposes. The study was approved by the St James's and Tallaght Hospitals Joint Research Ethics Committee (2011/35/04).

2.14. 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 posttest. 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

3. Characterisation of immune response mechanisms underpinning *S. aureus* nasal colonisation

3.1. Introduction

S. aureus nasal colonisation is a multi-faceted process which depends on a multitude of factors that allow the bacteria to adapt and persist within this niche. These include genetic factors, adaptation to nutrient availability, environmental factors including age and smoking and intra-species competition with other commensal organisms occupying the same niche e.g. *S. pneumoniae* (272), *Corynebacterium* species (273), *S. epidermis* (274). In terms of host: pathogen interactions during *S. aureus* nasal colonisation, the majority of research has focused on understanding bacterial factors that allow the bacteria to adhere within its niche. This research has identified the major bacterial factors and host ligands that facilitate adherence of *S. aureus* to two distinct sites with the nasal cavity. In the anterior nares, *S. aureus* binds cornified envelope protein loricrin via ClfB (95), while *S. aureus* has also been shown to adhere to SREC-I via an interaction with WTA (94) in the inner nasal cavity. Relatively little is known, however, about the host factors involved in controlling *S. aureus* nasal colonisation. Defects in local antimicrobial peptide (AMPs) activity have been linked with persistent *S. aureus* carriage (117, 275), suggesting that AMPs play a valuable role in the elimination of *S. aureus* from the nose. Furthermore, polymorphisms in several immune genes encoding Interleukin-4 (IL-4), TLR2, mannose-binding lectin, glucocorticoid receptor gene and C-reactive protein have all been linked to persistent *S. aureus* nasal carriage (101-104). These factors all highlight the fact that *S. aureus* cultivates a unique relationship with the host immune system during colonisation.

To-date, persistent carriage has only correlated with genetic polymorphisms in the hosts immune system, however, few mechanistic studies have been performed to define the local immune response occurring during *S. aureus* nasal colonisation. A recent study has suggested that elimination of *S. aureus* nasal carriage is dependent on a Th17-cell-mediated response locally within the nose. *S. aureus* clearance from the nose was shown to be dependent upon the induction of IL-1 β , IL-17 and the neutrophil chemo-attractant CXCL1 locally at this site. IL-17 was identified as a key mediator of *S. aureus* decolonisation through the ability of this cytokine to control CXCL1 driven recruitment of neutrophils to the site. Mice deficient in IL-17A displayed increased nasal bacterial burden compared with wild-type mice following nasal colonisation with *S. aureus*,

coupled with an impaired neutrophil influx to the nose (123). However, IL-17A deficiency did not result in the same increased level of bacterial burden observed in severe combined immuno-deficient (SCID) and β/δ -TCR KO mice following *S. aureus* nasal colonisation, suggesting that IL-17 is not solely responsible for controlling clearance of *S. aureus* from the nose during colonisation. This data suggests that other T-cell cytokines are involved in the elimination of *S. aureus* during nasal colonisation.

Similar to the intestinal mucosa, the nasal mucosa is continually exposed to a diverse ecosystem of microorganisms. Evidence from the intestinal mucosal setting has shown that commensal species have evolved mechanisms to suppress inflammatory responses and actively induce immune tolerance which facilitates their survival within this niche. Studies in germ-free mice have identified an important link between the intestinal micro-biota and the development of T-reg cells both locally in the gut and systemically. In the gut, Polysaccharide A (PSA) on the outer membrane of *Bacteroides fragilis*, an important gut commensal, signals via TLR2 on CD4⁺ cells to induce their differentiation into T-reg cells (276). These cells then secrete immunosuppressive IL-10 which results in immune tolerance towards *B. fragilis* (262, 277). Similarly, colonisation of germ-free mice with a cocktail of murine gut commensal Clostridia strains resulted in the induction of TGF- β and T-reg cells in the colon and subsequent resistance to colitis (278).

Although the impact of intestinal flora on the host immune regulatory machinery has been well investigated, little is known about how other commensal species might manipulate this at sites outside the gut such as the nasal cavity. *Streptococcus pneumoniae* is a commensal coloniser of the upper-respiratory tract of humans. It has been shown that during nasopharynx colonisation with *S. pneumoniae*, there was an induction of the anti-inflammatory cytokines IL-10 and TGF- β locally within the nasopharyngeal tissue. T-reg cells were also expanded in the nasopharyngeal tissue following *S. pneumoniae* nasopharynx colonisation. Inhibition of TGF- β led to a failure to induce T-reg cells but had no effect on IL-10 production, suggesting that in this model T-reg cells are not the main source of IL-10. This TGF- β -induced T-reg expansion facilitated bacterial survival by limiting the influx of neutrophils to the nasopharynx. Although the

role of IL-10 was not fully examined, an increase in IL-10 production was observed between days 1 and 3 in the nasopharynx of colonised mice. However, the main conclusion from this study was that TGF- β drives an expansion of T-regs in the nasopharyngeal tissue which in turn facilitates *S. pneumoniae* survival within this niche (265).

It is hypothesised that *S. aureus* is capable of promoting immuno-regulatory responses locally within the nasal cavity as a means to facilitate colonisation and persistence at this site. *S. aureus* has previously been shown capable of inducing IL-10 production in order to facilitate persistence during a biofilm model of infection (254), indicating that during chronic biofilm infection *S. aureus* can utilise IL-10 for its own survival. Interestingly, it has been debated that *S. aureus* forms a biofilm like state in the nose (279, 280); if this is the case, IL-10 could be at the forefront of a potential evasion strategy implemented by *S. aureus* in this nasal setting.

This current study initially aims to further decipher host mechanisms that control *S. aureus* nasal colonisation including Th17 cytokines IL-17 and IL-22. It will also investigate the potential of *S. aureus* to manipulate the hosts own regulatory machinery, specifically IL-10, to facilitate its persistence at the nasal site and will further establish if these immuno-regulatory mechanisms act upstream to control these effector T-cell responses. Finally, alternative immune regulatory mechanisms including TGF- β and T-regs that could be potentially manipulated by *S. aureus* during nasal colonisation will also be examined.

3.2. Results

3.2.1. Characterisation, generation and *in vivo* nasal testing of streptomycin resistant colonising strains.

Murine *S. aureus* nasal colonisation models are well-established; however in order to further improve this model so that colonisation can be established over an extended period of time, it was hypothesised that strains of *S. aureus* isolated from persistent carriers may colonise the mouse for prolonged periods. Seven *S. aureus* isolates from a cohort of persistent nasal carriers were kindly provided by Dr. Willem van Wamel, Department of Medical Microbiology and Infectious Diseases, Erasmus Medical Centre, Rotterdam, The Netherlands (281). These different strains of *S. aureus* (referred to as RD strains) were used to establish murine nasal colonisation in WT C57/BL6 mice.

Initially, it was important to confirm that these clinical isolates were *S. aureus*, and this was accomplished by culturing the strains on Mannitol salt agar (MSA) plates. As *S. aureus* is a known mannitol fermenter, it appears as yellow colonies and causes a colour change from red to yellow on the agar due to acidification as a product of fermenting mannitol. Results showed that there was a colour change observed on the MSA plates from five out of the six clinical strain isolates (**Table 1**). As RD48 did not ferment the mannitol, it therefore was not considered a *S. aureus* strain and was disregarded.

Streptomycin-resistant (Sm^{R}) *S. aureus* strains were required, as this allows the administration of streptomycin (Sm) supplemented water to mice, in order to eradicate normal flora from the nose and allows rapid identification of the colonising strain in selective media (282). In order to isolate Sm^{R} mutants of these *S. aureus* clinical strains, *S. aureus* clinical isolates were grown in TSB overnight, plated on Sm-supplemented plates and incubated overnight at 37°C. Spontaneous Sm^{R} colonies were then isolated and grown overnight on Sm-supplemented agar plates to confirm resistance. These Sm^{R} mutants were then grown on MSA to ensure no change in mannitol fermentation ability.

Having generated streptomycin-resistant mutants of the human colonising strains it was important to ensure that these Sm mutant strains were not significantly altered from the parental strain in terms of growth rate, colony shape or size and toxin production. To analyse colony shape, the parent and Sm mutant strains were grown on TSA, Sm supplemented TSA and sheep blood agar plates. This confirmed a lack of contaminants and that colony shape remained the same in all cultures (**Table 1**).

Growth rate was determined by culturing each strain in TSB and measuring the optical density of the bacteria over time. In the case of each strain, the growth rates of the Sm^R mutants were not significantly altered from the parental WT strains (**Figure 3.1A**) (**Table 1**).

To confirm that the generation of the Sm^R mutant strain did not adversely affect accessory gene regulator (*agr*) function, a delta toxin assay was performed. Delta toxin production can be used to assess *agr* function. The *agr* system controls many virulence factors of *S. aureus*. δ -toxin has weak activity on sheep blood but is synergistic with β -toxin. Where β -toxin and δ -toxin from the *S. aureus* strain meet, this generates a zone of clear haemolysis. Visualisation of a translucent area of haemolysis surrounding the bacteria is a measure of toxin production by the bacteria (**Figure. 3.1B**). The Sm^R mutant and parent strain were plated on sheep blood plates alongside a known α and δ -toxin producer (*S. aureus* strain Newman (283)) as a comparison and RN4220 which produces β -toxin only (284). Results demonstrate that RD9, RD32 or their Sm^R counterparts were not capable of α or δ toxin production. All other strains displayed varying degrees of α or δ toxin production (**Table 1**).

An interaction between the squamous cell envelope protein loricrin and the *S. aureus* surface protein ClfB has been defined as critically important for facilitating *S. aureus* nasal colonisation (95). To assess the colonisation potential of these *S. aureus* isolates, the abilities of both the parental and Sm^R strains to bind to recombinant loricrin in an *in vitro* binding assay was tested. Sm resistance did not impact upon the abilities of the RD strains to bind to recombinant loricrin, however none of the clinical isolate strains had a greater loricrin binding ability than the laboratory strain Newman (**Figure 3.2**).

Taken together these results demonstrate that the Sm mutation in the human isolates does not adversely affect colony size, shape, growth rate or toxin production or the ability of these strains to adhere to loricrin.

To investigate the colonising ability of the human colonising RD strains *in vivo*, groups of WT mice were inoculated with 2×10^8 CFU of Sm^R RD *S. aureus* strains, or *S. aureus* strain Newman Sm^R. On d10 post colonisation the nose was excised and the bacterial burden in the nares assessed. Two human colonising strains RD9 and RD21 were comparable to strain Newman in terms of their colonising ability with approximately 1712 CFU, 1779 CFU and 1641 CFU recovered from the noses of Newman, RD21 and RD9 colonised mice, respectively (**Figure 3.3A**). Looking at colonisation rate (determined by the number of mice colonised by *S. aureus*/total number of mice) however, only Newman had a 100 percent colonisation rate on d10. Strain RD44 also appeared to have a 100% colonisation rate; however, only 100 CFU were recoverable from the noses of RD44 colonised mice at this time point (**Figure 3.3B**). Taken together, it appeared that the human colonising strains were not superior to the laboratory strain Newman in terms of their ability to colonise the murine nose. Consequently, *S. aureus* Newman was only used throughout the rest of these studies.

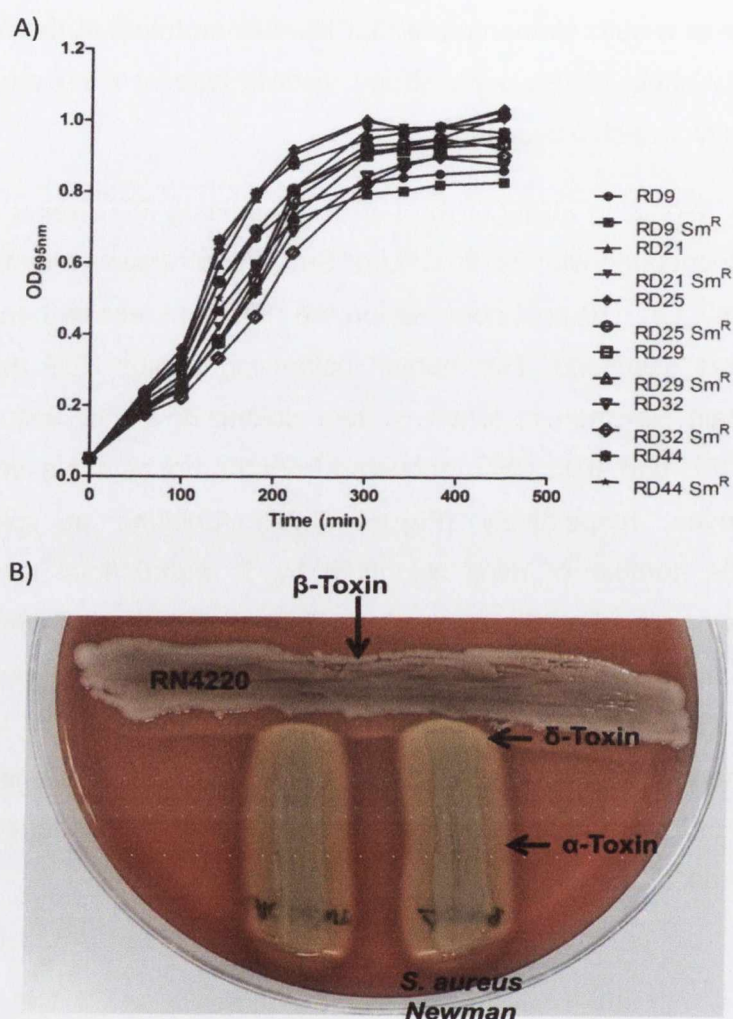


Figure 3.1. Validation of *S. aureus* growth rate and toxin production.

In order to measure *S. aureus* growth rate, bacterial strains were grown in TSB over the indicated period of time. The growth rate of *S. aureus* was estimated using optical density, recorded at 595nm using a spectrophotometer **(A)**. Bacterial strains were plated on sheep blood agar plates. Newman was used as a positive control as it is a known α and δ -toxin producer. Where β -toxin from RN4220 and δ -toxin from Newman met, there becomes a translucent area. The translucent area surrounding the bacteria is a measure of toxin production. The Sm^R RD strains and their parent counterpart were then compared with each other and to Newman, in terms of toxin production **(B)**. **Experiments performed in conjunction with Dr. Michelle Mulcahy, Host: Pathogen Interactions Group, School of Biochemistry and Immunology, TCD.**

Table 3.1. Human Sm^R clinical isolates did not differ from their parental strain post mutation.

Strain (Post-Sm ^R Mutation)	MSA Utilisation	Parent v Sm ^R Colony Shape	Toxin Production
RD9	Yes	Same	None
RD21	Yes	Same	α and δ toxin
RD25	Yes	Same	α and δ toxin
RD29	Yes	Same	α and δ toxin
RD32	Yes	Same	None
RD44	Yes	Same	α and δ toxin
RD48	No	N/A	N/A

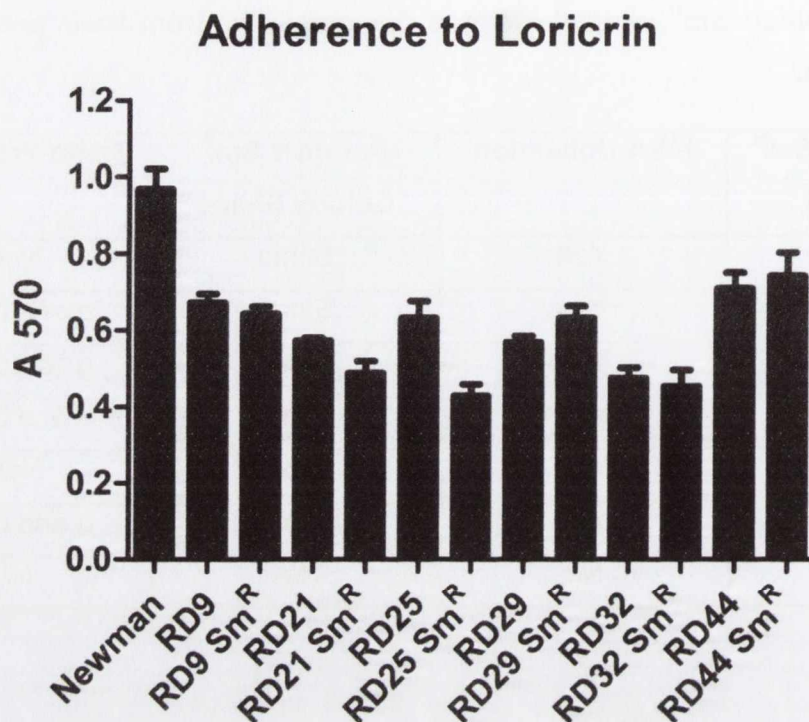


Figure 3.2. Examination of the loricrin binding abilities of *S. aureus* Newman, human colonising isolates (RD) and their Sm^R strains.

S. aureus Newman, human RD isolate strains and their Sm^R counterparts were added to 96-well plates containing immobilized recombinant mouse loricrin (1μM). Bacterial adherence was measured by staining with crystal violet and measurement of the absorbance at 570 nm. Values represent the mean ± SD of triplicate wells. **Experiments performed in conjunction with Dr. Michelle Mulcahy, Host: Pathogen Interactions Group, School of Biochemistry and Immunology, TCD.**

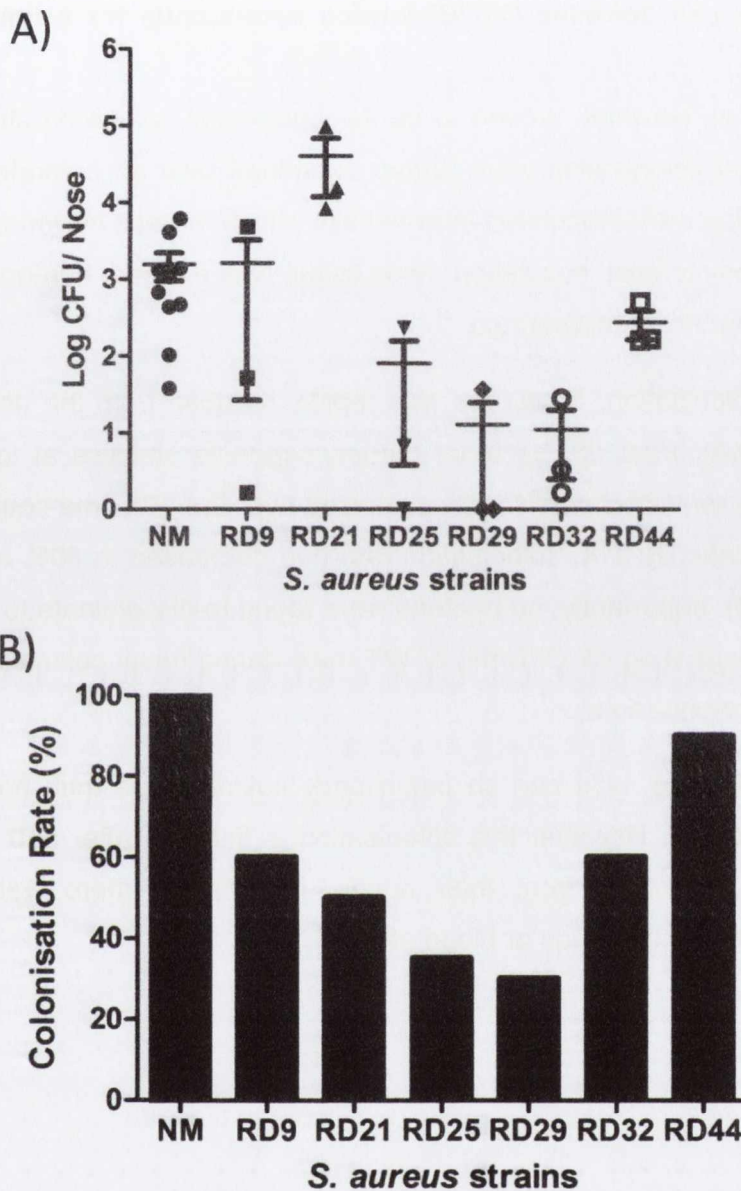


Figure 3.3. Nasal colonisation with *S. aureus* strain Newman and Sm^R RD isolates in C57/BL6 wild-type mice.

Groups of WT mice were inoculated intra-nasally with various *S. aureus* isolates (2×10^8 CFU). Mice were euthanised on d10 and bacterial burden in the nose quantified. Nasal homogenates were plated onto Sm supplemented agar to determine *S. aureus* CFU (**A**). Colonisation rate was determined by number of mice positive for *S. aureus*/ total number of mice (**B**). Results expressed as mean $\pm$ SEM CFU per nose (n=3-5, per group).

3.2.2. *S. aureus* can colonise C57/BL6 mice persistently for a limited period of time.

As *S. aureus* strain Newman proved to be the best nasal colonising strain at d10, the dynamics of nasal colonisation were further examined over an extended time-course. Groups of WT mice were inoculated intra-nasally with *S. aureus* Newman (2×10^8 CFU). At specific time points post inoculation, nose tissue was excised, homogenised and the number of CFU per nose enumerated.

Up to d3 post colonisation, *S. aureus* was rapidly reduced from the noses of the WT mice (**Figure 3.4A**). Post d3, bacterial burden began to stabilise at lower levels. All C57/BL6 animals remained persistently colonised over the 10d time course as seen by the colonisation rate. By d14, colonisation rate had decreased to 50% and was 0% by d21 (**Figure 3.4B**). Importantly, no bacteria were found to disseminate to the blood (Log <1 CFU/ml) or lungs (Log <1 CFU/ml) of WT mice during nasal colonisation at d1 post *S. aureus* nasal colonisation.

In conclusion, C57/BL6 mice can be persistently colonised via intra-nasal inoculation with *S. aureus* to d10. However this colonisation is finite as after d10 C57/BL6 mice began to clear *S. aureus* from their noses. Importantly, there was no bacterial dissemination to either the lungs or blood after d1.

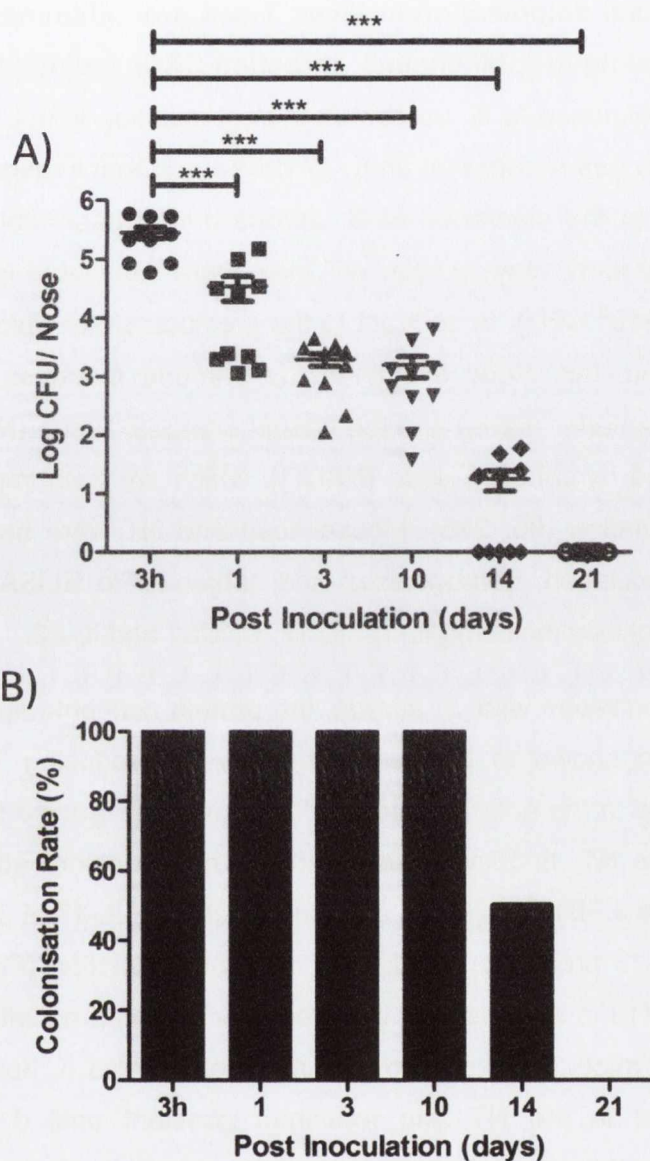


Figure 3.4. *S. aureus* is capable of colonising the noses of C57BL/6 mice persistently to d10.

Groups of WT mice were inoculated intra-nasally with *S. aureus* Sm^R Newman. Noses were harvested at various time points post-inoculation, homogenised, and plated on Sm supplemented agar to determine *S. aureus* CFU/nose (**A**). Colonisation rate was determined by number of mice positive for *S. aureus*/ total number of mice (**B**). Results expressed as mean $\pm$ SEM. n = 9-12 mice per group. Data pooled from 3 independent experiments. Statistical analysis was performed using a one-way ANOVA with Tukey post-test. *** $P \leq 0.001$.

3.2.3. *S. aureus* nasal colonisation induces T-cell pro-inflammatory cytokines IL-17 and IL-22 and the neutrophil chemo- attractant CXCL1 within the nasal tissue

The local immune response to *S. aureus* during colonisation is not well understood. A single previous study has shown that an IL-17 driven neutrophil response plays a major role in contributing to the clearance of *S. aureus* during nasal colonisation (123). In order to confirm that IL-17 plays a role, WT mice were inoculated intra-nasally with *S. aureus* Newman (2×10^8 CFU). In contrast to the previous study which only excised the nose for examination, this study examined the immune response in both the nose (encompassing the anterior nares) and the nasopharyngeal tissue (NT) (which includes the Nasal Associated Lymphoid Tissue (NALT)), which are both important sites for *S. aureus* nasal colonisation (95, 285). Mouse nose and NT were harvested at various time points post-inoculation, homogenised and subjected to ELISA to detect various cytokines and chemokines including IL-1 β , IL-17, CXCL1 and IL-22.

Following nasal colonisation with *S. aureus*, the protein concentrations of IL-1 β , which has previously been shown to be upstream of IL-17 producing T-cells (286), was substantially elevated in the nose of colonised mice at d3 (**Figure 3.5A**) compared with PBS controls. In the NT, IL-1 β was elevated at 3 h post colonisation compared with PBS controls (**Figure 3.5B**). There was also an induction of IL-17 at 3 h post inoculation in the nose. This IL-17 production peaked at d3 post inoculation (**Figure 3.6A**) before rapidly declining at d10 in the noses of colonised mice compared with PBS controls. In the NT of colonised mice, IL-17 protein was not present at 3 h, however by d1 IL-17 protein was induced in the NT and remained constant until d10 (**Figure 3.6B**). Discrepancy between the concentrations and peaks of both IL-17 and IL-1 β in the nose and NT likely reflects differences in cell phenotypes found in each location. For example, epithelial cells will be present in the nose which can make IL-1 β in response to *S. aureus* exposure (287). Concentrations of the neutrophil chemo-attractant CXCL1 were significantly increased 3 h post inoculation in both the nose (**Figure 3.7A**) and NT (**Figure 3.7B**) of colonised mice compared with PBS controls. At d1 the concentrations of CXCL1 in the nose and NT were substantially reduced but remained constant until d10.

In addition, this study also examined IL-22 production in both the nose and NT. IL-22 protein was induced in the nose at d1 and continued to increase, as significant concentrations of IL-22 protein were detectable in the nose at d10 post colonisation compared with PBS controls (**Figure 3.8A**). In contrast, there was a more rapid induction of IL-22 protein in the NT after 3 h which steadily declined until d10 post colonisation (**Figure 3.8B**) compared with PBS treated mice. This data, suggests that IL-22 may be an alternative Th17 cytokine which could potentially play an important role in controlling *S. aureus* nasal colonisation.

It was important to next establish the cellular sources of the cytokines IL-17 and IL-22 in the nasal cavity during *S. aureus* nasal colonisation. To facilitate this mice were colonised with Newman Sm^R, and at specific time points, the nose and NT was excised for analysis of total leukocyte infiltration. Leukocytes were also stained with a panel of well-defined surface markers to identify individual T-cell subsets by flow cytometry combined with intracellular cytokine staining for IL-17 and IL-22.

The total numbers of leukocytes were assessed in both the nose and NT during *S. aureus* nasal colonisation. There was a significant increase in the number of leukocytes infiltrating the nose at 3 h post colonisation compared with PBS controls. Similarly, at 3h and d1 post colonisation, there was also a significant increase in total leukocytes in the NT compared with PBS controls. The absolute numbers of leukocytes were much greater in the NT (**Figure 3.9A**) as compared with the nose (**Figure 3.9B**). Looking at the proportions of lymphocyte subsets present however, T-cells were not detected in the Nose (**Figure 3.10**), however CD4⁺, CD8⁺ and $\gamma\delta$ ⁺ T-cells were all expanded compared to PBS controls in the NT (**Figure 3.11A&B**). Therefore, only the NT was used to examine the T-cell response during *S. aureus* nasal colonisation.

During *S. aureus* nasal colonisation, IL-17 producing CD4⁺ and $\gamma\delta$ ⁺ T-cells were substantially expanded in the NT, peaking at d1 post colonisation. CD4⁺ T-cells were identified as the dominant source of IL-17. CD8⁺ T- cells did not produce any IL-17 during nasal colonisation (**Figure 3.12A&B**). This data supports previously published results suggesting that CD4⁺ T-cells produced IL-17 during nasal colonisation (123). In

addition, it also highlights a novel role for IL-17 producing $\gamma\delta$ T-cells during *S. aureus* nasal colonisation.

At d7 post colonisation (IL-22 mRNA expression was significantly upregulated in the nose at 168 h post colonisation (288)), IL-22 was predominantly produced by CD4⁺ T-cells (CD3⁺CD4⁺) and CD8⁺ T-cells (CD3⁺CD8⁺). IL-22 was also produced by a population of CD3⁻CD4⁺ cells, which potentially represent type-3 innate lymphoid cells (ILCs) (**Figure 3.13A&B**). However, the low numbers of these cells recoverable from the NT (~200 cells) precluded further phenotypic analysis of these cells. IL-22-producing $\gamma\delta$ ⁺ T-cells (CD3⁺TCR δ ⁺) were not detected at this time point (**Figure 3.13B**).

To conclude, Th17 cytokines IL-17 and IL-22 were found to be substantially induced within the NT during *S. aureus* nasal colonisation. CD4⁺ T-cells were producing both IL-17 and IL-22, however it will be important to investigate if these CD4⁺ T-cells are dual producers of both these cytokines. These data suggest that IL-17 and IL-22 might be produced at differential time-points as IL-17 appears to be produced at early stages during colonisation, while IL-22 is induced at later time-points. $\gamma\delta$ ⁺ T-cells also emerged as another IL-17 producing cell during *S. aureus* nasal colonisation.

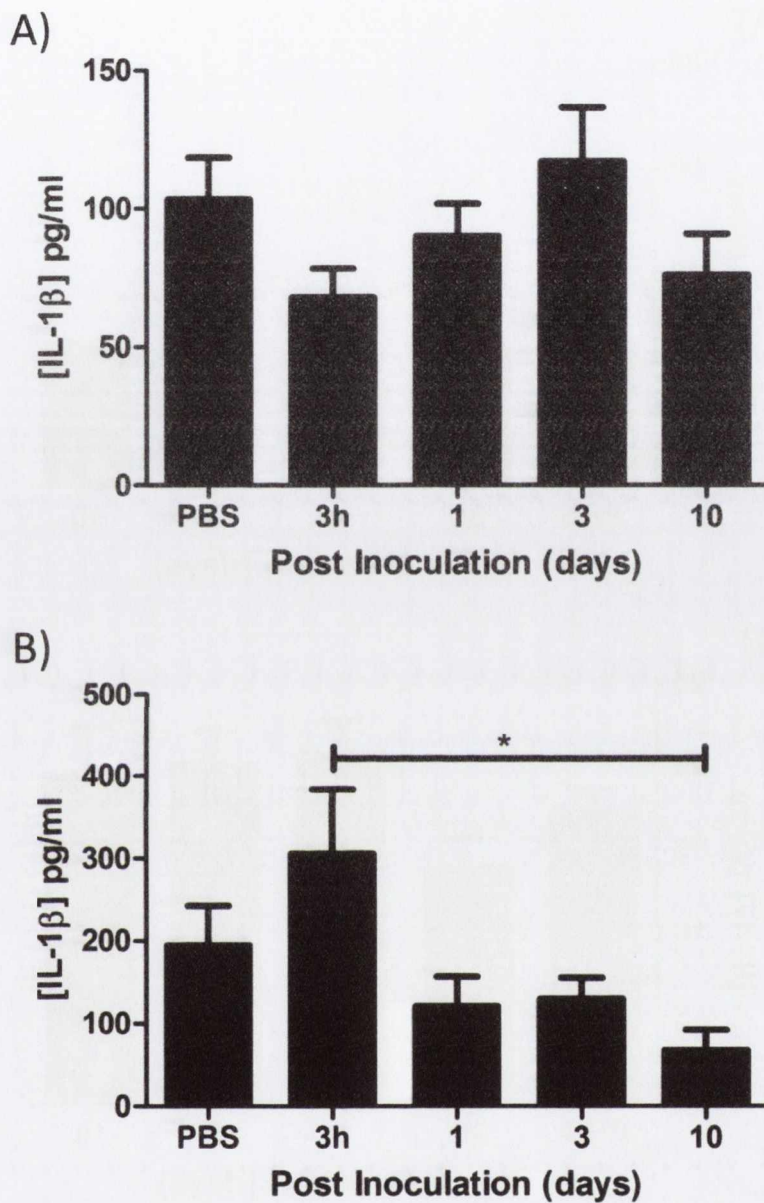


Figure 3.5. *S. aureus* nasal colonisation up-regulates IL-1 β locally in the nose and NT.

Groups of WT mice were inoculated intra-nasally with *S. aureus* (2×10^8 CFU) or were administered with PBS. At indicated time points post inoculation, Noses (**A**) and NT (**B**) were harvested, homogenised and concentrations of IL-1 β were measured by ELISA. Results expressed as mean $\pm$ SEM. $n = 9-12$ mice per group. Data pooled from 3 independent experiments. Statistical analysis was performed using a one-way ANOVA with Tukey's post-test. * $P \leq 0.05$

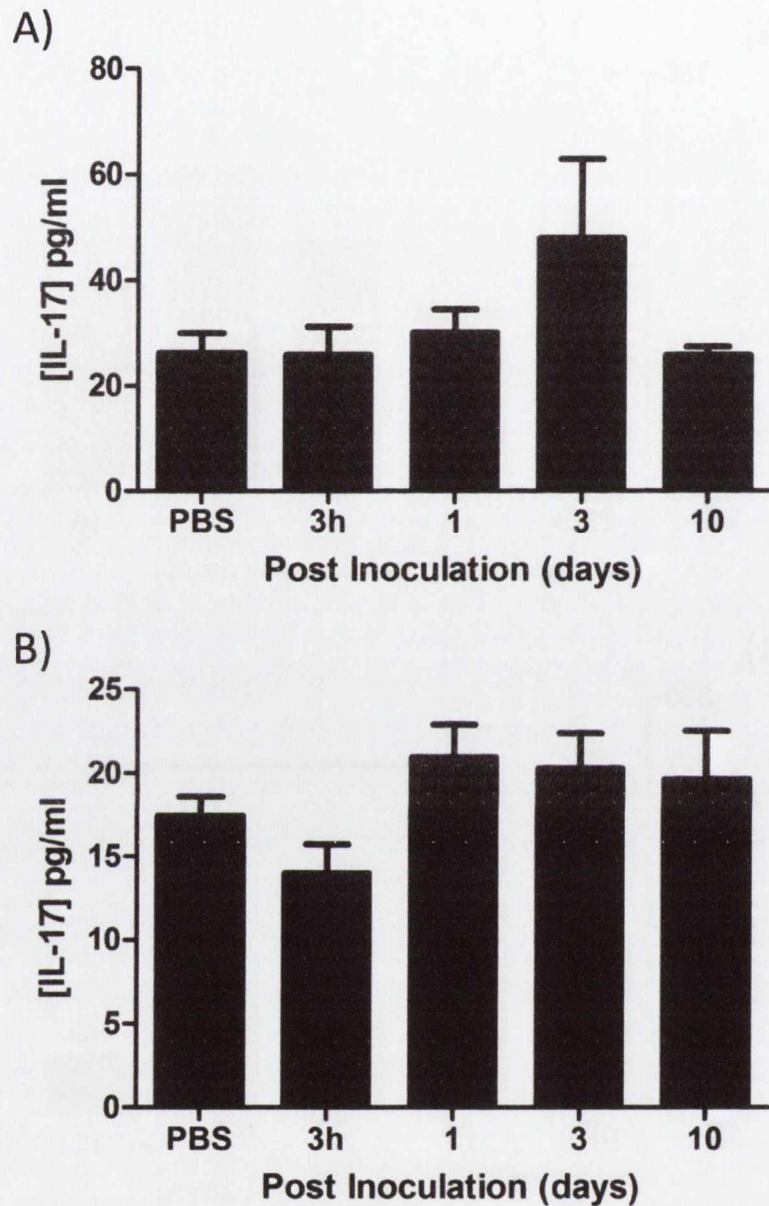


Figure 3.6. *S. aureus* nasal colonisation up-regulates IL-17 locally in the nose and NT.

Groups of WT mice were inoculated intra-nasally with *S. aureus* (2×10^8 CFU) or were administered with PBS. At indicated time points post inoculation, Noses (**A**) and NT (**B**) were harvested, homogenised and concentrations of IL-17 were measured by ELISA. Results expressed as mean $\pm$ SEM. $n = 9-12$ mice per group. Data pooled from 3 independent experiments. Statistical analysis was performed using a one-way ANOVA with Tukey's post-test. NS

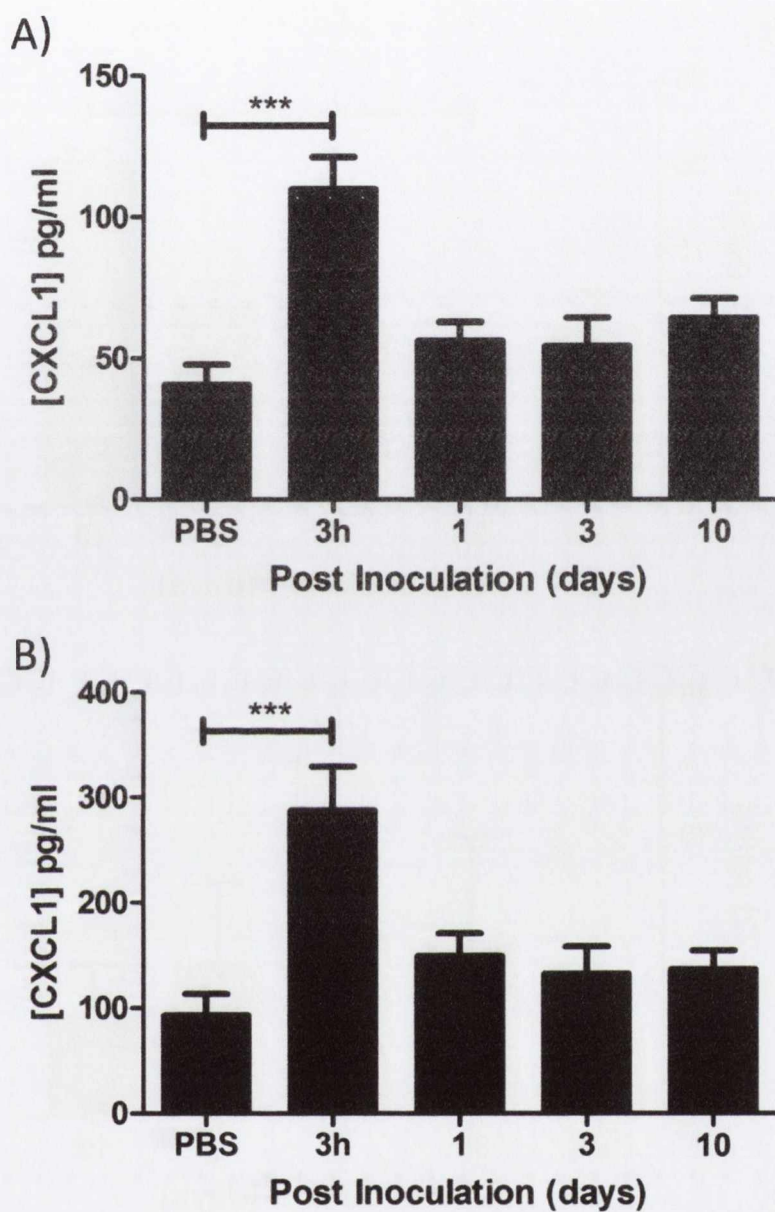


Figure 3.7. *S. aureus* nasal colonisation up-regulates CXCL1 locally in the nose and NT.

Groups of WT mice were inoculated intra-nasally with *S. aureus* (2×10^8 CFU) or were administered with PBS. At indicated time points post inoculation, Noses **(A)** and NT **(B)** were harvested, homogenised and concentrations of CXCL1 were measured by ELISA. Results expressed as mean $\pm$ SEM. $n = 9-12$ mice per group. Data pooled from 3 independent experiments. Statistical analysis was performed using a one-way ANOVA with Tukey's post-test. *** $P \leq 0.001$

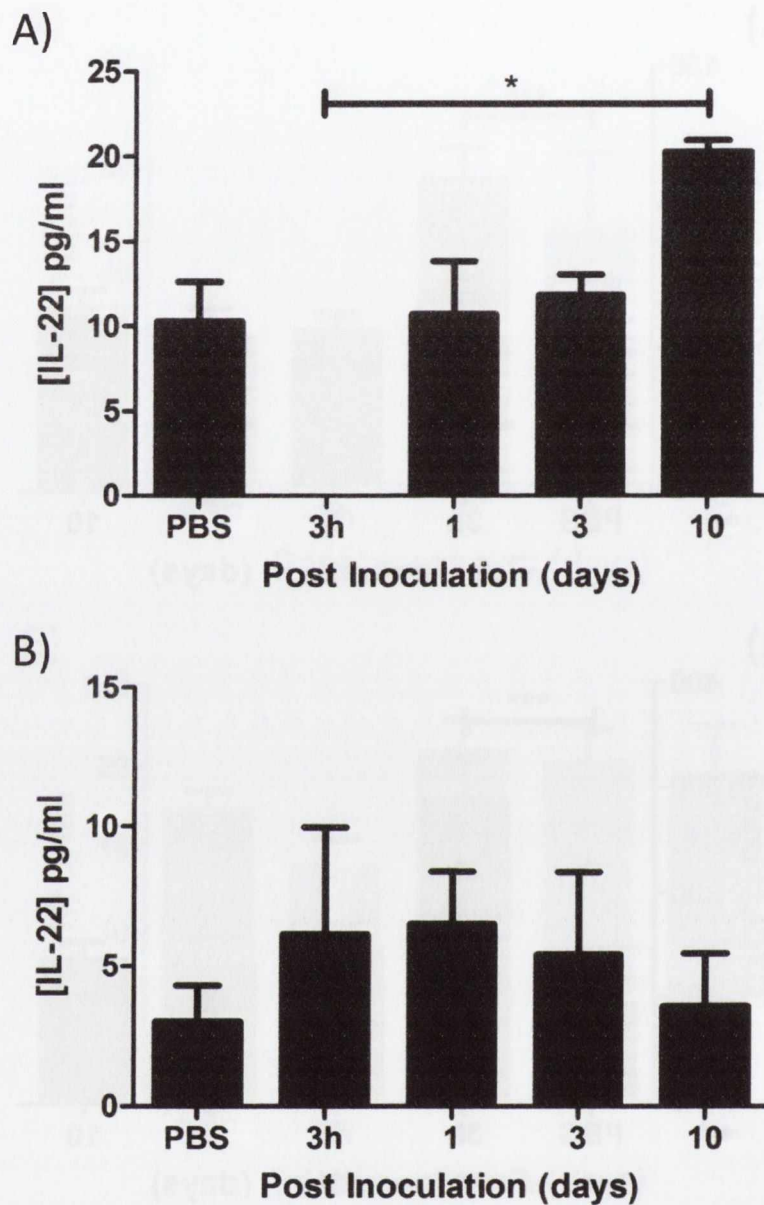


Figure 3.8. *S. aureus* nasal colonisation up-regulates IL-22 locally in the nose and NT.

Groups of WT mice were inoculated intra-nasally with *S. aureus* (2×10^8 CFU) or were administered with PBS. At indicated time points post inoculation, Noses (**A**) and NT (**B**) were harvested, homogenised and concentrations of IL-22 were measured by ELISA. Results expressed as mean $\pm$ SEM. $n = 9-12$ mice per group. Data pooled from 3 independent experiments. Statistical analysis was performed using a one-way ANOVA with Tukey's post-test. * $P \leq 0.05$

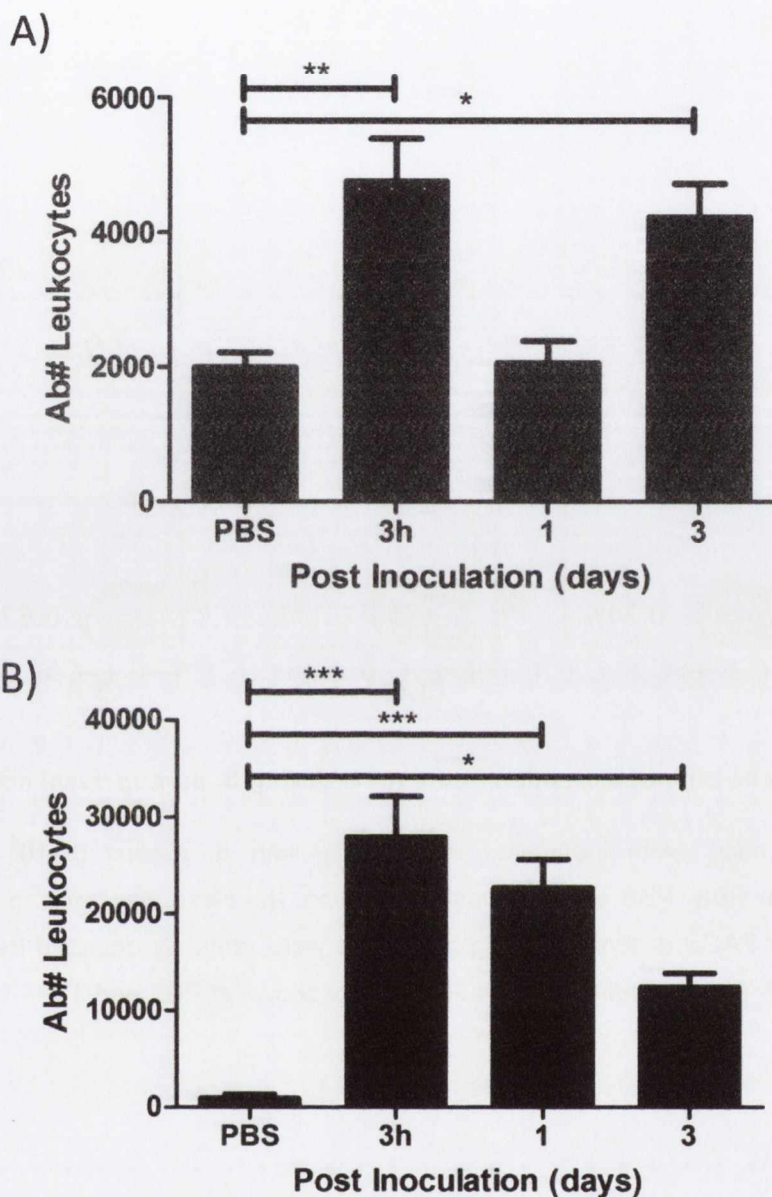


Figure 3.9. *S. aureus* nasal colonisation induces significant leukocyte influx into both the nose and NT

Groups of WT mice were inoculated intra-nasally with *S. aureus* (2×10^8 CFU) or were administered with PBS. At indicated time points post inoculation, Nose **(A)** and NT **(B)** was excised and leukocytes enumerated. Results expressed as mean $\pm$ SEM. $n = 9-12$ mice per group. Data pooled from 3 independent experiments. Statistical analysis was performed using a one-way ANOVA with Tukey's post-test. * $P \leq 0.05$ ** $P \leq 0.005$ *** $P \leq 0.001$

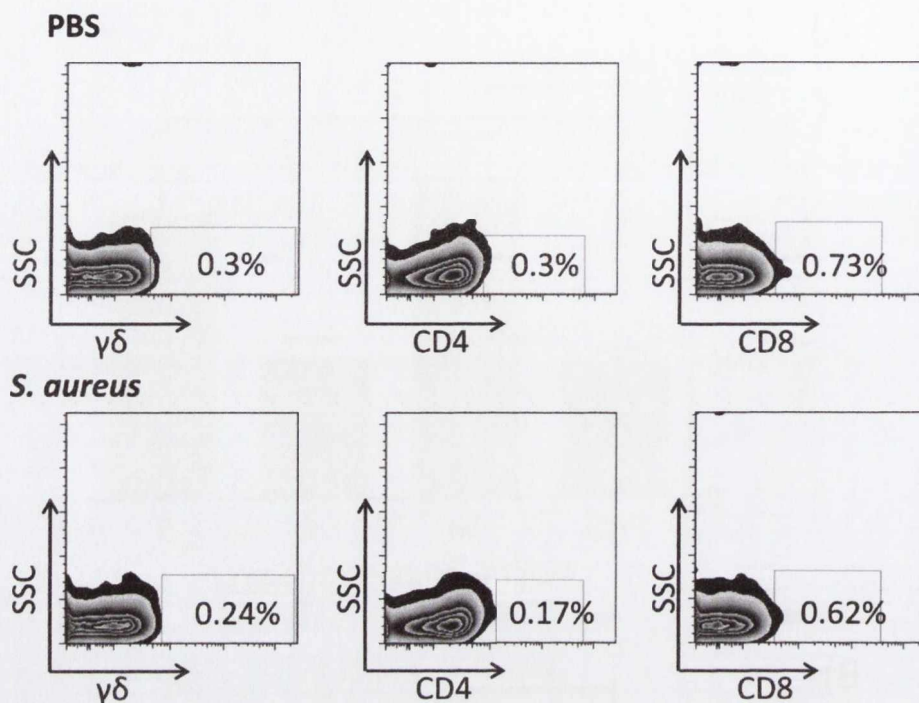


Figure 3.10. T-cells are not expanded in the nose during *S. aureus* nasal colonisation.

Groups of WT mice were inoculated intra-nasally with *S. aureus* (2×10^8 CFU) or were administered with PBS. PBS and 3 h post inoculation, the Nose was excised and leukocytes were isolated for FACS analysis. T-cell populations were primarily detected by populations of $\gamma\delta$, CD4 and CD8 T-cells. Representative FACS plots shown at PBS and 3 h

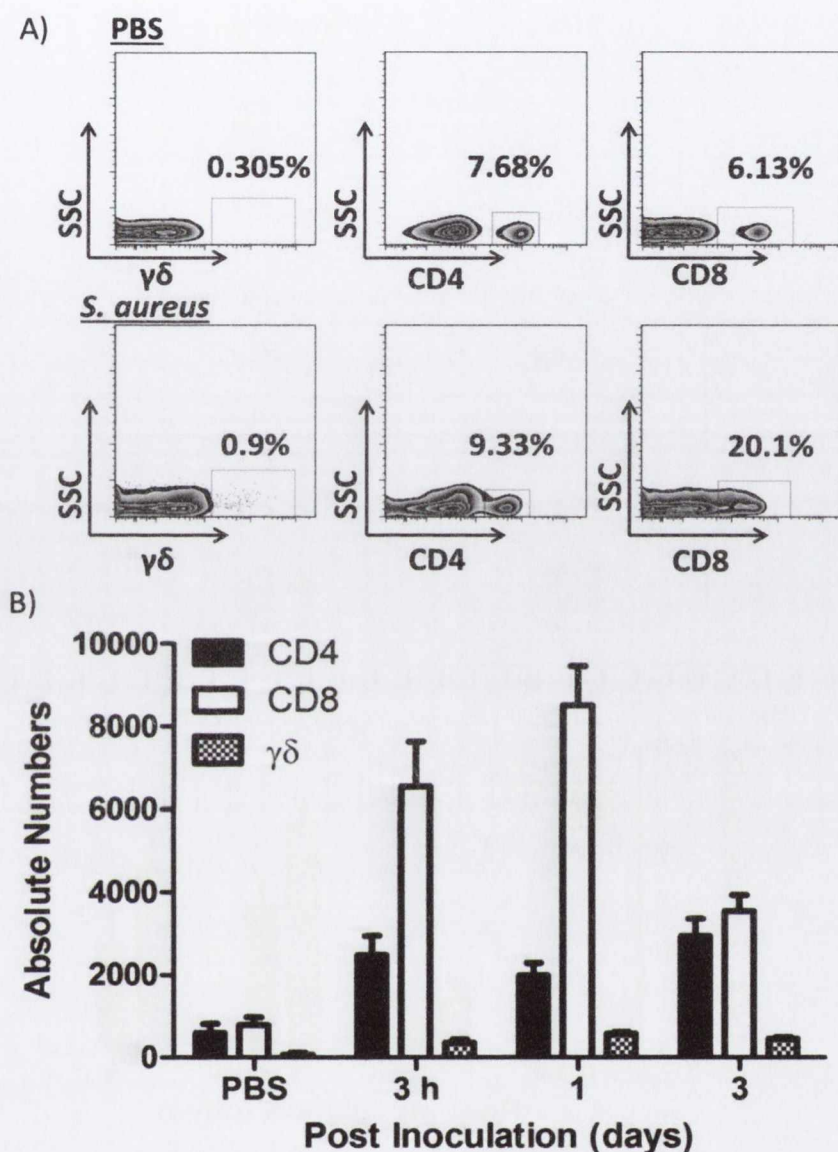


Figure 3.11. T-cells are expanded in the NT during *S. aureus* nasal colonisation.

Groups of WT mice were inoculated intra-nasally with *S. aureus* (2×10^8 CFU) or were administered with PBS. PBS and 3 h post-inoculation, the NT was excised and leukocytes were isolated for FACS analysis. T-cell populations were primarily detected by populations of $\gamma\delta$, CD4 and CD8 T-cells (A). Analysis of the absolute number of CD4, $\gamma\delta$ and CD8 T-cell subsets was then assessed (B). Results are expressed mean $\pm$ SEM with representative FACS plots shown for d1 ($n=4-5$, per group). Data are representative of two individual experiments.

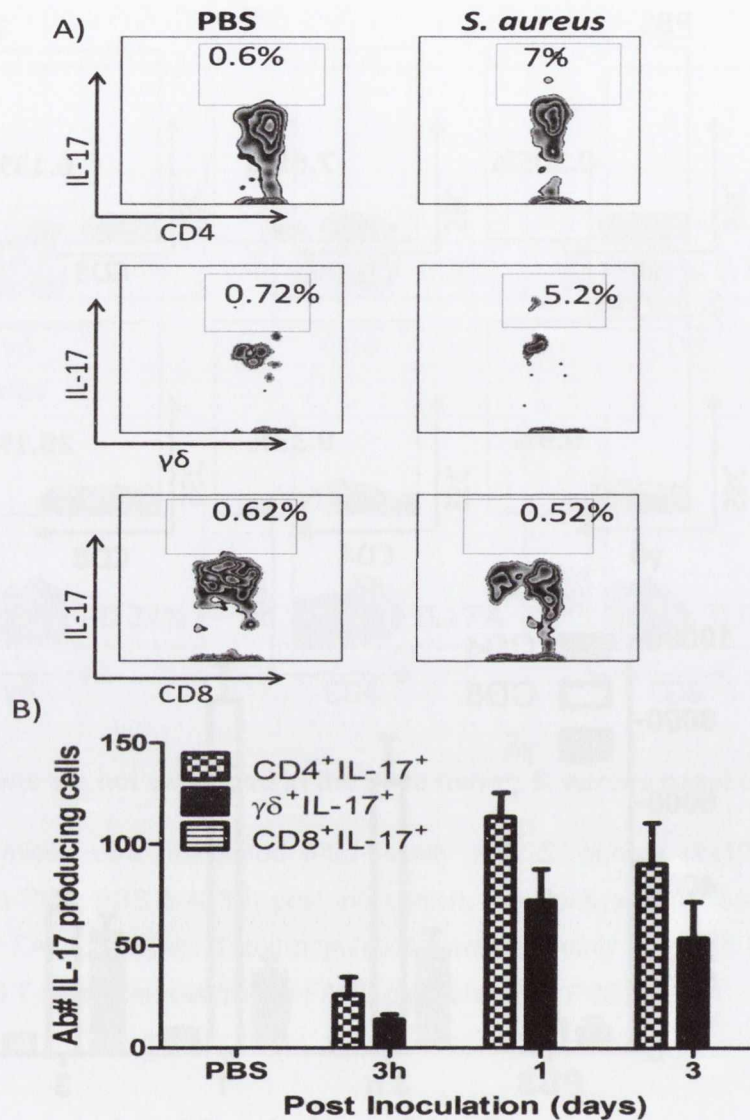


Figure 3.12. IL-17 is produced by $\gamma\delta^+$ and CD4⁺ T-cells in the NT during *S. aureus* nasal colonisation.

Groups of WT mice were inoculated intra-nasally with *S. aureus* (2×10^8 CFU) or were administered with PBS. At indicated time points post inoculation, cells isolated from the NT were cultured with PMA, Ionomycin and brefeldin A for 4 hrs and were surface stained with CD4, $\gamma\delta$ and CD8 before intracellular staining for IL-17 and analysis by flow cytometry (A). Analysis of the absolute number of IL-17 producing CD4, $\gamma\delta$ and CD8 T-cell subsets was then assessed (B). Results are expressed mean $\pm$ SEM with representative FACS plots shown for d1 ($n=4-5$, per group). Data are representative of two individual experiments.

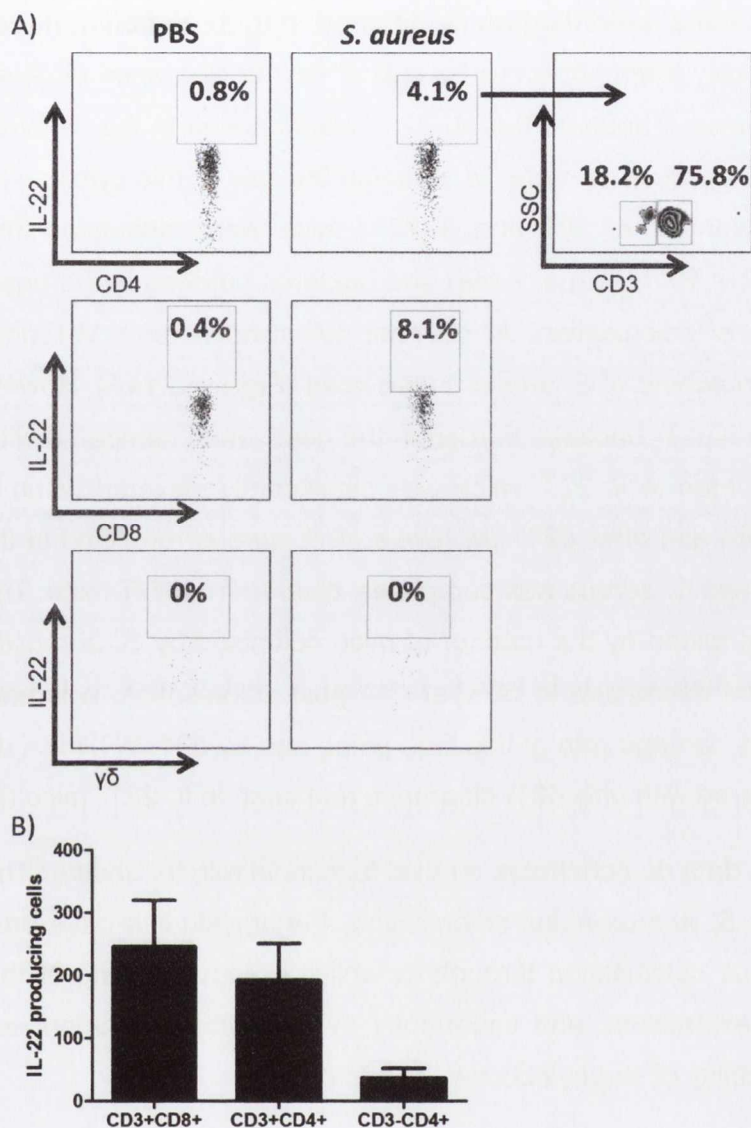


Figure 3.13. IL-22 was produced by CD4⁺, CD8⁺ and ILCs in the NT during *S. aureus* nasal colonisation.

Groups of WT mice were inoculated intra-nasally with *S. aureus* (2×10^8 CFU) or were administered with PBS. At day 7 post colonisation, cells isolated from the NT were cultured with brefeldin A and were surface stained with CD45, CD3, CD4, CD8 and $\gamma\delta$ before intracellular staining for IL-22 and analysis by flow cytometry (A). Analysis of IL-22 production by individual T-cell subsets was then assessed (B). Results are expressed as mean $\pm$ SEM with representative FACS plots shown ($n=4-5$, per group). Data are representative of two individual experiments.

3.2.4. *S. aureus* nasal colonisation is elevated in IL-22-deficient mice.

IL-17 has previously been shown to be critical for the clearance of *S. aureus* from the nose (123), however it appears that IL-22 is also induced in the nasal cavity during *S. aureus* nasal colonisation. In order to examine the role of this cytokine in controlling *S. aureus* nasal colonisation, WT and IL-22^{-/-} mice were inoculated intra-nasally with Newman Sm^R (2×10^8 CFU per nose) and bacterial burdens in the noses enumerated over the course of colonisation. At d3 post colonisation, both WT and IL-22^{-/-} mice retained similar numbers of *S. aureus* in the nose (**Figure 3.14A**). However, by d10, IL-22^{-/-} mice had a higher bacterial burden in the nose when compared with WT mice. By d14, bacterial burden in IL-22^{-/-} mice was significantly elevated when compared with WT mice ($P \leq 0.05$) and after d21, low levels of *S. aureus* remained in the noses of IL-22^{-/-} mice, whereas *S. aureus* was completely cleared from WT mice. The carriage rate in the nose (determined by the number of mice colonised by *S. aureus*/total number of mice) in WT mice decreased to 50% at d14 post colonisation, whereas IL-22^{-/-} mice maintained 100% carriage rate at this time point, and by d21, WT mice displayed 100% clearance compared with only 60% clearance rate seen in IL-22^{-/-} mice (**Figure 3.14B**).

In summary, this data demonstrates a novel functional role for another Th17 cytokine IL-22 in controlling *S. aureus* nasal colonisation. Further studies have proven that IL-22 controls *S. aureus* colonisation through its ability to induce killing of the organism by triggering AMP expression, and importantly by inhibiting attachment to the nares by controlling availability of staphylococcal ligands (288).

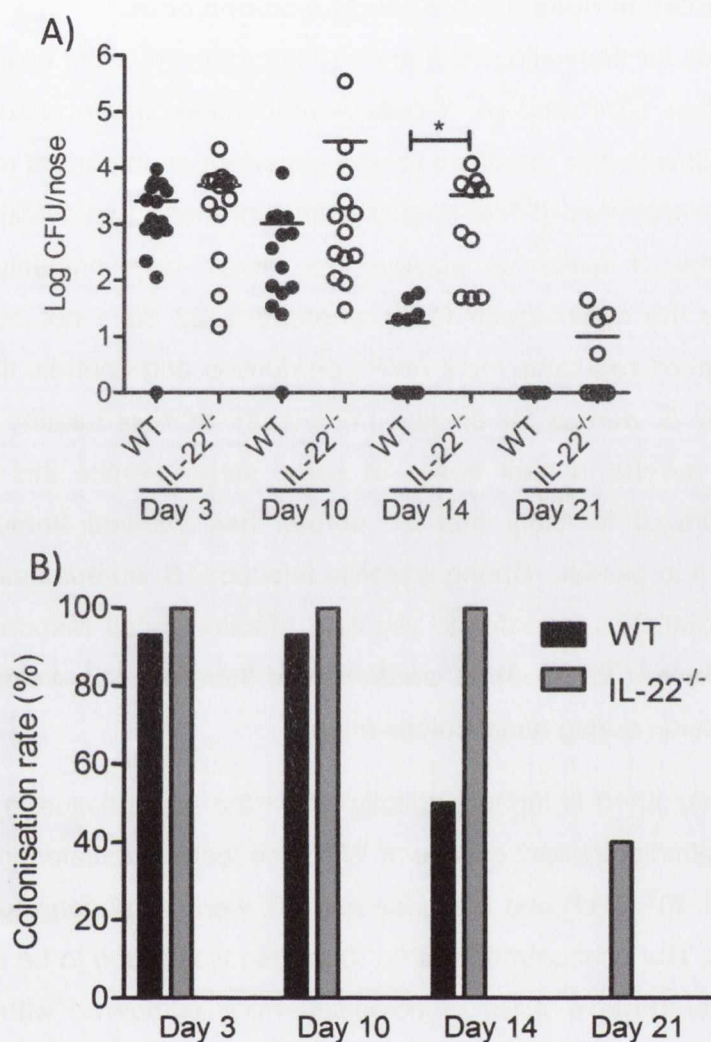


Figure 3.14. Bacterial burden is significantly elevated in the nasal cavity IL-22-deficient mice during *S. aureus* colonisation.

Groups of WT and IL-22^{-/-} mice were inoculated intra-nasally with *S. aureus* (2×10^8 CFU). At indicated time points post inoculation, the bacterial burden in the noses (A) was established. Results expressed as mean $\pm$ SEM CFU per nose ($n=10-15$, per group). Colonisation rate (B) as determined by the number of mice colonised by *S. aureus*/total number of mice was determined in WT and IL-22^{-/-} mice ($n=10-15$, per group). Statistical analysis was performed using a Kruskal-Wallis test. * $P \leq 0.05$. **Experiments performed in conjunction with Dr. Michelle Mulcahy, Host: Pathogen Interactions Group, School of Biochemistry and Immunology, TCD.**

3.2.5. IL-10 is induced in nose upon *S. aureus* colonisation.

Data presented thus far has validated that IL-17 is produced in the nose in response to *S. aureus*. In addition, CD4⁺ and $\gamma\delta$ ⁺ T-cells were identified as novel sources of this IL-17 production. IL-22 was also identified to play an important role in controlling *S. aureus* nasal colonisation. It has been shown that these two cytokines function by distinct mechanisms of action to achieve this. IL-17 acts primarily by promoting neutrophil influx to the nasal cavity (123) whereas IL-22 does not control neutrophil recruitment but instead regulates local AMPs production and controls the availability of ligands targeted by *S. aureus* for binding (123, 288). 80% of healthy people are still colonised with *S. aureus* in their noses at some stage despite this robust immune response. Therefore, it is likely that *S. aureus* has evolved immune-suppressive strategies to allow it to persist. During a biofilm infection, *S. aureus* has been shown to induce IL-10 from MDSCs in order to regulate effector T-cell responses in order to facilitate its persistence (257, 258). *S. aureus* could therefore use a similar mechanism of immune suppression during nasal colonisation.

To establish whether IL-10 is induced locally within the nasal tissue in response to *S. aureus* during nasal colonisation, groups of WT mice were inoculated intra-nasally with Newman Sm^R (2×10^8 CFU) and the nose and NT were homogenised and examined for IL-10 by ELISA. The concentration of IL-10 protein was found to be increased in the nose at d1 and d3 (**Figure 3.15**) of colonised mice compared with PBS controls. However, IL-10 was not produced in the NT of WT mice during nasal colonisation (≤ 1 pg/ml).

In order to determine the source of this IL-10, WT or IL-10 GFP reporter mice were infected with *S. aureus* (2×10^8 CFU) or sterile PBS as a control. As IL-10 protein concentrations were substantially induced in the nose at d3 post inoculation (**Figure 3.15**), this time point was chosen as the optimal time in order to determine the IL-10 source. However following analysis, no IL-10 GFP positive signal was detectable in the nose by flow cytometry (**Figure 3.16**). This is likely due to the very small numbers of leukocytes that are recoverable from the nose (**Figure 3.9**).

This data demonstrates for the first time, that *S. aureus* induces IL-10 production locally within the nose during nasal colonisation. Unfortunately however, it was not possible to identify the source of IL-10 within the nose.

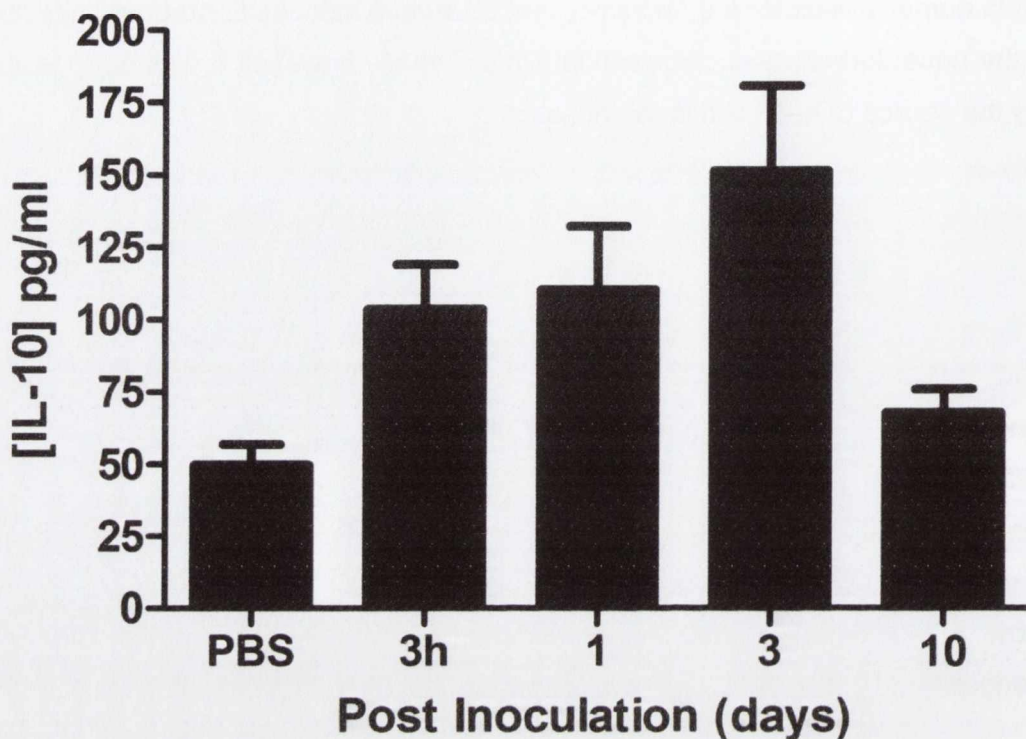


Figure 3.15. *S. aureus* nasal colonisation up-regulates IL-10 locally in the nose

Groups of WT mice were inoculated intra-nasally with *S. aureus* (2×10^8 CFU) or were administered with PBS. At indicated time points post inoculation, Noses were harvested, homogenised and concentrations of IL-10 were measured by ELISA. Results are expressed as mean protein concentration mean $\pm$ SEM. $n=9-12$, per group. Results are pooled from two individual experiments. Statistical analysis was performed using a one-way ANOVA with Tukey's post-test. NS

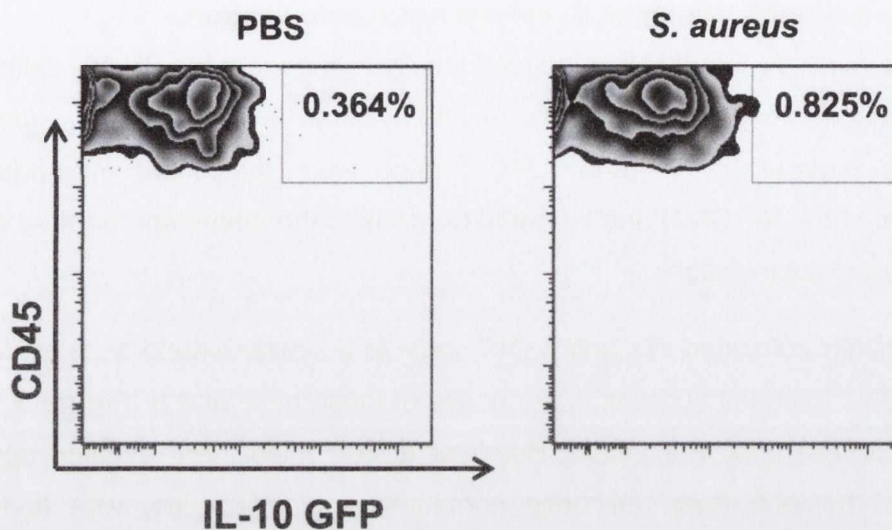


Figure 3.16. IL-10 was not detected in CD45⁺ leukocytes in the nose during *S. aureus* nasal colonisation.

Groups of IL-10 GFP-reporter mice were inoculated intra-nasally with *S. aureus* (2×10^8 CFU) or were administered with PBS. At d3 post colonisation, cells were isolated from the nose. Cells were surface stained with CD45 and analysed by flow cytometry. Data are representative of two individual experiments.

3.2.6. IL-10 facilitates sustained *S. aureus* nasal colonisation

Having demonstrated that IL-10 is induced locally within the nose, IL-10- deficient mice were then utilised to examine the role of this cytokine in controlling *S. aureus* nasal colonisation. Groups of WT and IL-10^{-/-} mice were inoculated intra-nasally with Newman Sm^R (2×10^8 CFU) and bacterial burdens in the noses enumerated at 3h, d1, d3 and d10 post colonisation.

S. aureus initially colonised WT and IL-10^{-/-} mice to a similar extent as is evidenced by the comparable bacterial burdens in the noses of these mice at 3 h (**Figure 3.17A**) and d1 (**Figure 3.17B**) post inoculation. However IL-10^{-/-} mice then revealed accelerated clearance of bacteria from the nose compared with WT mice, with IL-10^{-/-} mice demonstrating a statistically significant reduction in bacterial burden in the nose at d3 (**Figure 3.17C**) and d10 (**Figure 3.17D**) post colonisation compared with WT mice. In fact, by d10 post inoculation, IL-10^{-/-} mice had almost completely cleared *S. aureus* from their noses, whereas *S. aureus* was still present in the nasal cavities of WT mice. Importantly there was no increased bacterial dissemination in the lungs or blood of IL-10^{-/-} mice (Log CFU ≤ 1) compared with WT mice (Log CFU ≤ 1) at d1 post *S. aureus* nasal colonisation.

To investigate the direct effects of IL-10 in the nose, recombinant murine IL-10 (200 ng per nose) was administered to groups of WT and IL-10^{-/-} mice in conjunction with *S. aureus* intranasal inoculation. At d3 post colonisation, WT mice that received rIL-10 exhibited an increase in bacterial burden compared with WT mice treated with PBS (**Figure 3.18**). Similarly, administration of rIL-10 to IL-10^{-/-} mice increased the level of *S. aureus* bacterial burden (**Figure 3.18**). Taken together, this data identify IL-10 as one of the major factors facilitating *S. aureus* colonisation of the nose.

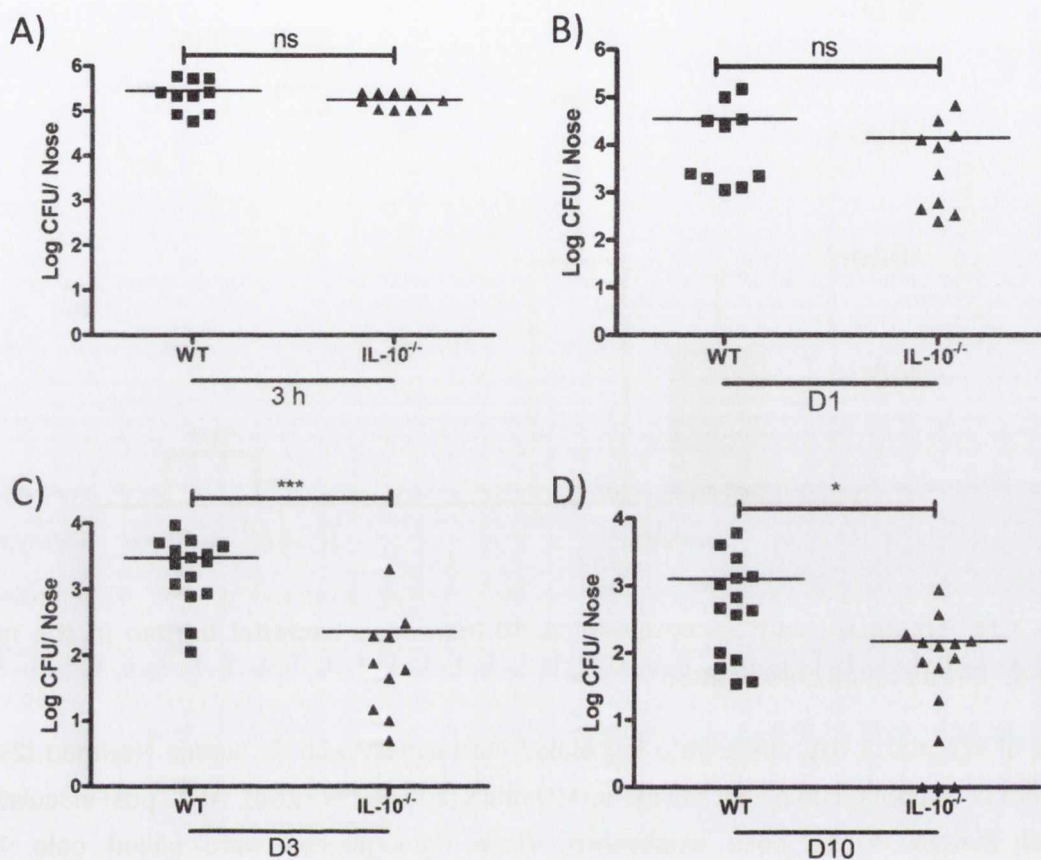


Figure 3.17. *S. aureus* nasal colonisation is reduced in IL-10^{-/-} mice.

Groups of WT and IL-10^{-/-} mice were inoculated intra-nasally with *S. aureus* Newman (2×10^8 CFU). At 3h (A), d1 (B), d3 (C) and d10 (D) post inoculation, bacterial burden in the nose established. Nose homogenates were plated onto TSA supplemented with streptomycin to obtain *S. aureus* CFU/nose. Results are expressed as Log CFU/ nose ($n=10-15$, per group), median indicated by bar. Results are pooled data from three independent experiments. Statistical analysis was performed using an unpaired t test. * $P \leq 0.05$ *** $P \leq 0.001$

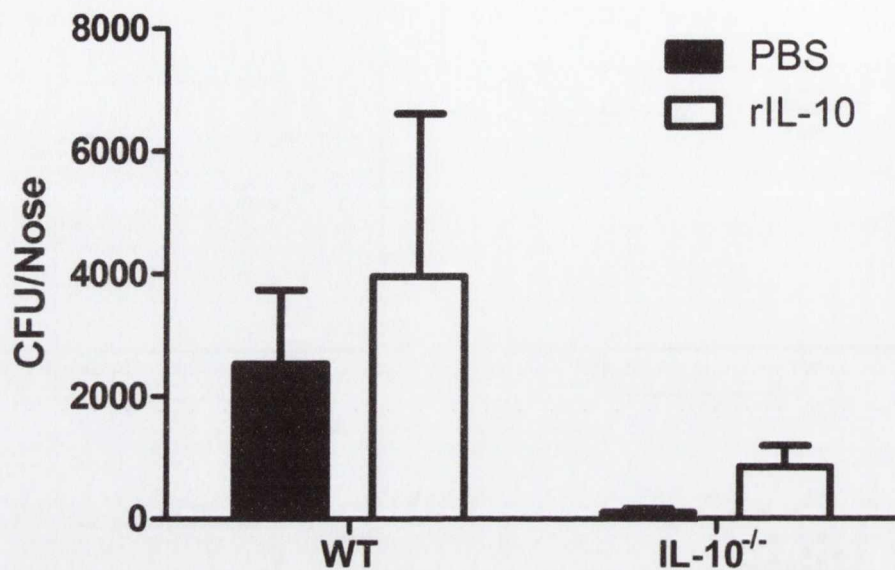


Figure 3.18. Treatment with recombinant IL-10 increases bacterial burden in the nose during *S. aureus* nasal colonisation.

Groups of WT and IL-10^{-/-} mice were inoculated intra-nasally with *S. aureus* Newman (2×10^8 CFU) with or without recombinant murine IL-10 added (200 ng per nose). At d3 post inoculation, bacterial burden in the nose established. Nose homogenates were plated onto TSA supplemented with streptomycin to obtain *S. aureus* CFU/nose. Results expressed as mean CFU per nose ($n=4$, per group). Statistical analysis was performed using an unpaired t test. NS

3.2.7. IL-10 acts upstream to regulate the IL-17 but not IL-22 axis during *S. aureus* nasal colonisation.

As observed, the induction of IL-10 locally within the nose appears to be critical in promoting *S. aureus* nasal colonisation. As shown, T-cell cytokines IL-17 and IL-22 are important mediators in the clearance of *S. aureus* during nasal colonisation (123, 288). In order to decipher if the role of IL-10 is upstream of IL-17 and IL-22 during nasal colonisation, groups of WT and IL-10^{-/-} mice were inoculated intra-nasally with Newman Sm^R (2×10^8 CFU) and both nose and NT were excised, homogenised and the supernatant examined for IL-1 β , IL-17, CXCL1 and IL-22 by ELISA.

In the nose, IL-1 β protein concentrations were higher in IL-10^{-/-} mice compared with WT mice at both 3 h and d1 post colonisation (**Figure 3.19A**) with a trend towards increased IL-1 β concentrations in the NT after d1 in the absence of IL-10 (**Figure 3.19B**) compared with WT mice. Importantly, IL-17 protein concentrations were significantly increased in the nose at 3 h of IL-10^{-/-} mice compared with WT (**Figure 3.20A**). In the NT, IL-17 concentrations were also significantly elevated at d1 and d3 post inoculation in IL-10^{-/-} mice compared with WT (**Figure 3.20B**). CXCL1 was significantly increased after 3 h in the nose (**Figure 3.21A**) and d1 in the NT (**Figure 3.21B**) in the IL-10^{-/-} mice compared with WT.

IL-22 protein concentrations were not affected in the nose at IL-10^{-/-} mice compared with WT mice during *S. aureus* nasal colonisation in either the nose (**Figure 3.22A**) or the NT (**Figure 3.22B**). Taken together it appears that during *S. aureus* nasal colonisation the induction of IL-10 acts to regulate the production of IL-17 but not IL-22 production.

As previously shown, IL-17 is produced by both CD4⁺ and $\gamma\delta^+$ T-cells in the nose. In order to investigate if IL-10 controls the IL-17 production by these T-cells, groups of WT and IL-10^{-/-} mice were inoculated intra-nasally with Newman (2×10^8 CFU). At 3 h, d1 and d3 post inoculation, the NT was isolated and leukocytes were stained with a panel of well-defined surface markers to identify individual T-cell subsets by flow cytometry combined with intracellular cytokine staining for IL-17.

The absolute numbers of $\gamma\delta^+$ T-cells were significantly elevated in the NT of IL-10^{-/-} mice compared with WT mice at d1 post colonisation (**Figure 3.23A**). CD4⁺ T-cells were also significantly expanded at d1 post inoculation when compared with WT mice (**Figure 3.23B**). IL-10^{-/-} mice also had a substantially greater numbers of CD8⁺ T-cells in the NT during nasal colonisation compared with WT mice (**Figure 3.23C**).

Examining cytokine production by these effector T-cell populations, $\gamma\delta^+$ cells were shown to be a novel secondary source of IL-17, however, the absence of IL-10 resulted in an increase in the absolute number of IL-17⁺ $\gamma\delta^+$ cell in the NT compared with WT mice (**Figure 3.24A**). Notably, at d3 post colonisation IL-17 production by CD4⁺ T-cells was significantly increased in the IL-10^{-/-} mice colonised with *S. aureus* compared with WT mice (**Figure 3.24B**). In summary, IL-10 appears to control the production of IL-17 by both CD4⁺ and $\gamma\delta$ T-cells during *S. aureus* nasal colonisation.

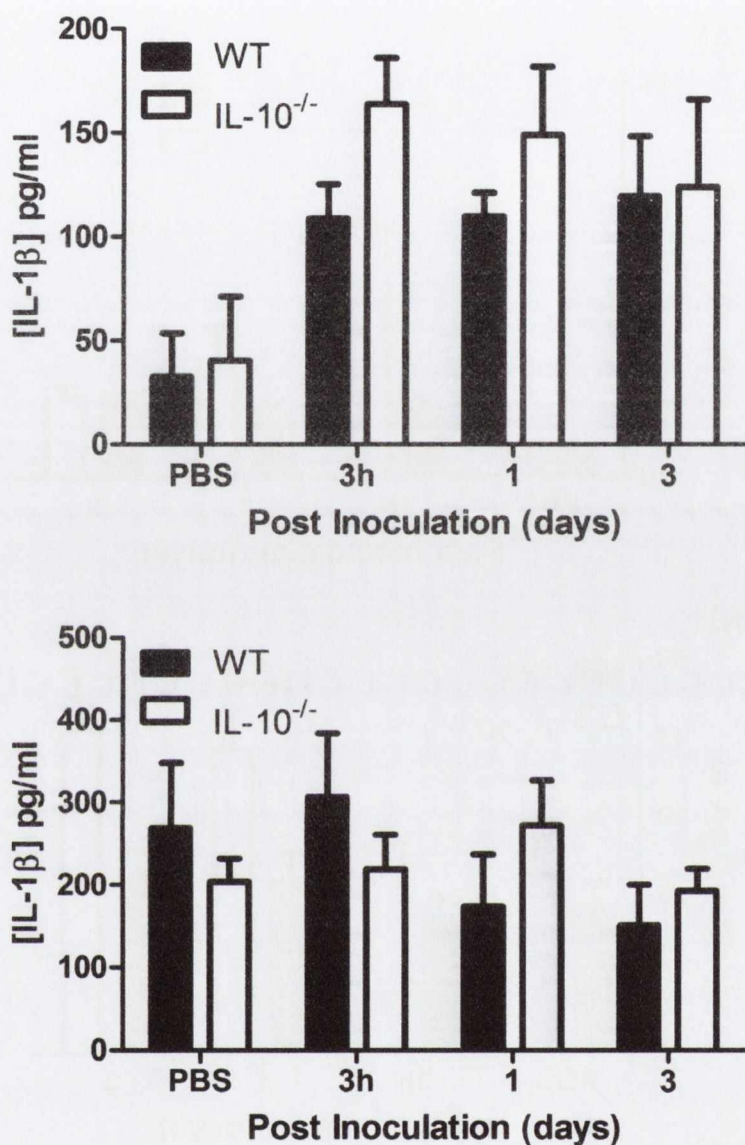


Figure 3.19. IL-10 controls pro-inflammatory IL-1 β production in the nose during *S. aureus* nasal colonisation.

Groups of WT and IL-10^{-/-} were inoculated intra-nasally with *S. aureus* (2x10⁸ CFU) or were administered with PBS. At indicated time points post inoculation, Noses (**A**) and NT (**B**) were harvested, homogenised and concentrations of IL-1 β were measured by ELISA. Results expressed as mean $\pm$ SEM of n=4-5 mice/group. Data are representative of two independent experiments. Statistical analysis was performed using two-way ANOVA with Bonferroni post-test. NS

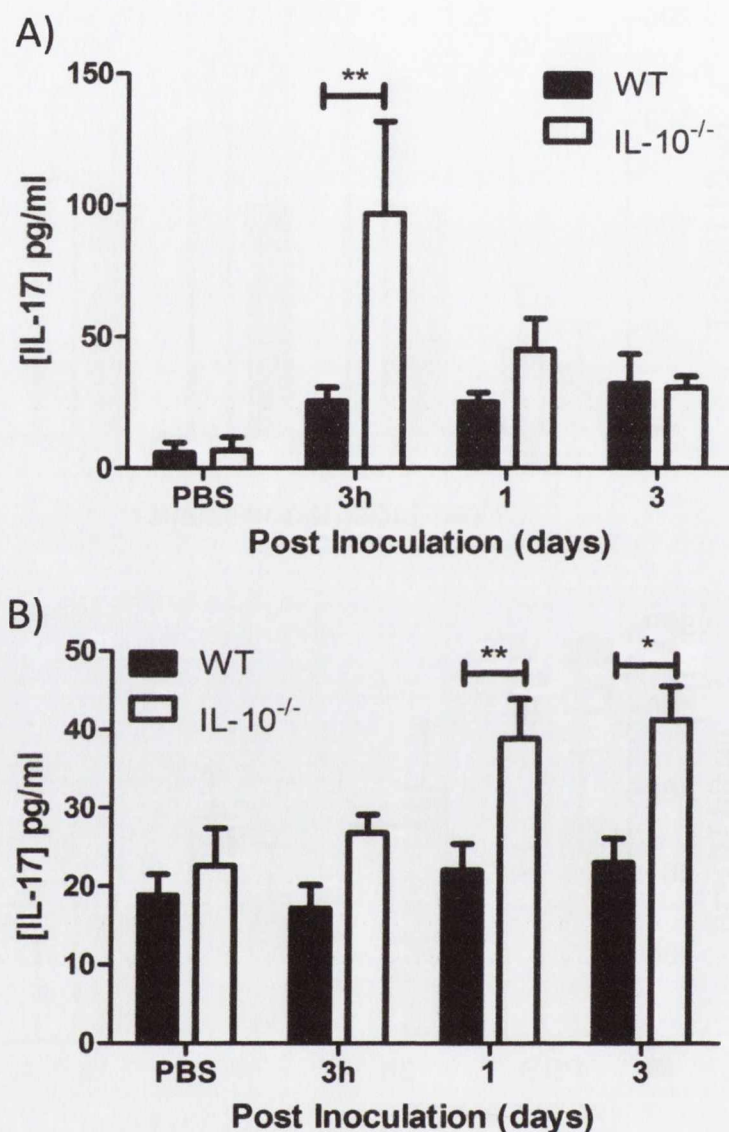


Figure 3.20. IL-10 controls pro-inflammatory IL-17 production in the nose and NT during *S. aureus* nasal colonisation.

Groups of WT and IL-10^{-/-} were inoculated intra-nasally with *S. aureus* (2x10⁸ CFU) or were administered with PBS. At indicated time points post inoculation, Noses **(A)** and NT **(B)** were harvested, homogenised and concentrations of IL-17 were measured by ELISA. Results expressed as mean ± SEM of n=4-5 mice/group. Data are representative of two independent experiments. Statistical analysis was performed using two-way ANOVA with Bonferroni post-test. **P*≤0.05, ***P*≤0.005

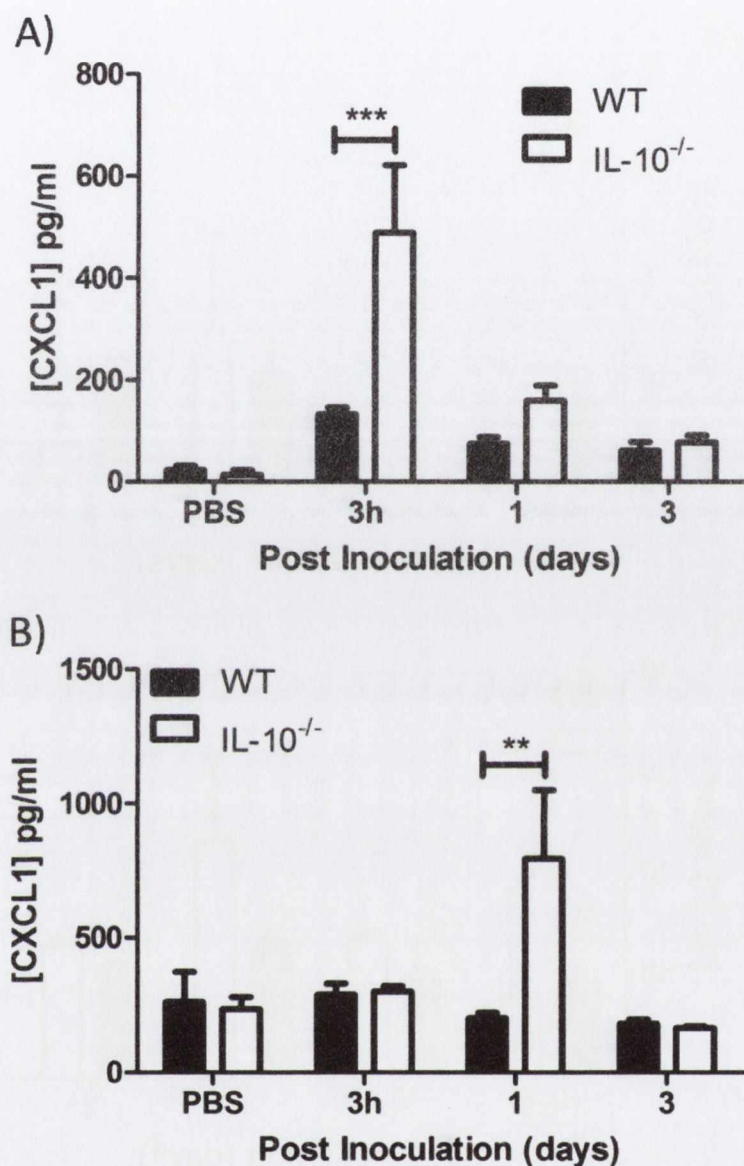


Figure 3.21. IL-10 controls neutrophil chemo-attractant CXCL1 production in the nose and NT during *S. aureus* nasal colonisation.

Groups of WT and IL-10^{-/-} were inoculated intra-nasally with *S. aureus* (2x10⁸ CFU) or were administered with PBS. At indicated time points post inoculation, Noses **(A)** and NT **(B)** were harvested, homogenised and concentrations of CXCL1 were measured by ELISA. Results expressed as mean ± SEM of n=4-5 mice/group. Data are representative of two independent experiments. Statistical analysis was performed using two-way ANOVA with Bonferroni post-test. ***P*≤0.005 ****P*≤0.001.

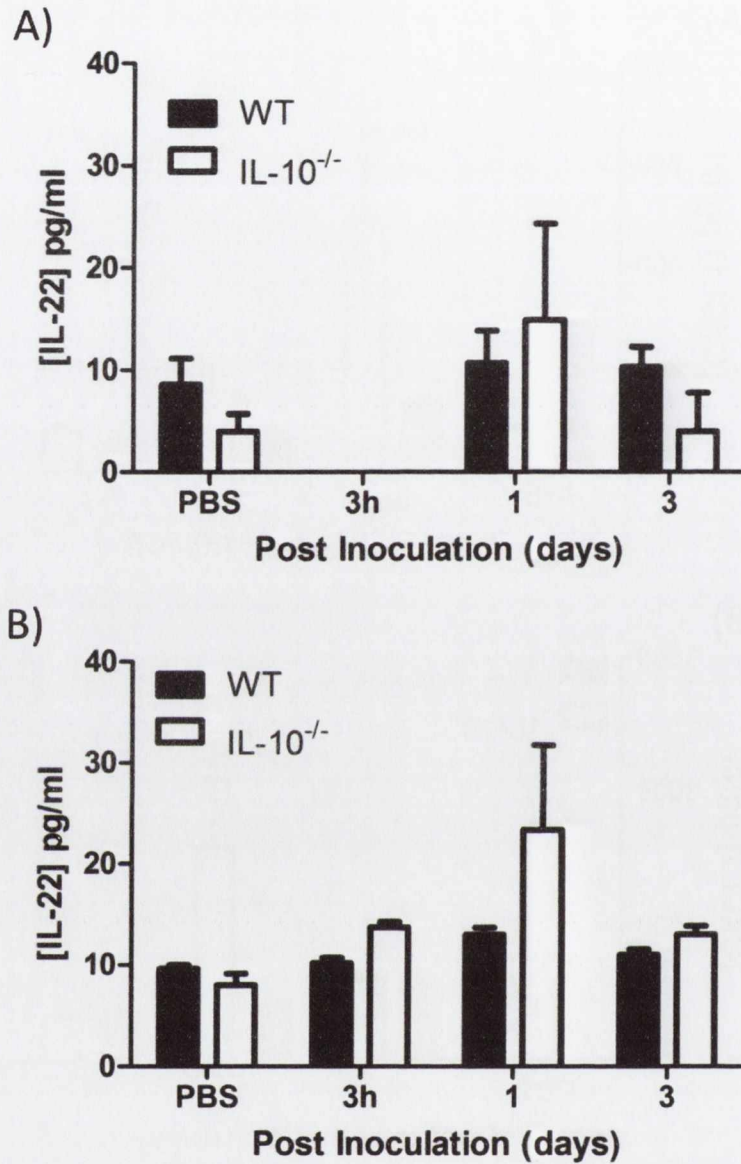


Figure 3.22. IL-10 does not control IL-22 production in the nose during *S. aureus* nasal colonisation.

Groups of WT and IL-10^{-/-} were inoculated intra-nasally with *S. aureus* (2x10⁸ CFU) or were administered with PBS. At indicated time points post inoculation, Noses (**A**) and NT (**B**) was harvested, homogenised and concentrations of IL-22 were measured by ELISA. Results expressed as mean ± SEM of n=4-5 mice/group. Data are representative of two independent experiments. Statistical analysis was performed using two-way ANOVA with Bonferroni post-test. NS

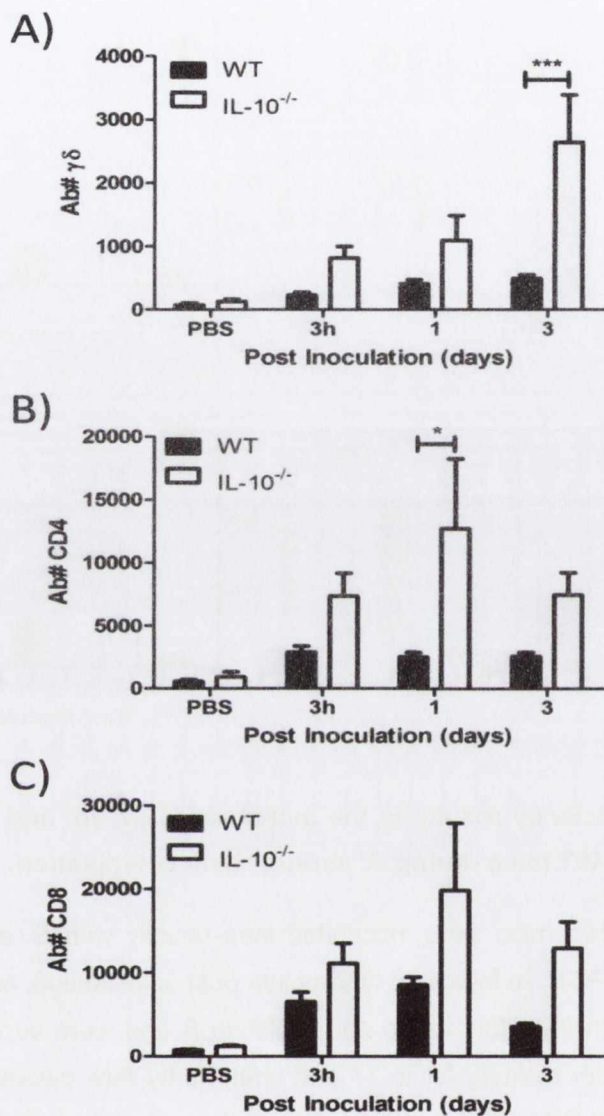


Figure 3.23. IL-10- deficiency results in an increase in the migration of $\gamma\delta$, CD4 and CD8 T-cell into the NT compared with WT mice during *S. aureus* nasal colonisation

Groups of WT and IL-10^{-/-} were inoculated intra-nasally with *S. aureus* (2x10⁸ CFU) or were administered with PBS. At indicated time points post inoculation, cells were isolated from NT, surface stained with fluorochrome-conjugated antibodies against $\gamma\delta$, CD4 and CD8 and analysed by flow cytometry. Total numbers of $\gamma\delta$ (A), CD4 (B) and CD8 (C) were enumerated. Results expressed as mean $\pm$ SEM, n= 4-5 mice/group. Data are representative of two independent experiments. Statistical analysis was performed using two-way ANOVA. * $P \leq 0.05$ *** $P \leq 0.001$.

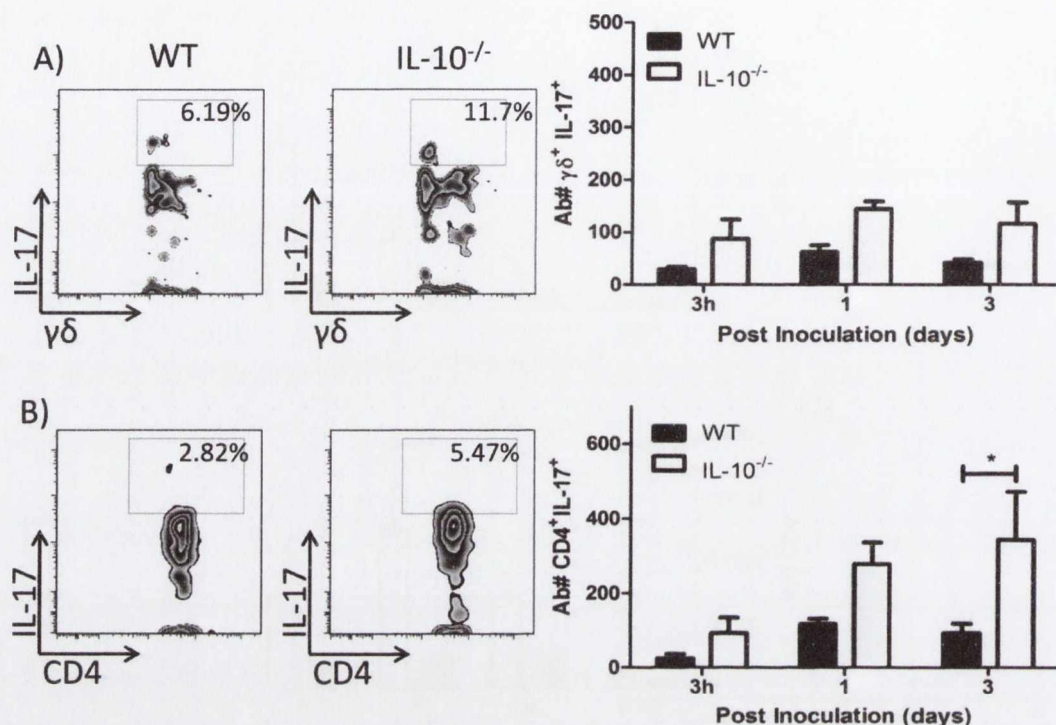


Figure 3.24. IL-10- deficiency results in the increased IL-17⁺γδ⁺ and IL-17⁺CD4⁺-T-cells in the NT compared with WT mice during *S. aureus* nasal colonisation.

Groups of WT and IL-10^{-/-} mice were inoculated intra-nasally with *S. aureus* (2x10⁸ CFU) or were administered with PBS. At indicated time-points post colonisation, cells were isolated from the NT and cultured with PMA/Ionomycin and brefeldin A and were surface stained with CD4 and γδ before intracellular staining for IL-17 and analysis by flow cytometry. Analysis of IL-17 production by γδ (**A**) and CD4 (**B**) T-cell subsets was then assessed. Results are expressed as mean ± SEM with representative FACS plots shown for d1 (*n*=4–5, per group). Data are representative of two individual experiments. Statistical analysis was performed using two-way ANOVA with Bonferroni post-test. **P*≤0.05

3.2.8. IL-10 controls the migration of neutrophils and macrophages to the nose and NT during *S. aureus* nasal colonisation.

IL-10 appears to act upstream of T-cells to regulate the production of IL-17, which has been shown to regulate neutrophil trafficking to the nasal cavity during *S. aureus* nasal colonisation (123), therefore the impact of IL-10 on phagocytic cell infiltration to both the nose and the NT was assessed at specific time points post colonisation in WT and IL-10^{-/-} mice. Groups of WT and IL-10^{-/-} mice were inoculated intra-nasally with Newman Sm^R (2×10^8 CFU) and at specific time points post colonisation cells were isolated from both sites and stained for phagocyte markers and analysed by flow cytometry (**Figure 3.25**).

There was increased neutrophil (CD11b⁺F480⁻Ly6G⁺) recruitment to the nose following *S. aureus* colonisation compared with PBS controls. Neutrophil recruitment was significantly greater in the nose at d1 post inoculation in IL-10^{-/-} mice compared with WT mice (**Figure 3.26A**). Neutrophils were significantly increased at d1 and d3 post inoculation in the NT of IL-10^{-/-} mice compared with WT mice (**Figure 3.26B**). Macrophage (CD11b⁺F480⁺Ly6G⁻) recruitment locally to the nose and NT was also increased following *S. aureus* colonisation compared with PBS controls. Macrophage recruitment was significantly increased in the nose at 3 h post inoculation in IL-10^{-/-} mice compared with WT mice (**Figure 3.27A**). Macrophages were significantly increased at d1 post inoculation in the NT of IL-10^{-/-} mice compared with WT mice (**Figure 3.27B**).

In conclusion, IL-10 controls the migration of both neutrophils and macrophages to both the nose and NT during *S. aureus* nasal colonisation.

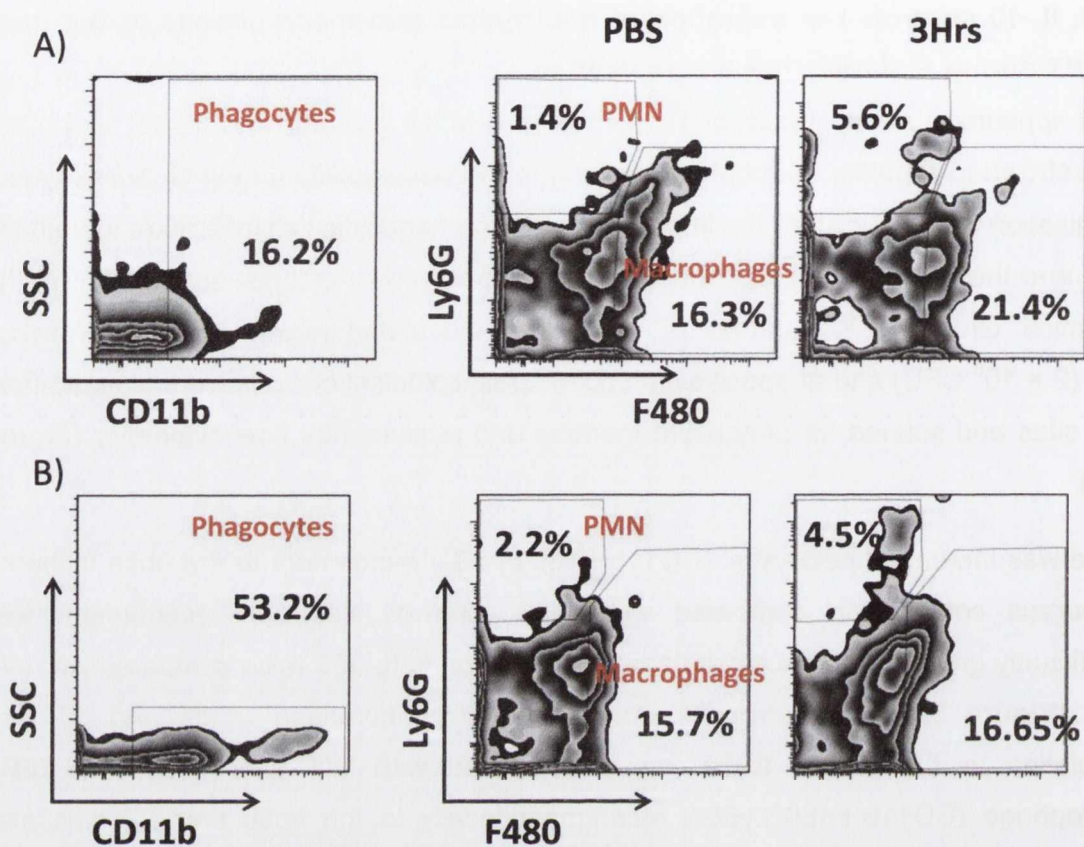


Figure 3.25. NT phagocyte staining panel during *S. aureus* nasal colonisation.

Groups of WT and IL-10^{-/-} mice were inoculated intra-nasally with *S. aureus* (2x10⁸ CFU) or were administered with PBS. Nose (A) and NT (B) cells were isolated, surface stained with fluorochrome-conjugated antibodies against CD11b, Ly6G and F480 and analysed by flow cytometry. Representative FACS plots are shown for PBS and 3 h post colonisation.

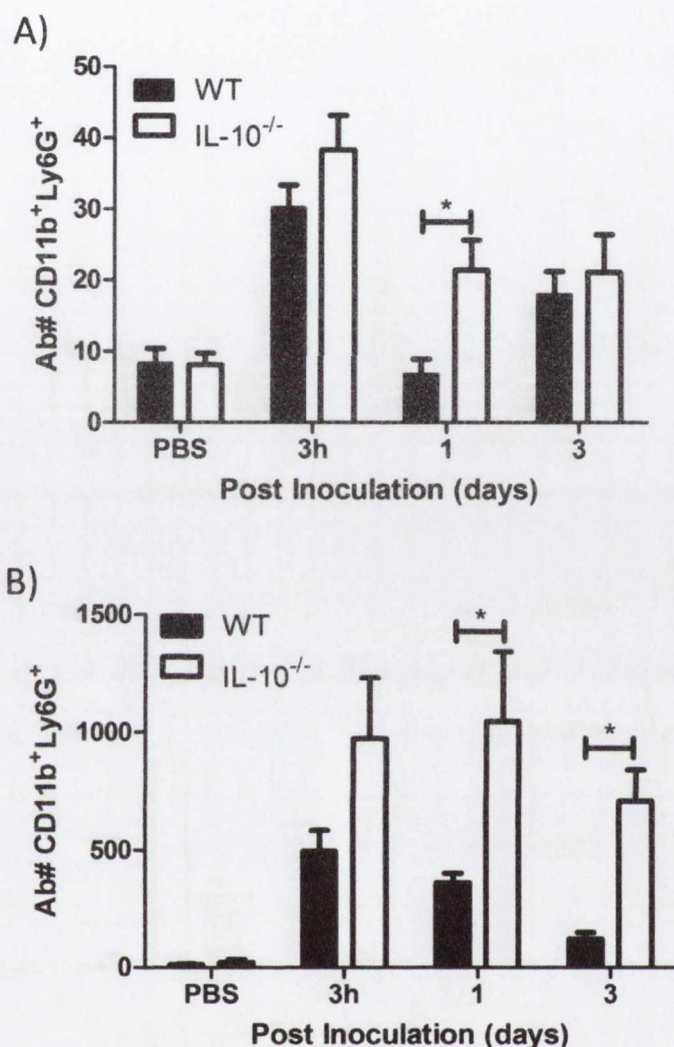


Figure 3.26. IL-10- deficiency results in a significant increase in the migration of neutrophils into both the NT and nose compared with WT mice during *S. aureus* nasal colonisation.

Groups of WT and IL-10^{-/-} mice were inoculated intra-nasally with *S. aureus* (2x10⁸ CFU) or were administered with PBS. Nose and NT were harvested from mice at the indicated times post-infection, surface stained with fluorochrome-conjugated antibodies against CD11b, Ly6G and F480 and analysed by flow cytometry. Absolute numbers of neutrophils (CD11b⁺Ly6G⁺) were assessed in the Nose (**A**) and NT (**B**). Results expressed as mean ± SEM, n= 4-5 mice/group. Data is representative of two independent experiments. Statistical analysis was performed using two-way ANOVA with Bonferroni post-test. *P≤0.05.

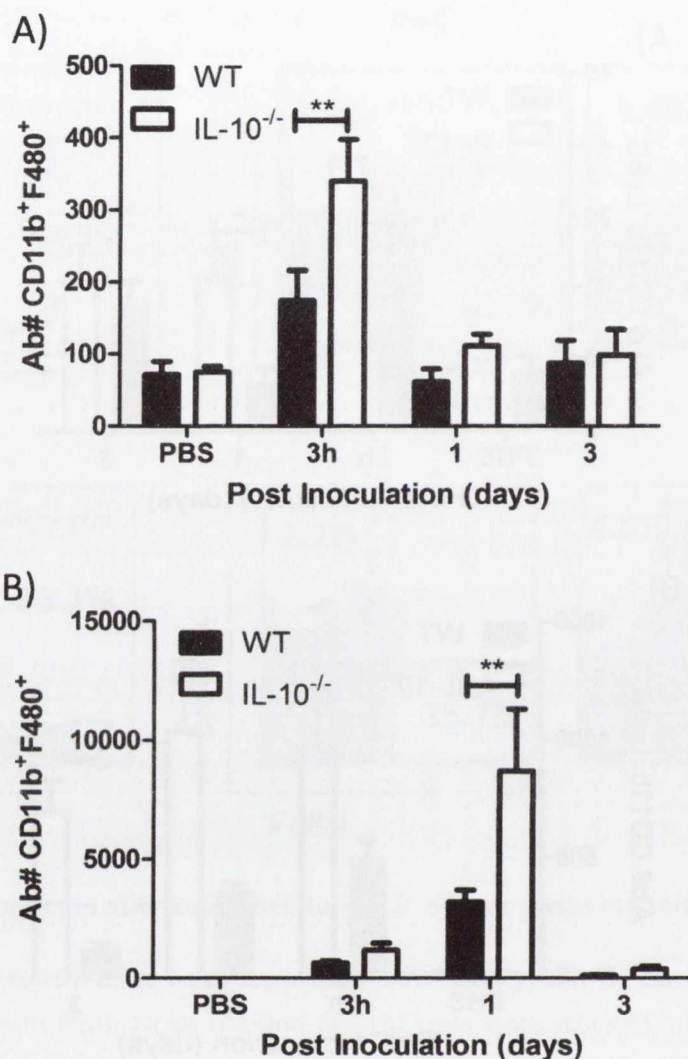


Figure 3.27. IL-10⁻ deficiency results in a significant increase in the migration of macrophages into the nose and NT compared with WT mice during *S. aureus* nasal colonisation.

Groups of WT and IL-10^{-/-} mice were inoculated intra-nasally with *S. aureus* (2×10^8 CFU) or were administered with PBS. Nose and NT were harvested from mice at the indicated times post-infection, surface stained with fluorochrome-conjugated antibodies against CD11b, Ly6G and F480 and analysed by flow cytometry. Absolute numbers of macrophages (CD11b⁺F480⁺) were assessed in the Nose (**A**) and NT (**B**). Results expressed as mean $\pm$ SEM, n= 4-5 mice/group. Data is representative of two independent experiments. Statistical analysis was performed using two-way ANOVA with Bonferroni post-test. ** $P \leq 0.005$

3.2.9. TGF- β is induced locally in the nasal cavity and T-regs are expanded in the NT during *S. aureus* nasal colonisation.

IL-10 is one aspect of a broad immune-modulatory arsenal that can potentially be induced by *S. aureus* to facilitate its persistence during nasal colonisation. In order to establish if other immune regulatory mechanisms such as TGF- β or T-regs can be induced and/or possibly play a role during *S. aureus* nasal colonisation, WT mice were intra-nasally inoculated with *S. aureus* (2×10^8 CFU) or PBS. At specific time points post challenge the concentrations of TGF- β in the nose and NT were measured by ELISA.

In the nose and NT an increase in TGF- β protein expression was also observed across all time points in the *S. aureus* colonised mice compared with PBS controls (**Figure 3.28A**) (**Figure 3.28B**).

TGF- β has been shown to play a role in promoting the expansion of T-reg cells during *S. pneumoniae* colonisation (265). To examine if T-regs were expanded during *S. aureus* nasal colonisation, WT mice were intra-nasally inoculated with *S. aureus* (2×10^8 CFU), leukocytes were isolated from the NT and stained for T-reg markers and analysed by flow cytometry (**Figure 3.29A**). The absolute number of CD4⁺CD25⁺FoxP3⁺ T-reg cells isolated from the NT was significantly increased at d3 post colonisation, compared with PBS treated controls (**Figure 3.29B**). It is possible that these T-regs could be a source of IL-10; however, as outlined previously, this study was unable to identify the cellular source of IL-10 within the NT. Alternatively, it is possible that *S. aureus* is also capable of activating or expanding T-reg cells as an alternative, IL-10 independent mechanism of immunosuppression that facilitates *S. aureus* colonisation within the nasal cavity

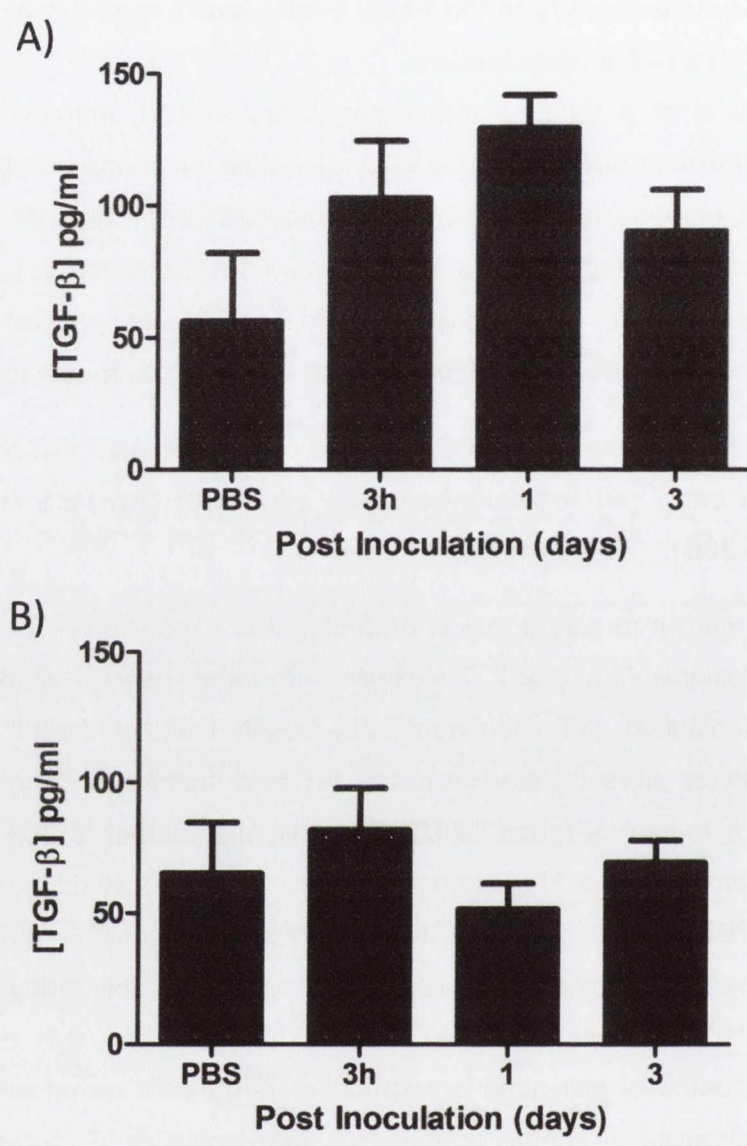


Figure 3.28. TGF-β production is induced in the nose and NT of WT mice during *S. aureus* nasal colonisation.

Groups of WT mice were inoculated intra-nasally with *S. aureus* (2×10^8 CFU) or were administered with PBS. At indicated time points post inoculation, Noses **(A)** and NT **(B)** were harvested, homogenised and concentrations of TGF-β were measured by ELISA. Results expressed as mean $\pm$ SEM. n = 9-12 mice per group. Data pooled from 3 independent experiments. Statistical analysis was performed using a one-way ANOVA with Tukey post-test. NS

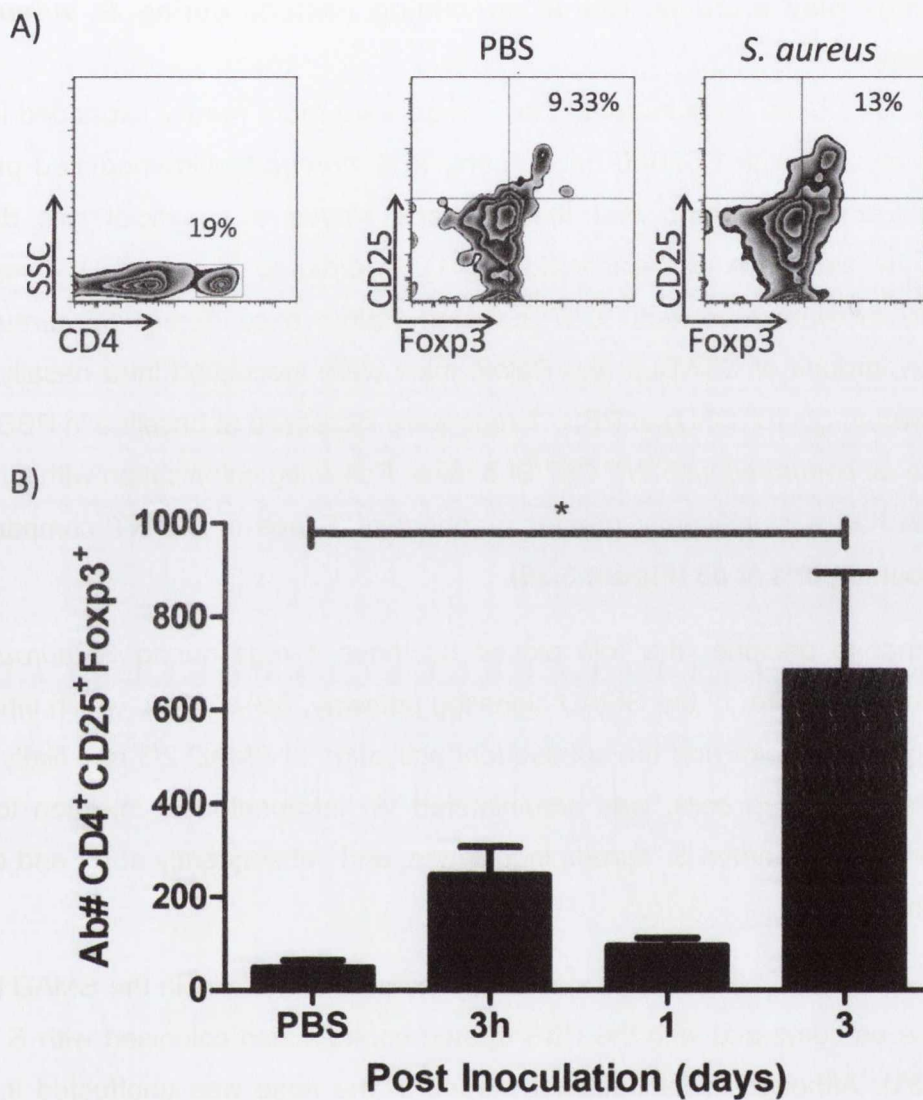


Figure 3.29. T-regs are significantly expanded during in the NT *S. aureus* nasal colonization.

Groups of WT mice were inoculated intra-nasally with *S. aureus* (2×10^8 CFU) or PBS. NT were harvested from mice at the indicated times post-inoculation, surface stained with fluorochrome-conjugated antibodies against CD4, CD25 and Foxp3 and analysed by flow cytometry at d3rs post colonisation **(A)**. Absolute numbers of T-regs (CD4⁺CD25⁺Foxp3⁺) were assessed in the NT **(B)**. Results expressed as mean $\pm$ SEM, $n = 4-5$ mice/group. Data is representative of two independent experiments. Statistical analysis was performed using a one-way ANOVA with Tukey post-test. * $P \leq 0.05$.

3.2.10. T-regs play a crucial role in containing bacteria during *S. aureus* nasal colonisation.

It has previously been demonstrated that T-regs were more readily expanded in Balb/c mice as compared with C57BL6 mice during a *S. aureus* biofilm-mediated prosthetic implant infection model and that these T-regs played a beneficial role during in promoting the clearance of the infection (241). In order to investigate if T-regs were differentially increased between C57/BL6 and Balb/c mice during *S. aureus* nasal colonisation, groups of C57/BL6 and Balb/c mice were inoculated intra-nasally with *S. aureus* Newman (2×10^8 CFU) or PBS. T-regs were increased at baseline in PBS treated Balb/c mice as compared with WT C57/BL6 mice. Following colonisation with *S. aureus*, Balb/c mice had a significantly greater numbers of T-regs in the NT compared with C57/BL6 counterparts at d3 (**Figure 3.30**).

In an attempt to decipher the role played by those T-regs during *S. aureus* nasal colonisation, an inhibitor of the SMAD signaling pathway, SB-431542, which inhibits the TGF-beta type I receptor and the subsequent activation of SMAD 2/3 and likely inhibits the expansion of T-reg cells, was administered via intraperitoneal injection to Balb/c mice 1 h prior to intranasal *S. aureus* inoculation, and subsequently at d1 and d3 post-colonisation.

By d6 post colonisation there was significantly increased mortality in the SMAD inhibitor treated mice as compared with the PBS treated controls also colonised with *S. aureus* (**Figure 3.31**). Although initial bacterial burden in the nose was unaffected in SMAD inhibitor treated mice at d3 (**Figure 3.32A**), by d6 there was an increase in bacterial burden in the nose of the surviving mouse. Importantly, there was also increased dissemination of *S. aureus* to the lungs at this time point in the surviving SMAD inhibitor treated mouse compared with PBS controls also colonised with *S. aureus* (**Figure 3.32B**).

Collectively, these preliminary results require careful conclusions, as TGF- β impacts a multitude of host homeostatic processes, however, this data suggests that T-regs may play a role in restricting *S. aureus* in its niche and thereby preventing invasive disease.

Future experiments are now required to evaluate the toxicity of SMAD inhibitor treatment in naïve mice.



Figure 1. Effect of SMAD inhibitor treatment on gene expression. The bar chart shows the relative expression of various genes after treatment with SMAD inhibitor. The y-axis represents the relative expression (fold change) and the x-axis represents the gene. The first bar (Gene 1) is at 1.0. The second bar (Gene 2) is significantly lower, around 0.4. The third bar (Gene 3) is around 0.6. The fourth bar (Gene 4) is around 0.5. The fifth bar (Gene 5) is around 0.4. The sixth bar (Gene 6) is around 0.5. The seventh bar (Gene 7) is around 0.4. The eighth bar (Gene 8) is around 0.5. The ninth bar (Gene 9) is around 0.4. The tenth bar (Gene 10) is around 0.5. Error bars are present on each bar.

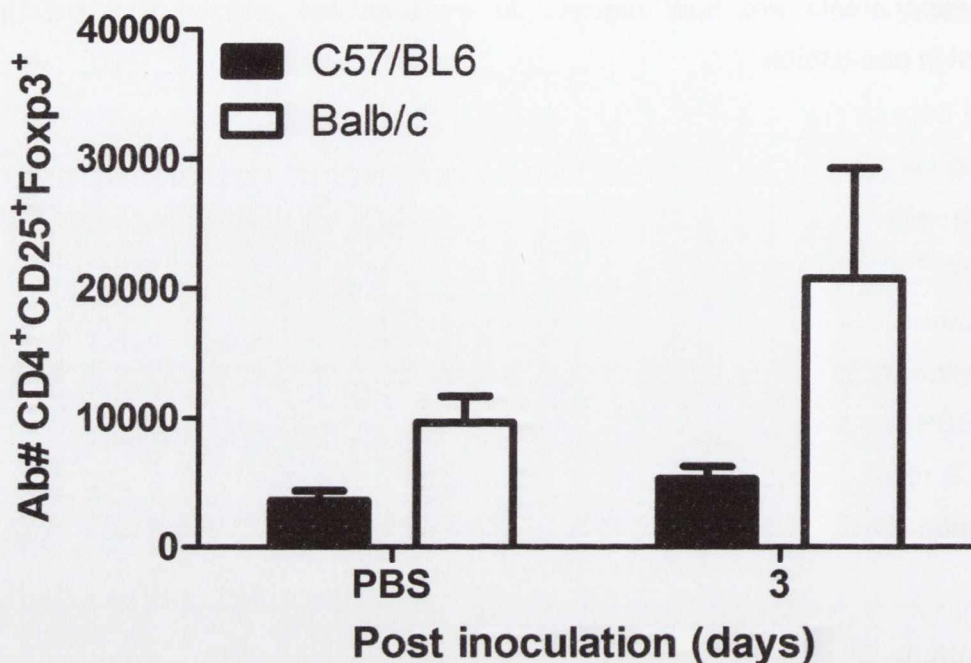


Figure 3.30. Balb/c mice demonstrate increase T-reg numbers locally in the NT as compared with C57/BL6 mice during *S. aureus* nasal colonisation.

Groups of C57BL/6 and Balb/c mice were inoculated intra-nasally with *S. aureus* (2×10^8 CFU) or PBS. NTs were harvested from PBS-treated mice or at d3 post-colonisation, stained with fluorochrome-conjugated antibodies against CD4, CD25 and Foxp3 and analysed by flow cytometry. Absolute numbers of T-regs (CD4⁺CD25⁺Foxp3⁺) were assessed in the NT. Results expressed as mean $\pm$ SEM, n= 9-10 mice/group. Data are pooled data from two independent experiments. Statistical analysis was performed using a two-way ANOVA with Bonferroni post-test. NS

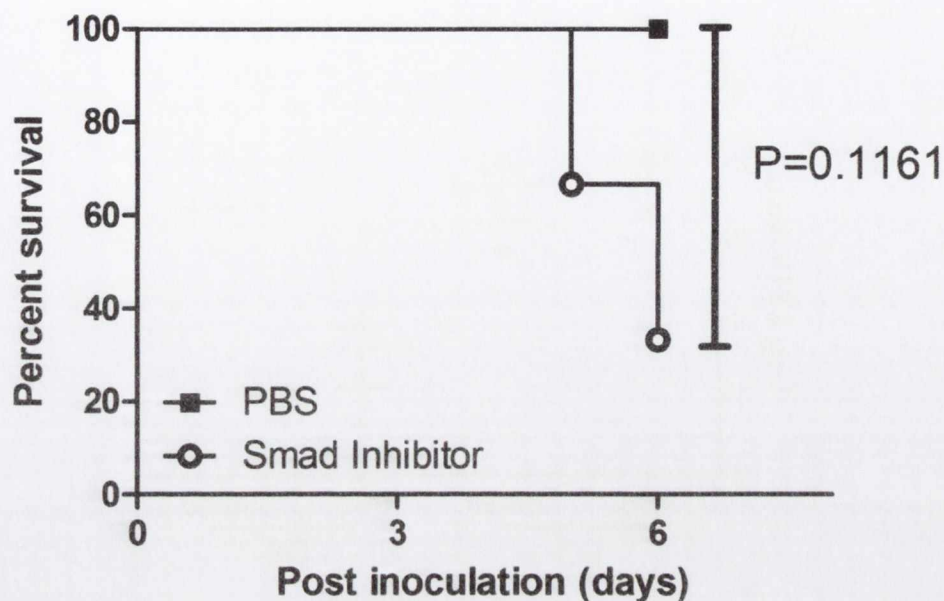


Figure 3.31. Inhibition of the SMAD2/3 signaling pathway results in increased mortality during *S. aureus* nasal colonisation.

Groups of Balb/c mice were inoculated intra-nasally with *S. aureus* (2×10^8 CFU). On days -1, 0 and 2 mice were given an intraperitoneal injection of SMAD inhibitor (0.05mg/kg) or PBS as a control. SMAD inhibitor-treated mice exhibited increased mortality by day 5 post-colonisation (n=3). Statistical analysis was performed using a Kruskal-Wallis test with Mantel-Cox test. $P=0.1161$.

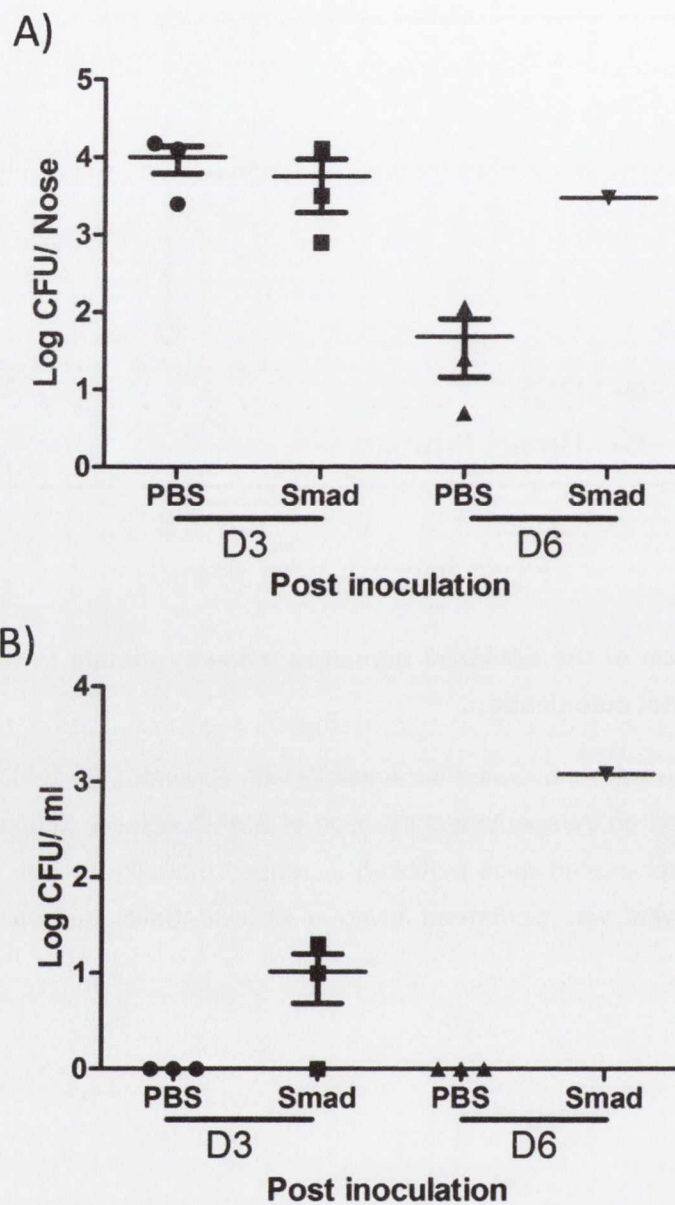


Figure 3.32. T-regs protect the host from bacterial dissemination to the lungs during *S. aureus* nasal colonisation.

Groups of Balb/c mice were inoculated intra-nasally with *S. aureus* (2×10^8 CFU). On days -1, 0 and 2 mice were given an intra-peritoneal injection of SMAD inhibitor. Noses (**A**) and lungs (**B**) were harvested at various days post-inoculation, homogenised, and plated on Sm-supplemented agar to determine *S. aureus* CFU. Results expressed as mean $\pm$ SEM. $n = 3-4$ mice per group.

3.3. Discussion

There is currently a dearth of data on the understanding of the host immune responses during *S. aureus* nasal colonisation. In order to address this, a murine model of *S. aureus* nasal colonisation was utilised to elucidate the immune responses crucial for bacterial clearance. To date, one previous study has shown that clearance of *S. aureus* from the nasal cavity is highly dependent on an IL-17-mediated response which was important in regulating neutrophil recruitment to the nose (123). However in this study, Archer *et al.* did not identify the source of IL-17 in the nose, but instead suggested that Th17 cells represented a likely source. This new research substantially advances previous work by identifying IL-17-producing cell populations at the site of colonisation. CD4⁺ T-cells were identified as the primary producers of IL-17 during *S. aureus* nasal colonisation. However, $\gamma\delta$ ⁺ T-cells also represented a novel minor population of T-cells also capable of IL-17 production in response to *S. aureus* during nasal colonisation. In the Archer study, deficiency in IL-17A did not lead to the same increased concentrations of bacterial burden observed when SCID and β/δ -TCR KO mice were intra-nasally colonised with *S. aureus* (123). This observation suggested a role for other Th17-associated cytokines in the elimination of *S. aureus* during colonisation. This research identifies that IL-22, another Th17 cytokine, is also significantly elevated in the nose during *S. aureus* nasal colonisation. During *S. aureus* nasal colonisation IL-22 is predominantly produced by CD4⁺ and CD8⁺ T-cells, while ILCs also potentially contribute to IL-22 production. Consistent with this, it was recently demonstrated that IL-22 is produced by both conventional CD3⁺ cells and also a population of CD3⁻ cells, potentially ILCs, which conferred protective during *S. aureus* pneumonia (76).

This study did not specifically determine whether IL-22-producing T-cells co-produce IL-17 during nasal colonisation, although these cells have been previously reported (289) and will be of further interest. Considering both studies and the research presented by Archer *et al.* it appears that both IL-17 and IL-22 function in concert to control *S. aureus* nasal colonisation, but operate through distinct mechanisms; IL-22 regulates bacterial burden through the induction of AMPs and by controlling keratinocyte differentiation and thus availability of *S. aureus* ligands within the nose but does not impact upon neutrophil recruitment (288), whereas IL-17 acts to exclusively to control the neutrophil-

mediated clearance of *S. aureus* during nasal colonisation (123). It was previously shown that IL-22 in combination with IL-17 synergistically induce β -defensin 2 and S100A9, while additively enhancing the production of S100A7 and S100A8 from keratinocytes *in vitro* (290). A transgenic mouse model deficient in both IL-17 and IL-22 would be required to definitively confirm this co-operative effect during *S. aureus* nasal colonisation. In addition, it will be important to ascertain the role played by these two cytokines during *S. aureus* colonisation in humans. Interestingly, data from a recent colonisation study performed in humans has shown that clearance of *S. aureus* from the human nares is predominantly associated with Th1-type cytokines in the nasal fluid whereas IL-17 concentrations were below the limit of detection (118). However, this human study did not measure IL-22 concentrations in the nasal fluid, and therefore it is not conclusive whether or not Th17 cells have a role in staphylococcal colonisation of humans.

The data presented would suggest that the host possesses a robust T-cell-dependent mechanism to limit *S. aureus* nasal colonisation; however despite this, the majority of the healthy population can still be colonised with *S. aureus* in their noses at various stages throughout life. Therefore, it is postulated that *S. aureus* must have mechanisms in place to facilitate its survival in the nose by suppressing the immune responses that are mediating its clearance. Data presented in this thesis demonstrate that the induction of the anti-inflammatory cytokine IL-10 is a key mechanism employed by the bacterium to achieve this. Following *S. aureus* nasal colonisation, IL-10 was produced locally within the nasal cavity. This IL-10 was produced within the nose but not the NT suggesting a tissue specific or cellular micro-environment preference for *S. aureus* to mediate its effects i.e. IL-10 is being produced directly at the site of colonisation. This study attempted to identify the source of IL10 by using IL-10 GFP reporter mice; however, was unable to do so because of a lack of sufficient leukocytes isolated from the nose. Other studies have speculated that macrophages represent the most likely source of IL-10 within the nasal sub-mucosa although this is based on their observations in *in vitro* studies only (254). Given that this FACS-based approach, failed to identify macrophages as the source of IL-10 *in vivo* during *S. aureus* nasal colonisation, other techniques including histology may be required to identify the IL-10

source. Intranasal treatment of mice with Clodronate liposomes to deplete macrophages (291) prior to colonisation may also be another strategy to determine whether macrophages are the primary source of IL-10 in the nose; if Clodronate-treated mice display similar bacterial burden to IL-10^{-/-} mice, this would indicate that macrophages are the source local of IL-10.

This study definitively proves that local induction of IL-10 is associated with persistence of *S. aureus* within the nose. IL-10^{-/-} mice exhibited an ability to clear *S. aureus* from the nose more rapidly than WT mice. The absence of IL-10 resulted in significantly greater numbers of T-cells including CD4, $\gamma\delta$ and CD8 T-cells in the NT during nasal colonisation. The absence of IL-10 also resulted in significantly increased production of the T-cell cytokine IL-17 in both the nose and the NT but did not appear to impact upon the production of IL-22. Consistent with this, it has been shown in other models, including *B. burgdorferi* subcutaneous infection, that IL-10 limits the production of IL-17 by CD4⁺ T-cells (292). IL-10 was previously shown to inhibit TCR-induced IFN- γ production but not IL-17 production from memory human T-cells (293). Therefore, IL-10 in certain circumstances appears capable of selectively inhibiting certain cytokine production over another by the same cell type. To conclude, *S. aureus* induced IL-10 acts to limit the migration and activation of T-cells, thereby aiding the bacteria to persist during colonisation.

The elevated production of IL-17 in the IL-10^{-/-} mice unsurprisingly resulted in greater neutrophil migration to both the nose and NT during nasal colonisation, which has previously shown to mediate bacterial clearance (123). Consistent with this, overexpression of IL-10 during *Pseudomonas aeruginosa*-induced lung inflammation resulted in reduced CXCL1 production and neutrophil migration, resulting in impaired bacterial clearance (294). Macrophages were also expanded during nasal colonisation with *S. aureus* and were increased in both the nose and the NT of IL-10^{-/-} mice as compared with WT mice. The role of macrophages was not fully examined in this study; however, as previously suggested, these cells could represent a population of IL-10-producing cells, or possibly are involved in the direct killing *S. aureus* (Explored in Chapter 4). As macrophages are a heterogeneous population of cells which can contain

both M1 and M2 subsets they most likely fulfil multiple functions in the context of *S. aureus* nasal colonisation and this will require further study. If *S. aureus* forms biofilms during nasal colonisation this could provide it with possible evasion mechanism as biofilms have been shown to skew the host-response towards an anti-inflammatory setting typified by the presence of M2 macrophages (295, 296)

In addition to IL-10, the host has other regulatory mechanisms that may also be manipulated by *S. aureus* in order to gain a survival advantage and facilitate persistence during nasal colonisation. During *S. pneumoniae* colonisation of the nasopharyngeal cavity, TGF- β was found to be essential for the expansion of T-reg cells, which in turn facilitated bacterial persistence (265). Analysis of both TGF- β and T-reg during *S. aureus* nasal colonisation led to the observation that TGF- β was induced both locally in the nose and NT. T-regs were also shown to be expanded in the NT of both C57/BL6 and Balb/c mice upon *S. aureus* nasal colonisation. Balb/c mice had greater levels of baseline T-regs in the NT compared with C57/BL6 mice and upon colonisation with *S. aureus*, T-regs were more expanded in Balb/c mice compared with C57/BL6 in the NT at d3, which would suggest that these mice may be better at promoting *S. aureus* persistence. Preliminary data suggest that this might be the case (31488 CFU/Nose vs 4564 CFU/ noses in the Balb/c compared with the C57/BL6 at d3 post *S. aureus* nasal colonisation).

Previously it has been shown that during a biofilm-mediated prosthetic implant infection model, BALB/c mice cleared infection more rapidly than C57BL/6 mice (241). The mechanism behind this effect was proposed to be that expansion of T-regs acted to constrain the local inflammatory response which may have caused tissue damage and bacterial adherence. This study also suggested that Th1 responses played a role in the development of chronic *S. aureus* infection by eliciting a more pro-inflammatory response. Therefore, this would suggest that a Th1 pro-inflammatory response would be disadvantageous in *S. aureus* clearance during nasal colonisation. However, considering that *S. aureus* infection is not the same colonisation, a Th1 response may have dissimilar effect in two different environments. Data from the recent human study has demonstrated that a Th1 type response was associated with bacterial clearance

(118), however in direct contrast Archer *et al.*, demonstrated that IFN- γ was in fact reduced during *S. aureus* nasal colonisation. Therefore, the true role of a Th1 response during *S. aureus* remains unknown.

In order to confirm a role for T-regs during *S. aureus* nasal colonisation, a SMAD2/3 inhibitor was used to block TGF- β signaling and subsequent T-reg development. Treatment of mice with the SMAD inhibitor systemically prior to nasal colonisation with *S. aureus* resulted in an increase in bacterial burden in the nose. However, in conjunction with this, an increased bacterial dissemination to the lungs and significantly increased host mortality was observed, suggesting that although T-reg expansion initially may facilitate bacterial persistence; it may also function to contain *S. aureus* with the nose during colonisation. Although preliminary, these results highlight that T-regs might be particularly important for preventing bacterial dissemination to the lungs and also the subsequent mortality of the host.

In summary, this research offers significant insight into the dynamics of *S. aureus* nasal colonisation. It validates previous data suggesting a role for IL-17-producing Th17 cells in the clearance of *S. aureus* during colonisation. In addition, IL-22 was identified as a novel cytokine which provided protection against *S. aureus* colonisation. IL-10 was identified as a novel effector of *S. aureus* immune evasion in this localised setting which provides the bacterium with a mechanism of limiting IL-17 responses, which in turn limits the subsequent migration of neutrophils. Finally, T-regs were found to also be expanded during nasal colonisation, however interestingly their role may not be to facilitate bacterial persistence, but actually to protect the host from bacterial dissemination, possibly indicating that in fact the host-*S. aureus* relationship is ultimately symbiotic, protecting both host and bug.

Chapter 4

4. Investigating the direct effects of *S. aureus* on phagocytic cells

4.1. Introduction

S. aureus has been shown to drive IL-10 production in order to facilitate its own persistence (254). During a localised model of chronic *S. aureus* orthopedic biofilm infection, MDSCs were identified as major producers of IL-10 (258). Heim *et al.* observed an expansion of MDSCs at this site of infection on day 7 post-infection, which was associated with bacterial persistence (257). In addition, during a chronic systemic infection MDSCs and to a lesser degree T-regs, were shown to facilitate *S. aureus* persistence by limiting T-effector cell activity. Therefore, during chronic infection it appears that *S. aureus* can utilise IL-10 to restrict the activation of T-effector cells for its own survival. As previously demonstrated in chapter 3, this strategy of inducing immunosuppression to facilitate persistence is not only observed during *S. aureus* infection but also during *S. aureus* commensal interaction with the host. IL-10 appears to be induced locally within the nose during *S. aureus* colonisation and this IL-10 functions to regulate the production of IL-17 effector CD4⁺ T-cells which subsequently controls the migration of phagocytic cells, specifically neutrophils, to the nasal site, thereby facilitating *S. aureus* persistence.

In Chapter 3, the source of IL-10 *in vivo* during *S. aureus* nasal colonisation was not identified. Previously published data suggests that there is preferential production of IL-10 by macrophages as compared with DCs in response to *S. aureus* peptidoglycan (PGN) (151). As macrophages are believed to be the primary cell type in the nose, they represent the most likely source of IL-10 during nasal colonisation and therefore a probable target for *S. aureus* (254). *S. aureus* isolates from persistent carriers were shown to be capable of simultaneously producing both IL-10 and TNF- α from PBMCs (152). This ability to independently produce both cytokines resulted from the activation of distinct signalling pathways downstream from TLR2. Activation of phosphoinositol 3-kinase (PI3K)-Akt-mTOR and extracellular signal-regulated kinase (ERK) was required for the IL-10 response while the TNF- α response was induced via p38 signaling. The *S. aureus* TLR2 ligands responsible for this dichotomy of signaling however remains unknown (152). This chapter aims to further expand on these studies by examining if

live colonising *S. aureus* strains are more effective than invasive *S. aureus* strains in terms of their ability to induce IL-10 production.

IL-10 was recently described to arrest phagosome maturation during Mtb infection of macrophages (249, 297). *In vitro* experiments demonstrated that blocking IL-10 enhanced phagosome maturation following infection of human macrophages with MtB, while the addition of recombinant IL-10 significantly inhibited this process compared with untreated cells. Signal transducer and activator of transcription (STAT) 3 is a transcription factor that has been demonstrated as being essential for almost all known functions of IL-10 (298). Phagosome maturation was also enhanced upon STAT3 inhibition, hence inhibition of Mtb-phagosome maturation by IL-10 may be in part mediated by STAT3 (249). Whether or not *S. aureus* induces IL-10 as a mechanism by which to directly inhibit killing by professional phagocytes, remains to be established.

This chapter will initially establish if colonising and invasive strains of *S. aureus* have a differential capacity to induce IL-10 and TNF- α production by phagocytes. It will then attempt to identify the mechanisms by which IL-10 acts on phagocytes to manipulate or inhibit their effector immune responses to *S. aureus*. Firstly examining the role played by IL-10 in controlling autocrine pro-inflammatory cytokine production by the phagocytes in addition to investigating whether IL-10 has a direct role on the ability of phagocytes to kill *S. aureus*.

4.2. Results

4.2.1. Murine and Human phagocyte phenotyping

In order to validate previous data demonstrating that *S. aureus* can stimulate IL-10 production from phagocytic cells, human and murine macrophages and dendritic cells were first generated from monocyte or bone marrow precursors. CD14⁺ cells were magnetically isolated from human buffy coats, a distinct CD14⁺ monocyte population was observed among these cells when analysed by flow cytometry (**Figure 4.1A**). These monocytes were then cultured in GM-CSF and IL-4 or M-CSF only for 6 days in order to convert them to dendritic cells and macrophages respectively. At the end of a 6 day culture, monocyte derived DCs had down-regulated CD14 and up-regulated the DC-specific C-type lectin, DC-SIGN (**Figure 4.1B**), monocyte-derived macrophages were identified as >95% CD14⁺ and CD11b⁺ cells, but largely negative for DC-SIGN (**Figure 4.1C**).

Bone marrow was isolated from C57/BL6 mice and cultured in the presence of 20% L929 (M-CSF). Following 6 days of culture, it was confirmed that 98% of cells were CD11b⁺F480⁺ confirming that they were mature macrophages (299) (**Figure 4.2**).

In summary, both murine BMMs, monocyte derived macrophages and dendritic cells were found to express the appropriate cell surface markers for their respective phenotypes.

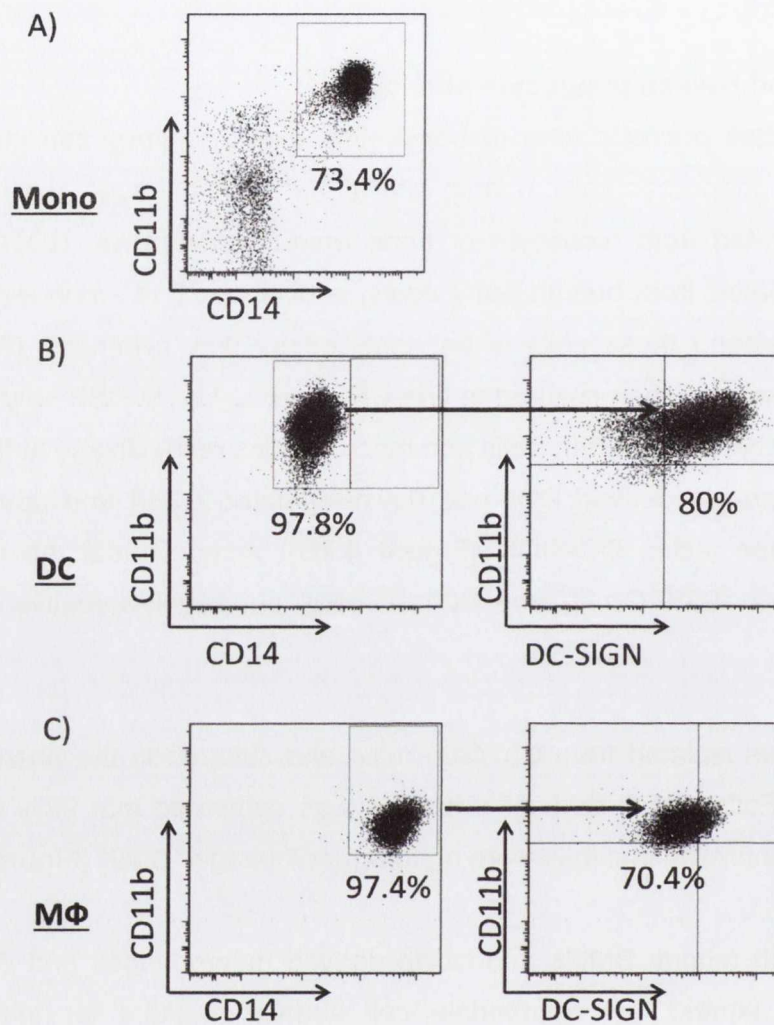


Figure 4.1. Monocyte purity and phagocyte phenotyping

CD14⁺ monocytes were initially magnetically sorted from buffy coats **(A)**. These CD14⁺ monocytes were then differentiated into monocyte-derived macrophages and DCs. These cells were stained for surface CD14, CD11b, CD11c, DC SIGN and analysed by flow cytometry. DCs were identified as CD11b⁺CD14^{int}DC-SIGN⁺ **(B)** Macrophages were identified as CD11b⁺CD14⁺DC-SIGN⁻ **(C)**. Representative FACS plots are shown above. (n=3-5 donors).

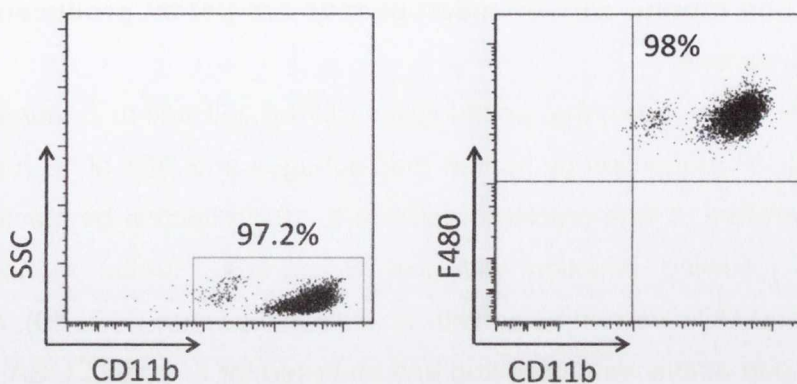


Figure 4.2. Murine bone-marrow derived macrophage phenotyping

Murine Bone-marrow derived macrophages were harvested on day 6 of culture in the presence of M-CSF and stained for cell surface markers CD11b and F480, and analysed by flow cytometry. Representative FACS plot is shown above (n=6).

4.2.2. Human and murine derived macrophages are potent producers of IL-10 in response to *S. aureus*.

Previous studies have shown that peptidoglycan in the cell wall of *S. aureus* is capable of stimulating IL-10 production by human macrophages and DCs at 18 h post-infection (151, 152). Therefore, it was crucial to validate IL-10 production by macrophages and dendritic cells following infection with live *S. aureus*. Human monocyte derived macrophages and DCs were infected with *S. aureus* Newman (MOI 10). At 18 h post-infection the supernatants were collected and analysed for IL-10 by ELISA. Both human monocyte derived macrophages and DCs were shown to produce IL-10 following infection with *S. aureus* (**Figure 4.3**). However, consistent with previously published studies (151), macrophages were more potent producers of IL-10 compared with their DC counterparts (**Figure 4.3**)

To confirm that murine macrophages are also capable of elevated IL-10 production, murine Bone Marrow derived Macrophages (BMM) were cultured and infected with *S. aureus* Newman strain at an MOI 10. At 18 h post-infection the supernatants were collected and analysed for IL-10 by ELISA. IL-10 production by BMM was significantly increased compared with medium treated controls at this time point (**Figure 4.4**)

In conclusion, *S. aureus* can induce IL-10-producing from both human and murine macrophages. Human macrophages were shown to produce significantly more IL-10 compared with DCs.

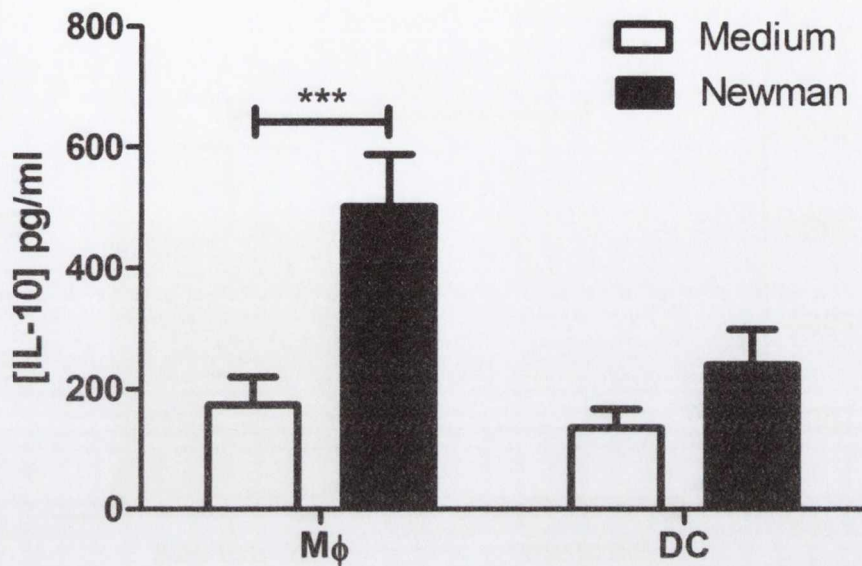


Figure 4.3. Human monocyte derived macrophages produce substantially more IL-10 compared with DCs.

Human monocyte derived macrophages and dendritic cells were infected with *S. aureus* strain Newman at an MOI 10. At 18 h post-infection IL-10 production was established by ELISA. Data expressed as mean $\pm$ SEM (n=5). Statistical analysis was performed using a two-way ANOVA with Bonferroni post-test. *** $P \leq 0.001$

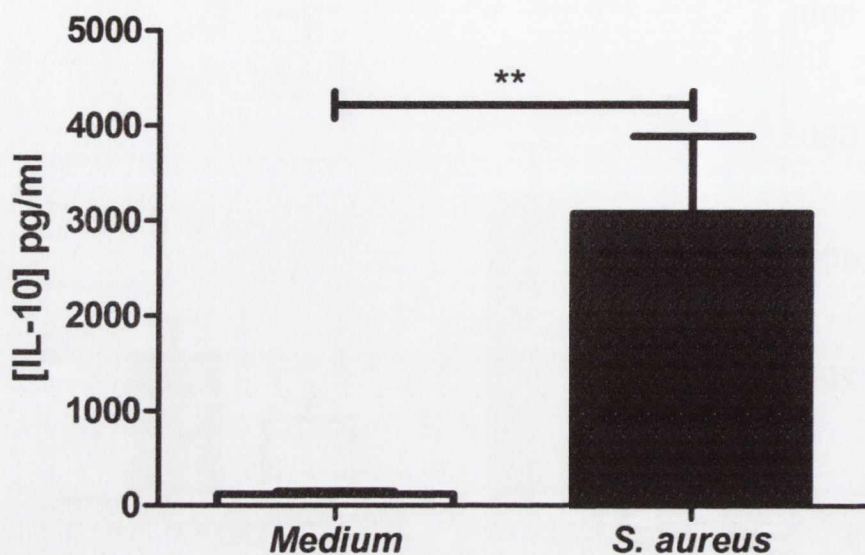


Figure 4.4. Murine bone marrow derived macrophages produce IL-10 upon infection with *S. aureus*.

Murine BMM were infected with *S. aureus* strain Newman at an MOI 10. At 18 h post-infection IL-10 production was established by ELISA. Data expressed as mean $\pm$ SEM. (n=5-8). Statistical analysis was performed using an unpaired t-test. ** $P \leq 0.005$

4.2.3. *S. aureus* isolates from persistent carriers simultaneously produces both IL-10 and TNF- α from macrophages.

As macrophages were identified as the primary producer of IL-10 in response to *S. aureus*, they were used in subsequent experiments. In order to establish if *S. aureus* colonising strains induce simultaneous production of both IL-10 and TNF- α , murine BMMs and human monocyte derived macrophages were infected with *S. aureus* strains isolated from persistent carriers (RD strains) at an MOI 10. At 3 and 18 h post-infection, supernatants were collected and analysed for IL-10 and TNF- α by ELISA.

IL-10 production from BMMs was rapidly induced in response to the colonising strains at 3 h post stimulation, compared with medium treated controls. RD9 induced more IL-10 than RD21 and RD25 at 3 h. However, RD9 stimulated BMMs produced significantly less amounts of IL-10 at 18 h compared with 3 h post stimulation. RD25 stimulated BMMs produced the greatest amount of IL-10 at 18 h post stimulation (**Figure 4.5A**). Human monocyte derived macrophages were not as responsive as BMMs and did not produce any significant concentrations of IL-10 in response to the colonising strains of *S. aureus* (**Figure 4.5B**).

At 3 h post stimulation, colonising *S. aureus* strains rapidly induce TNF- α production from BMMs compared with medium treated control. RD9 produced significantly more TNF- α from BMMs at 3 h compared with 18 h post stimulation. RD21 and RD25 also rapidly produced substantially more TNF α from BMMs at 3 h compared with 18 h post *S. aureus* stimulation (**Figure 4.6A**). Infection of human monocyte derived macrophages, with colonising strains induced a low TNF- α response at both 3 and 18 h post stimulation (**Figure 4.6B**).

Overall this data demonstrates that colonising stains of *S. aureus* can induce both IL-10 and TNF- α simultaneously from murine macrophages. These colonising strains elicit a far more robust response from BMMs in terms of both IL-10 and TNF- α production compared with human monocyte derived macrophages which produced no IL-10 and only low concentrations of TNF- α in response to the colonising strains. As previously

shown, Peres *et al* also demonstrated that certain nasal isolates were poor IL-10 inducers of PBMCs (152).

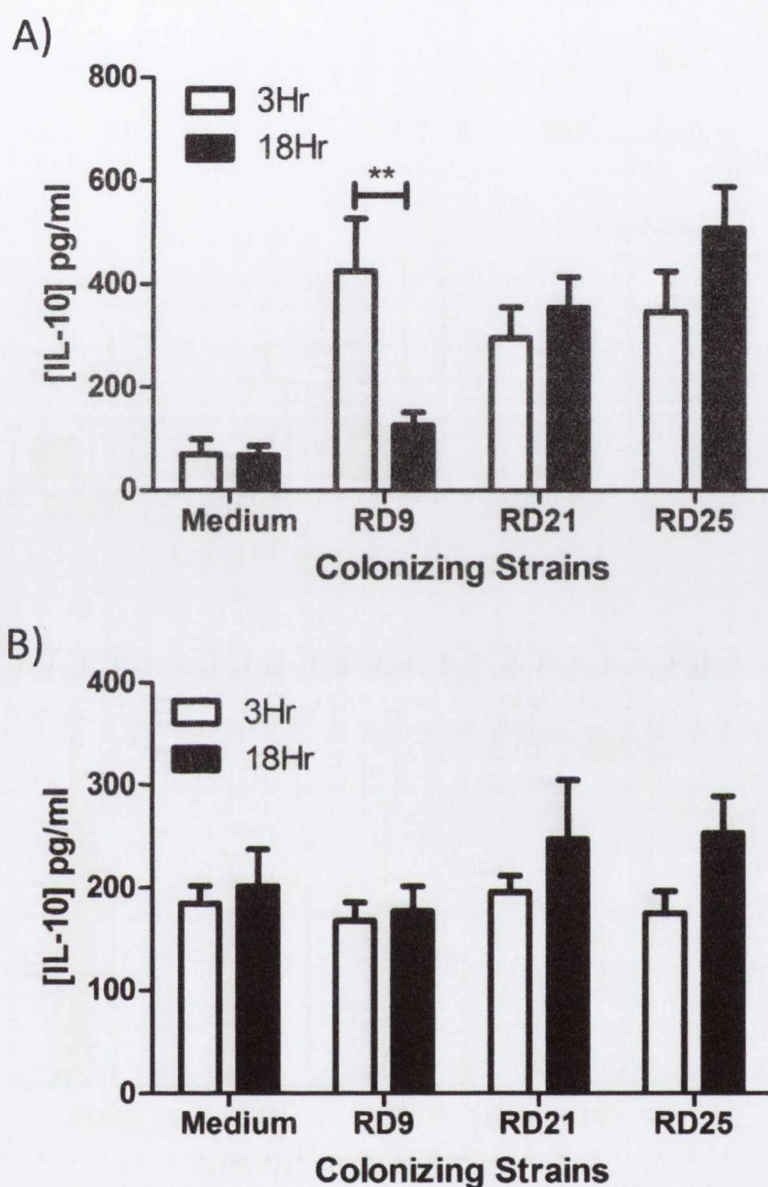


Figure 4.5. *S. aureus* colonising strains induce IL-10 production from BMMs but not human monocyte derived macrophages.

BMMs (A) and human monocyte derived macrophages (B) were infected with persistent human colonising strains of *S. aureus* RD9, 21, and 25 at an MOI 10. At 3 and 18 h post-infection the supernatants were collected. Analysis of IL-10 concentration in the supernatants was performed by ELISA. Data expressed as mean $\pm$ SEM. (n=3-4). Statistical significance was determined using a two-way ANOVA with Bonferroni post-test. * $P \leq 0.05$ ** $P \leq 0.005$

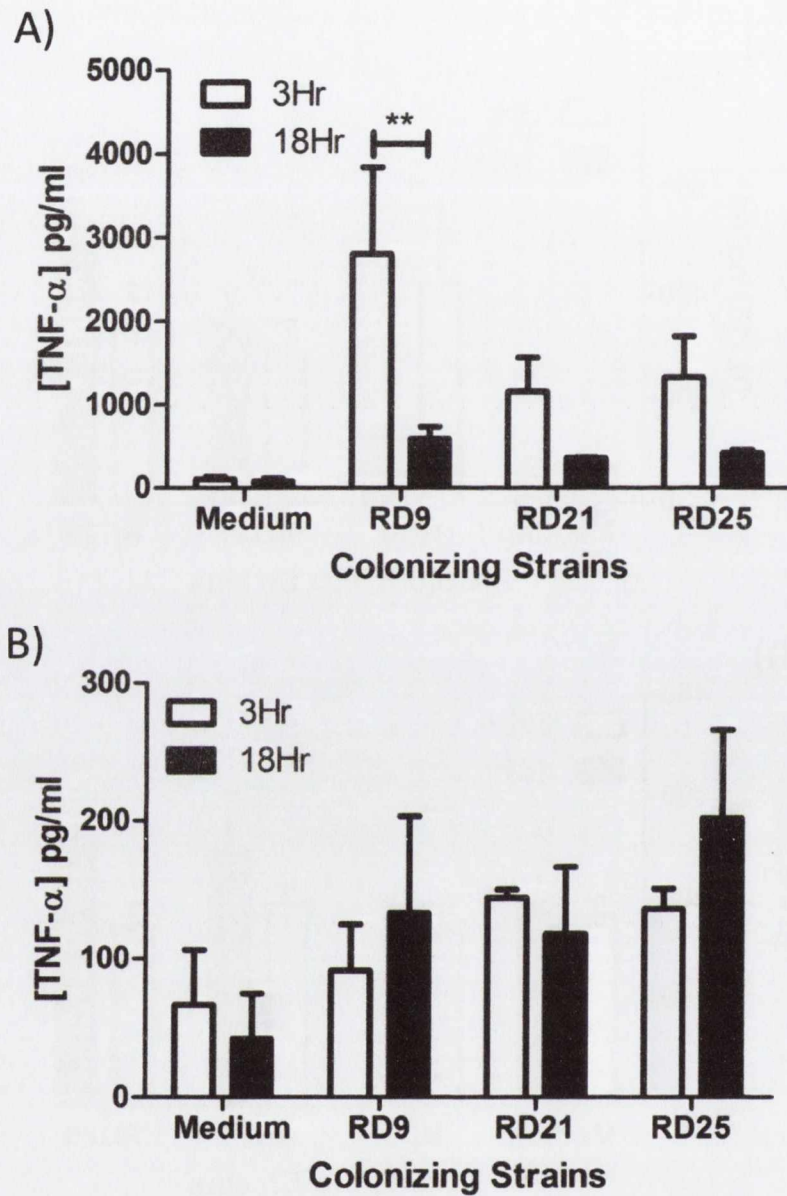


Figure 4.6. *S. aureus* colonising strains induce TNF- α production from BMMs and low TNF- α production from human monocyte derived macrophages.

BMMs (A) and human monocyte derived macrophages (B) were infected with persistent human colonising strains of *S. aureus* RD9, 21, and 25 at an MOI 10. At 3 and 18 h post-infection the supernatants were collected. Analysis of TNF- α concentration in the supernatants was performed by ELISA. Data expressed as mean $\pm$ SEM. (n=3-4). Statistical significance was determined using a two-way ANOVA with Bonferroni post-test. * $P \leq 0.05$ ** $P \leq 0.005$

4.2.4. Persistent carrier *S. aureus* strains demonstrate differential patterns of IL-10 and TNF- α induction compared with invasive USA300 *S. aureus* strain

As shown, *S. aureus* isolated from persistent carriers can induce the simultaneous production of IL-10 and TNF- α , at least from murine macrophages. To establish if an invasive strain of *S. aureus* behaved similar to the colonising strains, *S. aureus* strain USA300 was included. Murine BMMs and human monocyte derived macrophages were infected with *S. aureus* colonising strains in addition to USA300 (invasive strain) at an MOI 10. The laboratory strain Newman which was identified in chapter 3 as the best nasal coloniser of mice was also included. At 3 and 18 h post-infection, supernatants were collected and analysed for IL-10 and TNF- α by ELISA

Interestingly, at 3 h post-infection, the invasive strain USA300 and *S. aureus* strain Newman induced less IL-10 production by BMM as compared with the colonising strains (**Figure 4.7A**). However, by 18 h post-infection, the pattern of IL-10 production had reversed as the colonising strains no longer induced significant concentrations of IL-10 while the invasive strain USA300 and strain Newman now induced appreciable concentrations of IL-10. In particular, *S. aureus* strain Newman induced a strong IL-10 response at this time point (**Figure 4.7B**). Similar to the colonising strains, the invasive strain USA300 also failed to induce any IL-10 production by human monocyte derived macrophages at 3 (**Figure 4.8A**) or 18 (**Figure 4.8B**) h post-infection. Interestingly however, *S. aureus* strain Newman although incapable of inducing any IL-10 production at 3 h (**Figure 4.8A**), induced significant concentrations of IL-10 production by human monocyte derived macrophages at 18 h (**Figure 4.8B**) post-infection.

The invasive strain USA300 failed to induce any TNF- α production by BMM at 3 h post-infection which was in contrast to the colonising strains which induced significant concentrations of TNF- α at this time-point (**Figure 4.9A**). By 18 h, however USA300 and Newman were inducing significant concentrations of TNF- α production by which time TNF- α production in response to the colonising strains was significantly reduced (**Figure 4.9B**).

The TNF- α response in human monocyte derived macrophages, to all strains of *S. aureus* was relatively low at 3 h post-infection with the greatest response induced by the invasive strain USA300 (**Figure 4.10A**). By 18 h, USA300 was the only strain capable of inducing TNF- α production by the human monocyte derived monocytes and induced substantial concentrations of TNF- α compared with either *S. aureus* Newman or the colonising strains following stimulation (**Figure 4.10B**). IL-23 or IL-12 were not detected in the supernatant of murine macrophages stimulated with either colonising or invasive strains.

From this data, it can be concluded that colonising and invasive *S. aureus* strains induce differential patterns of cytokine production. BMM in particular respond to colonising *S. aureus* strains with the production of both IL-10 and TNF- α rapidly. In contrast to colonising strains, the invasive strain USA 300 induces substantially more of these cytokines at later time-points. The invasive strain USA300 is also a more potent TNF- α inducer than colonising strains in human macrophages at later time points. However, neither the colonising strains nor the invasive strain induced any IL-10 production by human macrophages. Only the laboratory strain Newman was capable of any IL-10 production by human macrophages and simultaneously induced TNF- α production by these cells.

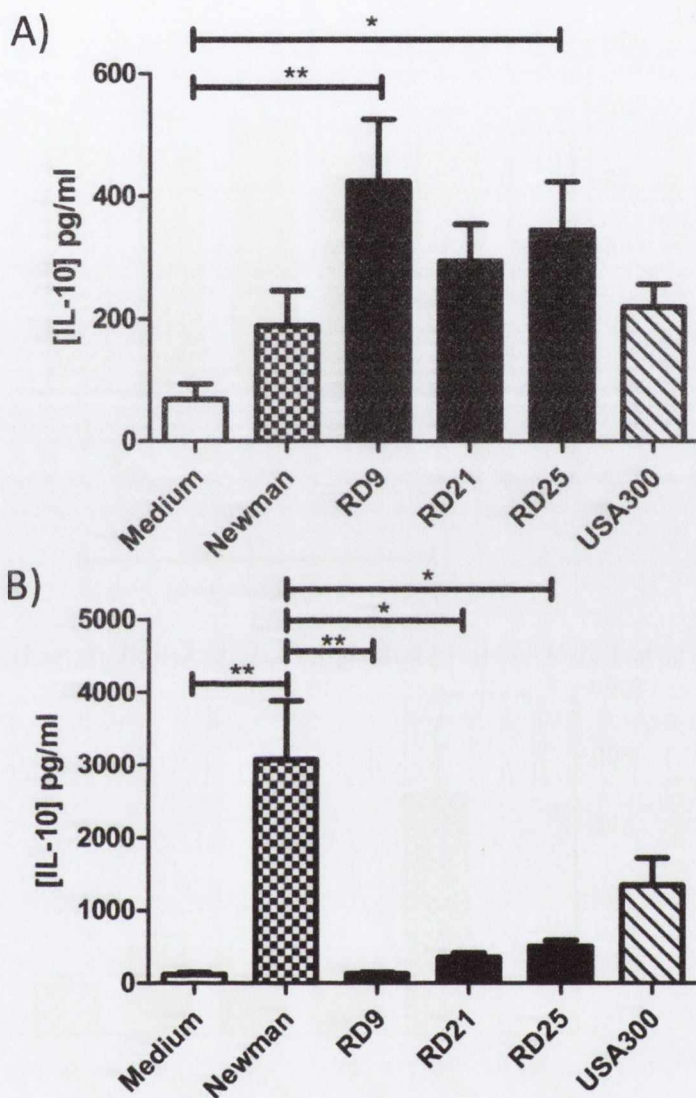


Figure 4.7. Colonising and invasive USA300 demonstrate differential patterns of IL-10 production by BMM.

BMMs were infected with the invasive *S. aureus* strain USA300 or the human persistent colonising strains RD 9, 21, 25 (data same as that shown in Figure 4.5) or Newman at an MOI 10. At 3 **(A)** and 18 **(B)** h post-infection the supernatants were collected. Analysis of IL-10 concentration in the supernatants was performed by ELISA. Data expressed as mean $\pm$ SEM. (n=4). Statistical significance was determined using a one-way ANOVA with Tukey's comparisons test. * $P \leq 0.05$ ** $P \leq 0.005$

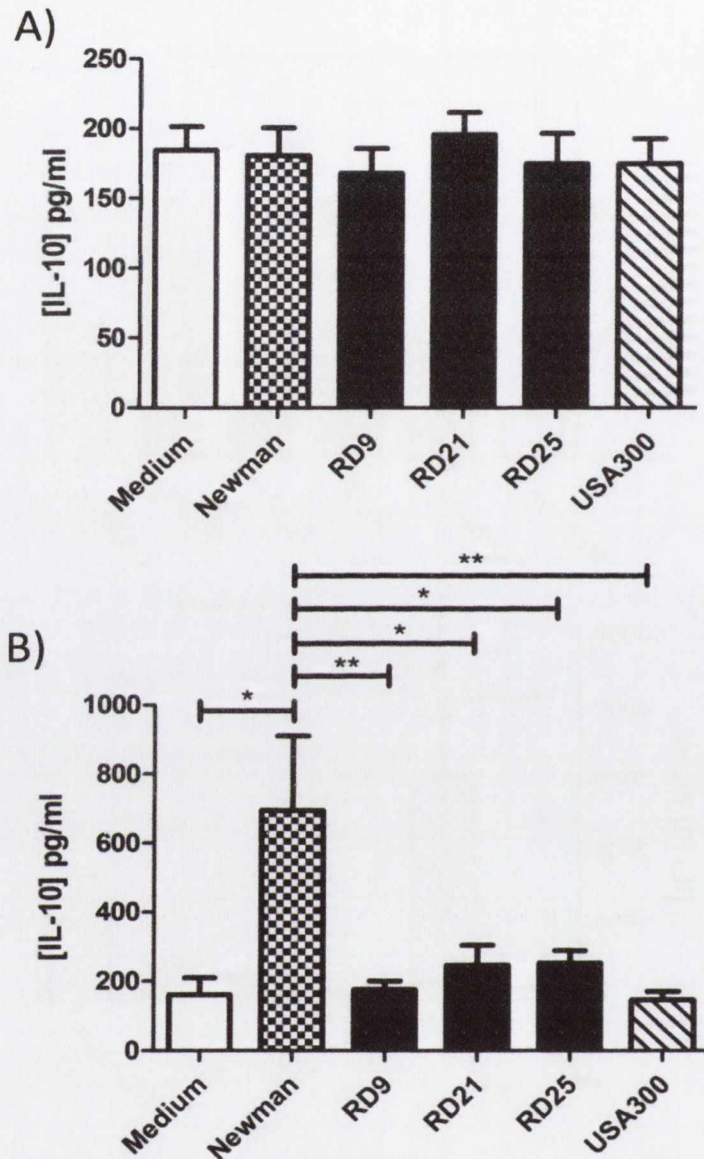


Figure 4.8. Colonising and invasive USA300 do not IL-10 production by human monocyte derived macrophages

Human Monocyte derived macrophages were infected with the invasive *S. aureus* strain USA300 or the human persistent colonising strains RD 9, 21, 25 (data same as shown in Figure 4.6) or Newman at an MOI 10. At 3 **(A)** and 18 **(B)** h post-infection the supernatants were collected. Analysis of IL-10 concentration in the supernatants was performed by ELISA. Data expressed as mean $\pm$ SEM. (n=4). Statistical significance was determined using a one-way ANOVA with Tukey's comparisons test. * $P \leq 0.05$ ** $P \leq 0.005$

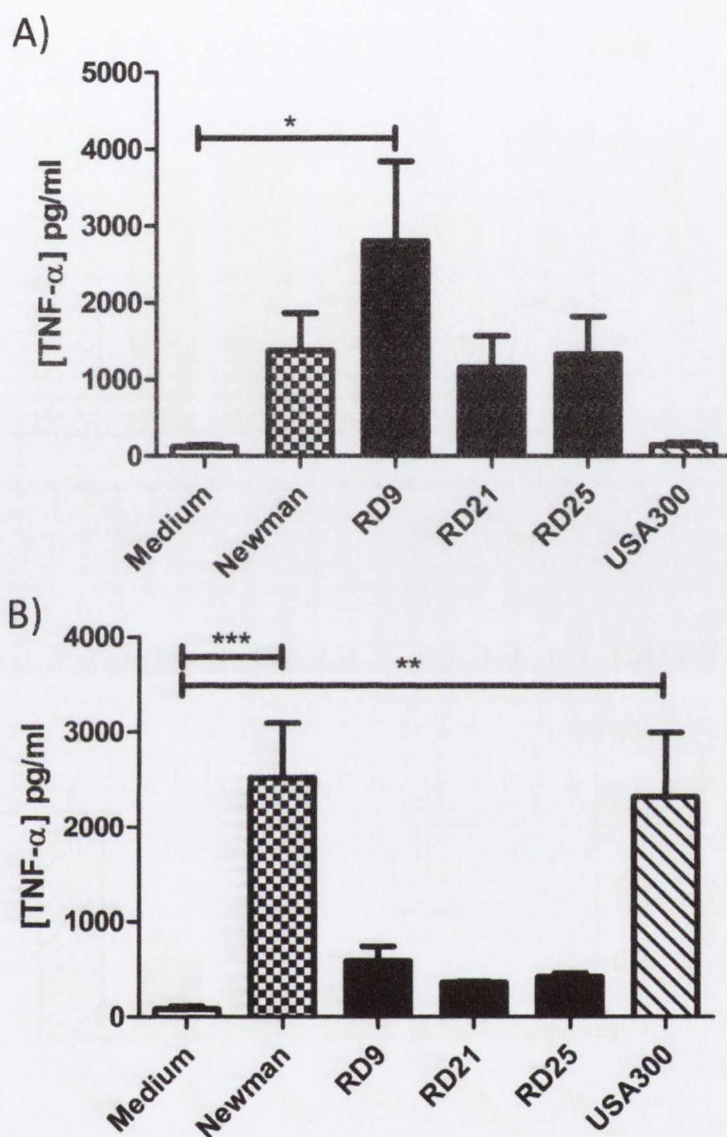


Figure 4.9. Colonising and invasive *S. aureus* strains induce contrasting TNF- α production from BMMs at different time-points.

BMMs were infected with the invasive *S. aureus* strain USA300 or the human persistent colonising strains RD 9, 21, 25 (data same as shown in Figure 4.7) or Newman at an MOI 10. At 3 **(A)** and 18 **(B)** h post-infection the supernatants were collected. Analysis of TNF- α concentration in the supernatants was performed by ELISA. Data expressed as mean $\pm$ SEM. (n=4-5). Statistical significance was determined using a one-way ANOVA with Tukey's comparisons test. * $P \leq 0.05$ ** $P \leq 0.005$

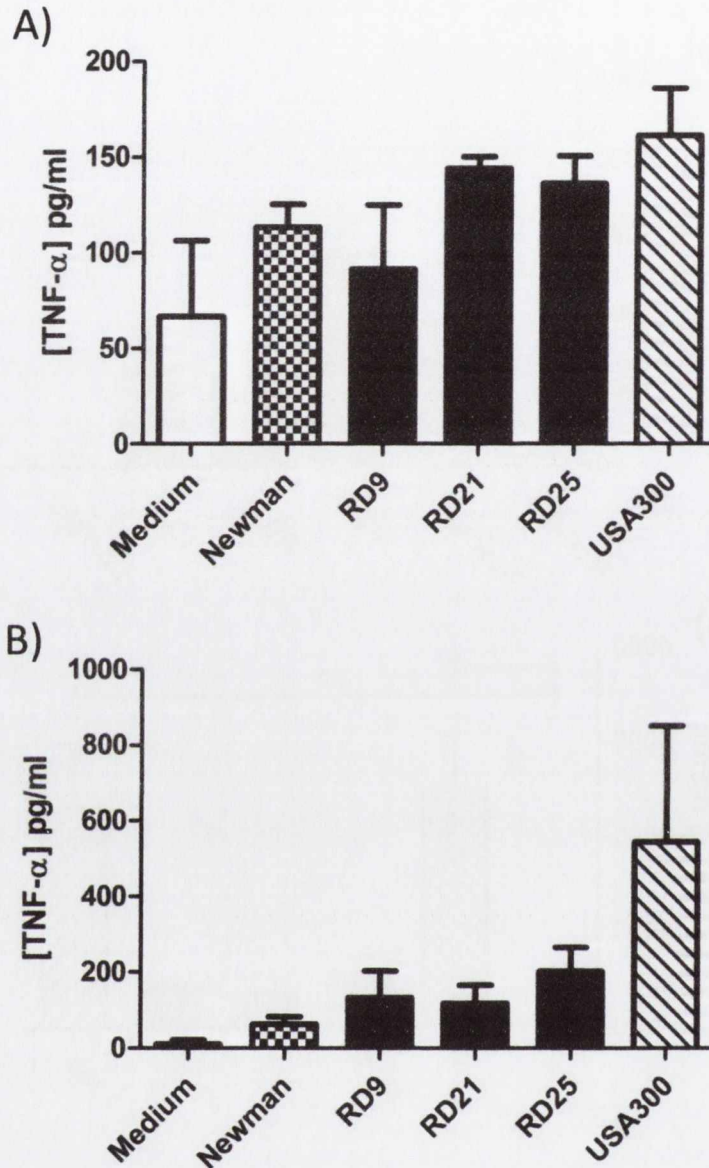


Figure 4.10. Colonising and invasive *S. aureus* strains induce TNF-α production from Monocyte derived macrophages.

Human Monocyte derived macrophages were infected with the invasive *S. aureus* strain USA300 or the human persistent colonising strains RD 9, 21, 25 (data shown in Figure 4.7) or Newman at an MOI 10. At 3 (A) and 18 (B) h post-infection the supernatants were collected. Analysis of TNF-α concentration in the supernatants was performed by ELISA. Data expressed as mean $\pm$ SEM. (n=4). Statistical significance was determined using a one-way ANOVA with Tukey's comparisons test. * $P \leq 0.05$ ** $P \leq 0.005$

4.2.5. *S. aureus* driven IL-10 production regulates TNF- α production from macrophages in an autocrine manner.

As previously demonstrated both colonising and invasive *S. aureus* strains have the ability to induce the simultaneous production of IL-10 and TNF- α from murine macrophages. However the pattern of cytokine induction was distinct between these strains as colonising strains favoured an early induction of both IL-10 and TNF- α , whereas invasive strain USA300 preferentially induced a later induction of these cytokines. Interestingly however, *S. aureus* Newman demonstrated a unique profile of cytokine induction. Newman induced late IL-10 and TNF α production from BMMs. It was also the only *S. aureus* strain to induce late IL-10 production from human macrophages. Therefore, as Newman was shown to be the most robust inducer of IL-10 from BMM and human macrophages, in addition to being the best murine nasal colonising strain in Chapter 3, it was used in subsequent experiments.

It was important to investigate if IL-10 directly regulated the production of TNF- α by macrophages in an autocrine manner. BMMs and human monocyte derived macrophages were cultured with anti-murine IL-10 (20 μ g/ml) and anti-human IL-10 (20 μ g/ml) respectively 1 h prior to infection with *S. aureus* Newman (MOI 10). At 18 h post-infection the supernatants were collected and analysed for TNF- α by ELISA.

Inhibition of IL-10 using the anti-IL-10 antibody resulted in a significant increase in TNF- α production from BMMs (**Figure 4.11**) and a substantial increase in TNF- α from monocyte derived macrophages (**Figure 4.12**) at 18 h post-infection. This data demonstrates that IL-10 acts to directly control the amount of TNF- α produced by macrophages *in vitro*.

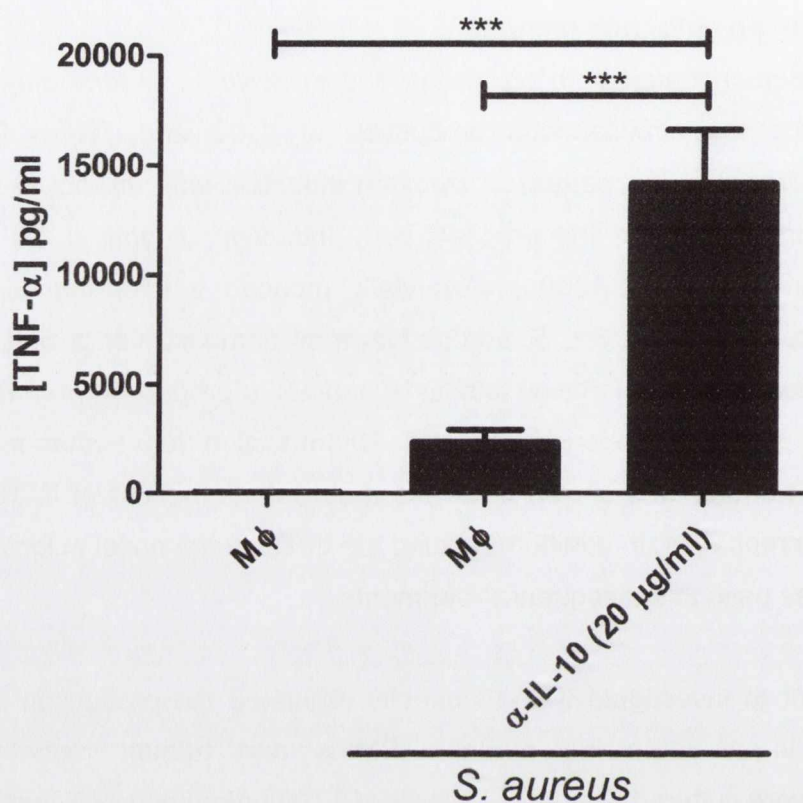


Figure 4.11. IL-10 controls murine macrophage production of TNF-α production following *S. aureus* infection.

BMMs were treated with mouse anti-IL-10 antibody (20 μg/ml) 1 hr prior to infection with *S. aureus* (Newman). At 18 h post-infection the supernatants were collected. Analysis of TNFα concentration in the supernatants was performed by ELISA. Data expressed as mean ± SEM. (n=7). Statistical significance was determined using a one-way ANOVA with Turkey's comparisons test. *** $P \leq 0.001$.

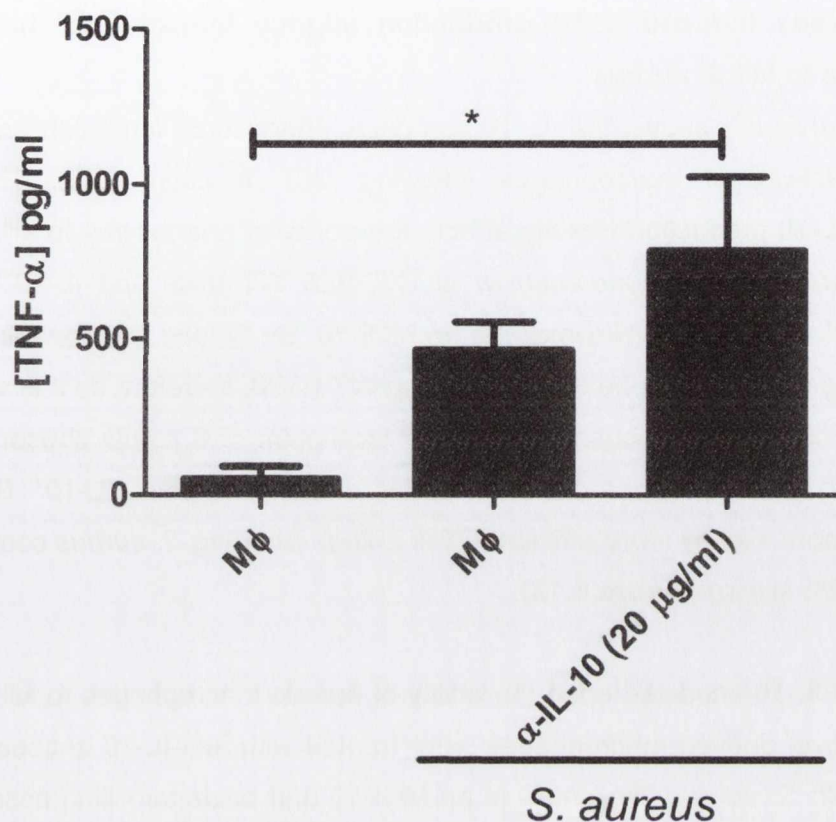


Figure 4.12. IL-10 controls human monocyte derived macrophage production of TNF- α production following *S. aureus* infection.

Human monocyte derived macrophages were treated with human anti-IL-10 antibody (20 μ g/ml), 1 h prior to infection with Newman. At 18 h post-infection the supernatants were collected. Analysis of TNF- α concentration in the supernatants was performed by ELISA. Data expressed as mean $\pm$ SEM. (n=3-4). Statistical significance was determined using a Kruskal-Wallis test with Dunn's Multiple comparisons test, * $P \leq 0.05$.

4.2.6. *S. aureus* induced IL-10 production slightly impacts on the ability of macrophages to kill *S. aureus*.

It has been previously shown that IL-10 can delay phagosome formation and therefore bactericidal activity of macrophages following MtB infection (249). Therefore to determine if IL-10 production directly affects the ability of phagocytes to kill *S. aureus*, BMM were isolated from bone marrow of C57/BL6 WT mice and IL-10^{-/-} mice and infected with live *S. aureus* (Newman) at an MOI 10. By 18 and 24 h post-infection the bacterial killing was found to be 90-100% using WT BMM, therefore as it is very difficult to improve upon this, consequently an earlier time-point of 6 h was chosen to assess bacterial killing in the absence of IL-10. At this earlier time-point, IL-10^{-/-} BMMs were shown to be more slightly more efficient (78% killing) at killing *S. aureus* compared with WT BMMs (62% killing) (**Figure 4.13**).

To establish if IL-10 impacted upon the ability of human macrophages to kill *S. aureus*, human monocyte derived macrophages were treated with anti-IL-10 antibody 1 h prior to infection with *S. aureus* (Newman) at an MOI 10 and bacterial killing assessed after 18 h. In the presence of anti-IL-10 the macrophages demonstrated a small increase in their capacity to kill *S. aureus* (84% killing) when compared with untreated controls (65% killing) (**Figure 4.14**).

Overall this data suggests that IL-10 may have a minor role in delaying or inhibiting macrophage killing of *S. aureus*. An earlier time-point such as 3 h would probably provide a better time-point to examine to initial effect of IL-10 on bacterial killing. Future experiments are required to further investigate the actual mechanism by which IL-10 impedes *S. aureus* killing by macrophages and to establish if this is due to a delay in phagolysosome maturation similar to that observed with MtB.

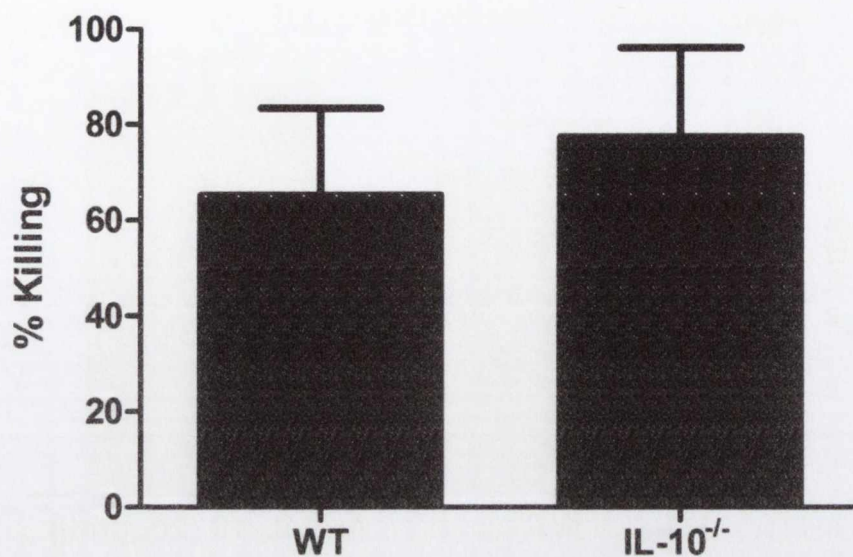


Figure 4.13. *S. aureus*-induced IL-10 did not significantly impact bacterial killing of the bacteria by murine BMMs.

Murine WT and IL-10^{-/-} BMMs were infected with *S. aureus* Newman. At 6 h post-infection killing of *S. aureus* was established by plating serial dilutions of each inoculum on TSA plates at 37°C. Total bacterial killing was determined as the % reduction of CFU in wells containing bacteria and phagocytes as compared with wells containing bacteria only. Data expressed as mean ± SEM (n=4). Statistical significance was determined using a Mann Whitney t-test. NS

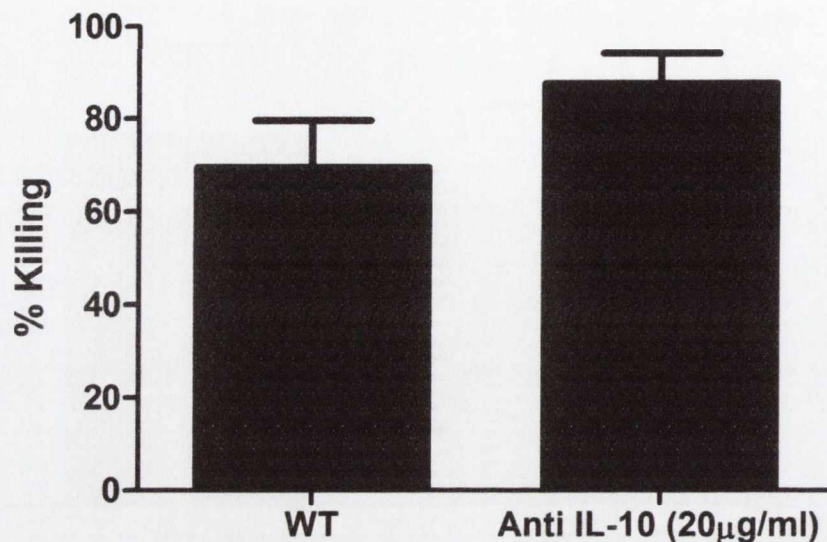


Figure 4.14. *S. aureus* induced IL-10 did not significantly impact bacterial killing of the bacteria by human macrophages.

Human monocyte derived macrophages were treated with anti-IL-10 monoclonal antibody 1 h prior to infection with *S. aureus* strain Newman. At 18 h post-infection killing of *S.aureus* was established by plating serial dilutions of each inoculum on TSA plates at 37°C. Total bacterial killing was determined as the % reduction of CFU in wells containing bacteria and phagocytes as compared with wells containing bacteria only Data expressed as mean ± SEM (n=5). Statistical significance was determined using a Mann Whitney t-test. NS (**Experiment performed by Sinead McKeon as part of her M.Sc. project**)

4.3. Discussion

Macrophages and DCs play crucial roles in coordinating the immune response to *S. aureus* and they are highly influential in establishing the microenvironment that sets up subsequent adaptive responses. This data demonstrates that *S. aureus* live infection induces greater IL-10 production from macrophages compared with DCs *in vitro*. This is in agreement with previous research showing that staphylococcal peptidoglycan (PGN) preferentially induces IL-10 responses in monocyte-derived macrophages (MΦs) as compared with DCs (151). Others have documented a protective role for both macrophages and dendritic cells during *S. aureus* infection in mice. Depletion of macrophages or DCs was associated with increased mortality during *S. aureus* systemic infections as a consequence of uncontrolled inflammation (12, 300) possibly due to a lack of IL-10 production. It has previously been shown that both murine macrophages (12) and DCs can effectively kill *S. aureus in vitro*, however this bacterial killing is highly dependent on the *S. aureus* strain (301).

In agreement with Peres *et al* (152), which showed that *S. aureus* isolates from persistent carriers have been shown to induce production of both TNF- α and IL-10 in human PBMCs, this study also demonstrates that *S. aureus* isolates from persistent carriers were shown to induce the simultaneous production of both TNF- α and IL-10 from murine macrophages. Colonising strains were then compared with a well-known invasive strain USA300 in order to compare the abilities of these contrasting strain types to induce IL-10 and/ or TNF α production from both human and mouse macrophages. Similar to colonising strains, USA300 was also capable of inducing both IL-10 and TNF- α from macrophages but interestingly this response was more delayed. Colonising strains produced greater concentrations of IL-10 and TNF- α at 3 h post-infection with *S. aureus* stimulation as compared with the invasive strain, while after 18 h the invasive *S. aureus* USA300, although capable of IL-10 production, appeared to favour TNF- α production. *S. aureus* Newman which is a well-known lab strain was also examined, as during Chapter 3 it emerged as the best coloniser of mice. Interestingly, Newman was the only strain capable of inducing IL-10 production by both murine and human

macrophages, albeit at the later time point. In contrast to the invasive strain USA300, Newman was not a potent inducer of TNF- α in human cells.

The early induction of IL-10 by colonising *S. aureus* strains suggests that these strains would be very successful nasal colonisers. As shown in chapter 3 however this is not the case, as Newman was the best coloniser *in vivo*. This is likely due to the robust capacity of this strain to induce IL-10 production by macrophages. The preferential induction of pro-inflammatory TNF- α by the invasive strain USA300 likely adds to its pathogenicity *in vivo*.

As this data has shown, murine macrophages can simultaneously induce both IL-10 and TNF α following stimulation with the colonising, invasive and Newman strains (**Section 4.2.3.**). Peres *et al* has previously shown that this ability of *S. aureus* to induce both pro- and anti-inflammatory responses from PBMC was very much dependent on TLR2 signaling. As of yet unidentified *S. aureus* TLR2 ligands in the *S. aureus* cell wall, are capable of inducing either pro- or anti-inflammatory signaling pathways in macrophages through their ability to activate unique downstream signaling pathways. Selective signaling through phosphoinositol 3-kinase (PI3K)-Akt-mTOR and extracellular signal-regulated kinase (ERK) was required for the anti-inflammatory response, while signaling through p38 was required for the pro-inflammatory response (152). Whether, this mechanism of signaling is uniform for all cell types upon engagement with *S. aureus* is unknown.

In chapter 3, *S. aureus* was shown to induce to the production of IL-10 locally within the nose which facilitated bacterial persistence at that site. As previously stated, macrophages have been suggested to be the dominant cell type in the nose. This chapter has demonstrated that murine macrophages stimulated with *S. aureus* can simultaneously induce both IL-10 and TNF- α production, therefore it was important to examine if IL-10 production was having an autocrine effect on the production of TNF α by macrophages stimulated with live *S. aureus*. Inhibition of IL-10 production by treatment with an anti-IL-10 antibody resulted in increased production of pro-

inflammatory TNF- α production in both human and mouse macrophages at 18 h post-infection. It has previously been demonstrated *in vitro*, that blocking IL-10 production by LPS activated macrophages results in increased TNF- α production by those macrophages (302). To conclude, this data demonstrates that IL-10 can have a direct role on macrophages, by inhibiting their production of TNF- α , this suggests that IL-10 could act locally on macrophages within the nose to dampen down pro-inflammatory responses and thereby facilitate bacterial persistence as demonstrated in Chapter 3. As shown during nasal colonisation, IL-10 functions to dampen down CD4⁺IL-17⁺ T-cell responses during nasal colonisation, IL-12 and IL-23 were not detected in supernatants from *S. aureus* stimulated BMMs, however production of these cytokines has been previously shown to be pre-dominantly from DCs. However, TNF- α release alone from macrophages, could lead to increased recruitment and activation of other inflammatory cells (303) *in vivo* which would result in increased bacterial clearance from the nose during nasal colonisation as observed in Chapter 3.

IL-10 has been shown capable of facilitating bacteria persistence during an Mtb infection by blocking phagosome maturation (249). However, the role of IL-10 in controlling phagocyte killing of *S. aureus* killing has not been explored. Consequently, this study investigated the direct effects of IL-10 on the ability of phagocytes to kill *S. aureus*. Inhibition of IL-10 in BMM and human monocyte derived macrophages resulted in a small but appreciable (~20%) increase in the ability of these cells to kill *S. aureus*. This data would suggest that IL-10 may have the capacity to directly interfere with *S. aureus* killing by macrophages. However a mechanism behind this effect was not defined. It is possible that IL-10 promotes intracellular survival of *S. aureus* within macrophages. It has previously been shown that *S. aureus* strains with high levels of Agr activity are capable engaging autophagosomes within phagocytes in order to facilitate intracellular survival (301), consequently it would be necessary to confirm using gentamicin killing assays if treatment with anti-IL-10 resulted in an accumulation of *S. aureus* intracellularly within the macrophage. The role of IL-10 in autophagy development is unclear as some studies show that IL-10 inhibits autophagy (304), while more recent literature suggests that it may promote it (305). Therefore, as *S. aureus* can

induce IL-10 production and if IL-10 promotes autophagy, this would help provide a mechanism for the increased bacterial killing in the absence of IL-10. As previously demonstrated, *S. aureus* manipulates autophagy as a mechanism to facilitate its intracellular survival (249). Mtb induced IL-10 has been shown capable of arresting the progression of phagolysosome maturation after 3 h in a STAT3 dependent manner, which can facilitate the growth and persistence of the bacteria for an extended period within the macrophage (249), therefore it would be important to examine killing of *S. aureus* at earlier time-points and to establish if IL-10 was inhibiting phagolysosome maturation in macrophages following phagocytosis of *S. aureus*.

In summary, this chapter expands on previous research by examining the dynamics of pro- and anti- inflammatory cytokine responses induced by contrasting *S. aureus* strains. It validates previous data demonstrating that colonising *S. aureus* strains from persistent carriers have the ability to induce both IL-10 and TNF- α from macrophages. In addition, it reveals a distinction between colonising strains and the invasive strain USA300 which was identified as a potent activator of TNF- α . Ultimately, it appears that each *S. aureus* strain type is unique and expresses a multitude of ligands on their cell surface that can engage the host cells to drive immune responses that suit itself in its own environment. In addition the mechanisms by which *S. aureus* induced IL-10 exert its immunosuppressive effects were revealed to be two-fold. Induction of IL-10 was identified as a mechanism used by *S. aureus* of dampening down pro-inflammatory cytokine production by macrophages in an autocrine manner, while IL-10 was also shown to directly interfere with the bacterial killing capacity of macrophages. These alternative effects of IL-10 on macrophages provide additional mechanisms by which IL-10 can directly dampen pro-inflammatory responses and evade bacterial killing, which all results in an ability to persist *in vivo* as shown in Chapter 3 during nasal colonisation.

Chapter 5.

5. Interleukin-10 plays opposing roles during acute *S. aureus* systemic and localised infections

5.1 Introduction

Immune regulation plays a critical role in protecting the host from the pathology associated with bacterial infection. IL-10 is a prototypic immuno-regulatory cytokine that is necessary for controlling the production of pro-inflammatory cytokines both *in vitro* and *in vivo* (138, 306-308). Systemic infection with *E. coli* (309) *T. gondii* (310), *T. cruzi* (311) and *P. chabaudi* (312) in IL-10-deficient mice, resulted in excessive production of pro-inflammatory cytokines including TNF- α and IFN- γ , which contributed to increased host immunopathology, morbidity and mortality. Neutralisation of TNF- α or IFN- γ in these mice attenuated organ damage and reduced mortality (309, 312).

Although immune regulation is critical for protection of the host, certain bacteria can manipulate these immunosuppressive mechanisms in order to facilitate persistence during infection. Phagocytosis of *M. tuberculosis* by macrophages results in IL-10 production which in turn has the capacity to block phagosome maturation in a STAT3-dependent manner promoting intracellular bacterial survival (249), in addition to inhibiting a Th1 response, thereby facilitating its persistence in the lung (313). Similarly, *B. pertussis* can promote IL-10 production by both macrophages and dendritic cells through expression of the virulence factors filamentous hemagglutinin (FHA) and adenylate cyclase toxin (ACT). This IL-10 then inhibits IL-12p70 production by DCs, and these DCs direct naive T-cells towards a regulatory phenotype (245-247). CD25⁻Foxp3⁺ T-regulatory (T-reg) cells were expanded in *B. pertussis*-infected mice and these cells work with IL-10-secreting T-reg-cells in the lungs to subvert bacterial clearance by down regulating Th1 responses (314). Following intra-nasal challenge with *S. pneumoniae*, induction of the anti-inflammatory cytokine TGF- β was found to promote local T-reg expansion, thereby facilitating bacterial survival within the nasopharyngeal tissue.

Immune-regulation therefore functions as a "double-edged sword"; on the one hand protecting the host against potentially fatal excessive pro-inflammatory responses, while also facilitating bacterial persistence by dampening down local pro-inflammatory responses. *S. aureus* has been shown to manipulate host immuno-regulatory mechanisms to facilitate persistence during biofilm and chronic systemic infections and

IL-10 appears to be at the forefront of this evasion strategy (254). In a systemic model of chronic *S. aureus* bloodstream infection, kidney abscesses were still apparent on day 56 post-infection due to impaired T-cell responses (255), subsequently this immunosuppression was attributed to the expansion of myeloid derived suppressor cells (MDSCs) and to a lesser extent T-regs in the infected mice compared with uninfected controls (256). Although both IL-10 and TGF- β were produced, immunosuppression was demonstrated to be primarily dependent upon cell-cell contact which inhibited effector T-cell responses (256). Adoptive transfer of MDSCs exacerbated systemic infection in recipient mice following *S. aureus* intra-venous challenge (256). Similarly, during chronic *S. aureus* orthopaedic biofilm infection, Heim *et al.* observed an expansion of IL-10-producing MDSCs at the site of infection, which was associated with bacterial persistence (257, 258). Targeted depletion of these cells resulted in an enhanced pro-inflammatory response that resulted in increased bacterial clearance (258, 259).

It appears therefore, that during chronic infection, *S. aureus* can manipulate host immune-regulatory machinery to facilitate its own survival. However, depending on the local micro-environment the mechanisms of immuno-suppression exerted by regulatory cells may differ. This current study investigated the importance of IL-10 during acute *S. aureus* infection in the context of both systemic and local infection. The data presented in this chapter aims to prove the hypothesis that the induction of IL-10 at contrasting infection sites, with different cellular micro-environments, can have a major influence on disease outcome for both the host and the bacteria.

5.2. Results

5.2.1. IL-10 is rapidly induced both locally and systemically following acute *S. aureus* peritoneal infection.

Using a previously described model of acute systemic infection induced by *S. aureus* peritoneal challenge (69), the production of IL-10 both locally and systemically was investigated. Groups of wild-type (WT) mice received a single intra-peritoneal injection of *S. aureus* (1×10^8 CFU) or sterile PBS as a control. At specific time points post-infection peritoneal fluid and sera were collected and IL-10 concentrations quantified by ELISA. IL-10 was significantly elevated at 3 h post-infection, both locally in the peritoneal cavity (**Figure 5.1A**) and systemically in the blood (**Figure 5.1B**) and had returned to baseline by 24 h post-infection.

In order to determine the source of the IL-10 producing cells, WT (IL-10GFP negative controls) or IL-10 GFP reporter mice were infected with *S. aureus* (1×10^8 CFU) or sterile PBS as a control. At 3 h post-infection, approximately 2.5% of the total peritoneal cells were IL-10 GFP positive as assessed by flow cytometry. Of this IL-10 GFP⁺ population, three distinct subsets of IL-10 producing cells were identified; CD11b^{int}CD19⁺ cells potentially representing regulatory B-cells, CD11b^{high} F480⁺ macrophages and a population of CD11b⁺ Ly6g⁺ Ly6c⁺ MDSC cells (**Figure 5.2A**). Further analysis of the IL-10GFP⁺ CD11b^{int}CD19⁺ population confirmed that these cells were primarily CD5a⁺CD1d⁻ and thus represented B1a regulatory B-cells (B-reg) with a smaller population of IL-10 producing CD5a⁻CD1d⁻ B1b B-cells. Considering the absolute numbers of IL-10 producing cells, it was observed that B1a cells represent the primary source of IL-10 within the peritoneal cavity at this early time point, (**Figure 5.2B**). No IL-10 producing CD4⁺ T-cells were found at this time point. By 12 h the GFP signal within the peritoneal cavity was below the level of detection. IL-10 GFP signal was undetectable in CD45⁺ leukocytes isolated from the blood 3 h post-infection (**Figure 5.3**), likely due to the low concentrations of IL-10 that are present at this site as evidenced by the ELISA data (**Figure 5.1B**).

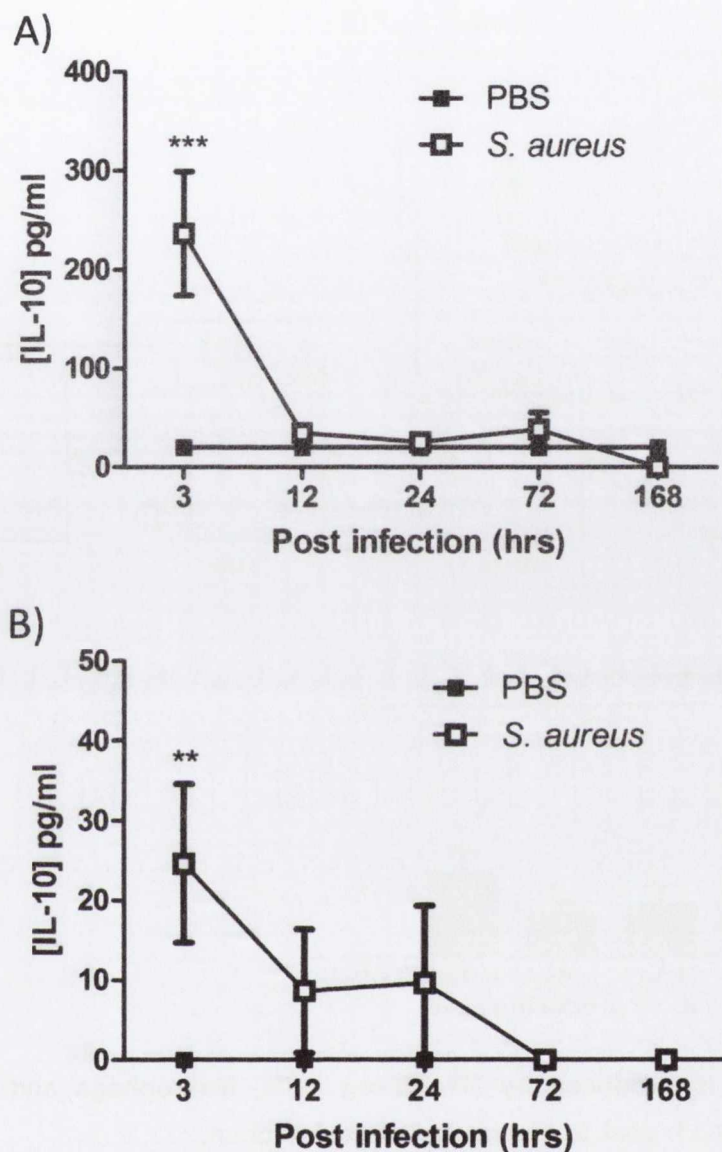


Figure 5.1. *S. aureus* acute peritoneal infection induces local and systemic IL-10 production.

Groups of WT mice received an intra-peritoneal injection of *S. aureus* (Newman, 1×10^8 CFU). At specific time points post-infection the peritoneal cavity was lavaged and blood was collected to isolate sera. IL-10 concentrations in the peritoneal lavage fluid (A) and the sera (B) were measured by ELISA. Results are expressed as mean $\pm$ SEM (n=4-5). Data is representative of 2 individual experiments. Statistical analysis was performed using a two-way ANOVA with Bonferroni post-test. ** $P \leq 0.005$ *** $P \leq 0.001$.

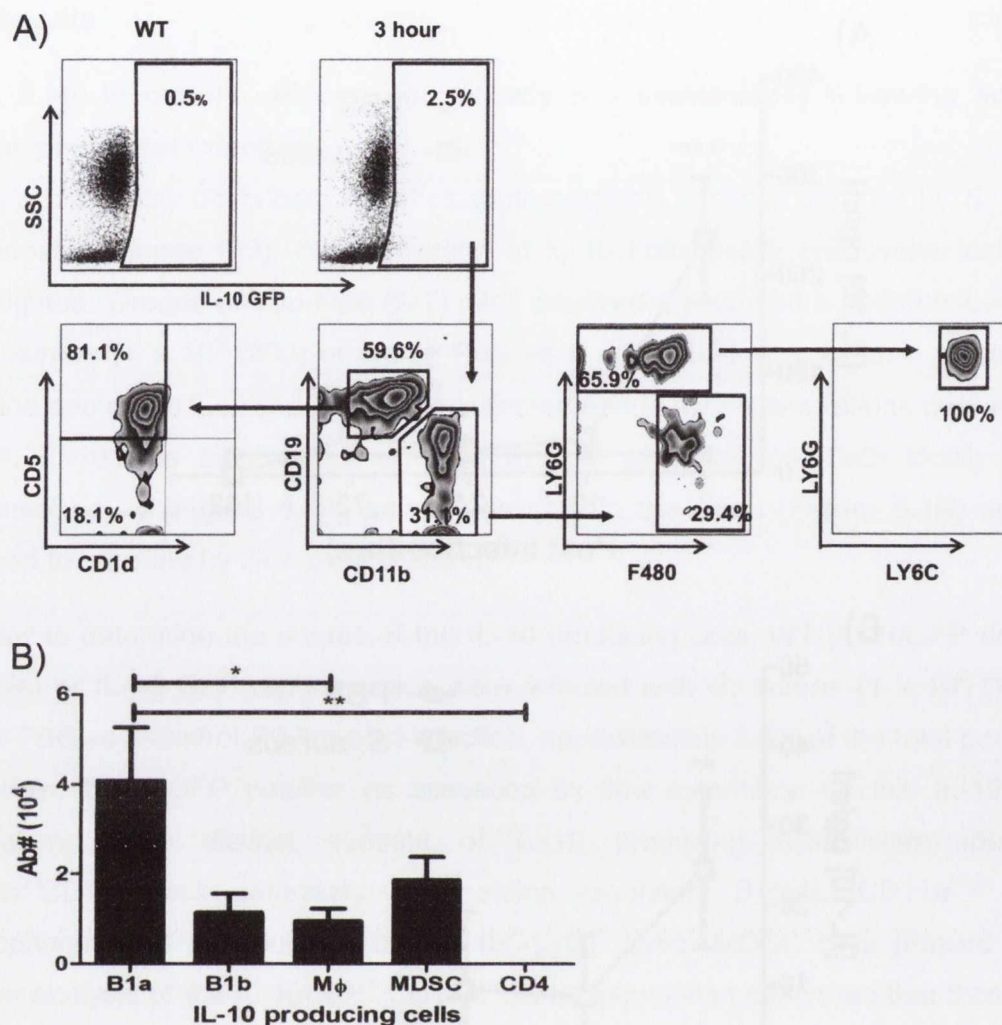


Figure 5.2. IL-10 is produced by B1a B-reg cells, Macrophage and MDSCs in the peritoneal cavity at 3 h post *S. aureus* peritoneal infection.

Groups of WT and IL-10 GFP-reporter mice received an intra-peritoneal injection of *S. aureus* (Newman, 1×10^8 CFU). At 3 h post-infection leukocytes were isolated from the peritoneal cavity for analysis of IL-10 production by individual subsets. The major sources of GFP⁺IL-10 expression were a population of CD11b⁺ cells comprising of CD11b^{int}CD19⁺CD5⁺ B1a cells, CD11b⁺Ly6G⁺Ly6C⁺ and CD11b⁺F480⁺ Macrophages (**A**). Values are expressed as mean absolute numbers of IL-10 producing cells isolated from the peritoneal cavity $\pm$ SEM ($n=4-6$, per group) (**B**) with representative FACS plots shown. Data are pooled from 2 individual experiments. Statistical analysis was performed using a one-way ANOVA with Tukey's post-test. * $P \leq 0.05$ ** $P \leq 0.005$ *** $P \leq 0.001$.

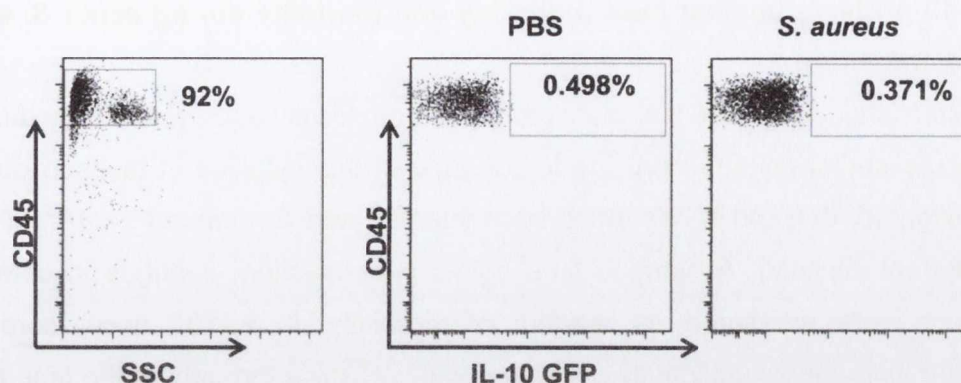


Figure 5.3. IL-10 production was not detected in CD45⁺ leukocytes in the blood 3 h post *S. aureus* peritoneal infection.

Groups of IL-10 GFP-reporter mice received an intra-peritoneal injection of *S. aureus* (Newman, 1×10^8 CFU). At 3 h post-infection leukocytes were isolated from the blood for analysis of IL-10 production by individual subsets. IL-10 production by CD45⁺ subsets was then assessed in the blood of which no GFP signal was found. Data is representative of 2 independent experiments (n=6).

5.2.2. IL-10 protects against host morbidity and mortality during acute *S. aureus* peritoneal infection.

Having established that IL-10 is rapidly produced both locally and systemically during *S. aureus* acute peritoneal infection, the importance of this cytokine in disease outcome was investigated. WT and IL-10^{-/-} mice were infected with *S. aureus* (1 x 10⁸ CFU) via intra-peritoneal injection. At various time points post-infection, changes in core body temperature were measured as readout of morbidity. In IL-10^{-/-} mice, core body temperature increased significantly compared with WT mice throughout the time course of infection (**Figure 5.4A**). Using an alternative invasive strain of *S. aureus* PS80 (301) at a higher challenge inoculum (5 x 10⁸ CFU) increased mortality in the IL-10^{-/-} mice was observed following *S. aureus* intra-peritoneal challenge. By 72 h post-infection there was 70% mortality in the IL-10^{-/-} as compared with 0% mortality in the WT mice (**Figure 5.4B**).

Having established the importance of IL-10 in protecting the host from significant morbidity, the importance of IL-10 for controlling bacterial clearance *in vivo* was examined. Groups of WT and IL-10^{-/-} mice were administered *S. aureus* (1 x 10⁸ CFU) via intra-peritoneal injection and bacterial burden in the blood, peritoneal cavity, kidney, spleen and liver assessed. IL-10^{-/-} mice had higher levels of bacterial burden in the blood at 3 h post-infection (**Figure 5.5A**). *S. aureus* was not found to be present in the blood at any timepoint later than 3 h. By 168 h post-infection WT mice had effectively cleared the infection (≤1 Log CFU remaining at the site of infection and within the organs). However, clearance of infection was impaired in the IL-10^{-/-} mice. At 168 h, there was slightly higher bacterial burden found locally in the peritoneal cavity (**Figure 5.5B**) in the IL-10^{-/-} mice compared with WT mice. In the peripheral organs IL-10^{-/-} mice displayed elevated bacterial burdens in the kidney (**Figure 5.5C**), spleen (**Figure 5.5D**) and liver (**Figure 5.5E**) at 72 and 168 h days post-infection.

Taken together this data concludes that IL-10 plays an overall protective role during *S. aureus* acute systemic peritonitis, preventing bacterial dissemination to peripheral organs and associated morbidity and mortality.

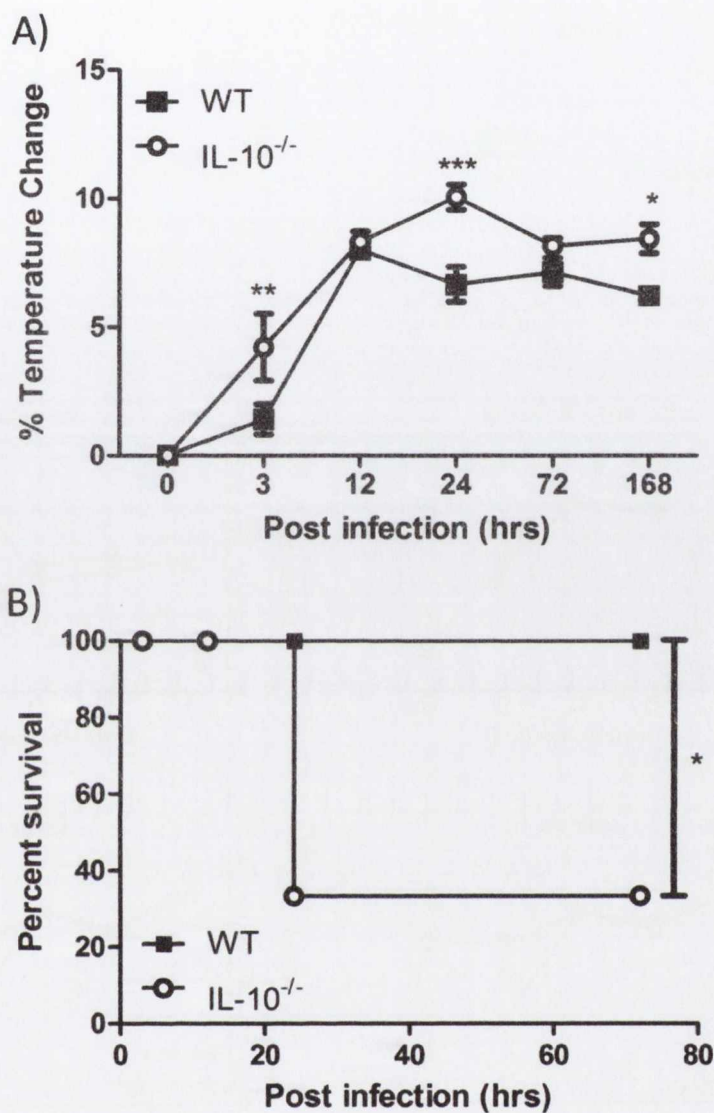


Figure 5.4. IL-10^{-/-} mice show increased morbidity and mortality during acute *S. aureus* peritoneal infection.

Groups of WT and IL-10^{-/-} mice received an intra-peritoneal injection of *S. aureus* (Newman, 1 x 10⁸ CFU). Rectal body temperatures were recorded immediately before infection and at specific time-point throughout the course of the infection (**A**). Statistical analysis was performed using two-way ANOVA. Groups of WT and IL-10^{-/-} mice received an intra-peritoneal injection of *S. aureus* (PS80, 5 x 10⁸ CFU) which resulted in significant mortality of IL-10 mice by 24 h post-infection (**B**). Results are expressed as mean±SEM (n=4-5) Statistical analysis was performed using a Kruskal-Wallis test with Mantel-Cox test. *P≤0.05 **P≤0.005 ***P≤0.001.

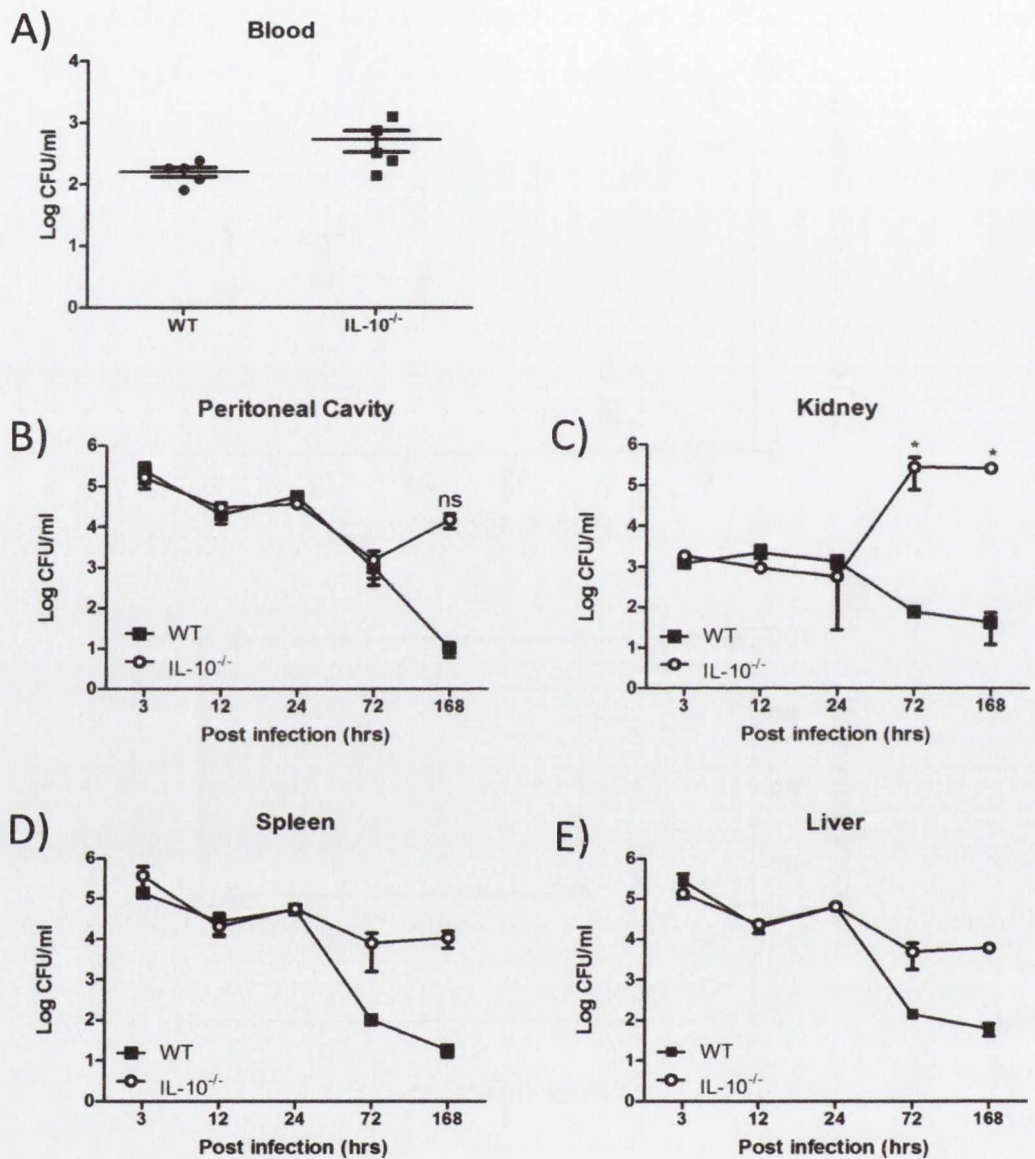


Figure 5.5. IL-10^{-/-} mice demonstrate increased systemic dissemination of bacteria during *S. aureus* induced acute peritoneal infection.

Groups of WT and IL-10^{-/-} mice received an intra-peritoneal injection of *S. aureus* (Newman, 1 x 10⁸ CFU). Bacterial burden in the blood was assessed at 3 h post-infection (**A**). At the indicated time points post challenge, the bacterial burden in the Peritoneal Cavity (**B**), Kidneys (**C**), Spleen (**D**) and Liver (**E**) were assessed. Results are expressed as mean±SEM Log CFU/ml (n=4-5). Data is representative of 2 individual experiments. Statistical analysis was performed using a two-way ANOVA with Bonferroni post-test. *P≤0.05

5.2.3. IL-10 is critical for controlling the production of inflammatory cytokines during acute *S. aureus* peritoneal infection.

IL-10 has previously been shown to be critical for constraining excessive inflammatory responses during infection with other organisms (309), therefore the requirement for IL-10 in controlling both local and systemic immune responses to *S. aureus* during acute peritonitis was examined. WT and IL-10^{-/-} mice were challenged with *S. aureus* (1 x 10⁸ CFU) via intra-peritoneal injection and local and systemic cytokine and chemokine concentrations assessed in the peritoneal lavage fluid and sera over time. In the absence of IL-10 pro-inflammatory cytokine concentrations were significantly elevated as compared with WT mice.

Locally within the peritoneal cavity, significantly elevated concentrations of the pro-inflammatory cytokines IL-1 β (**Figure 5.6A**), TNF- α (**Figure 5.6B**), IL-17 (**Figure 5.6C**), IFN- γ (**Figure 5.6D**), IL-22 (**Figure 5.6E**) and the neutrophil chemo-attractant CXCL1 (**Figure 5.6F**) were detected in the IL-10^{-/-} mice as compared with the WT mice.

Similarly, significantly higher concentrations of pro-inflammatory cytokines IL-17 (**Figure 5.7A**), IFN- γ (**Figure 5.7B**) and chemokine CXCL1 (**Figure 5.7C**) were also observed in the sera of IL-10^{-/-} mice as compared with WT mice.

This data clearly indicates that IL-10 acts to constrain excessive pro-inflammatory cytokine production during *S. aureus* acute systemic peritonitis.

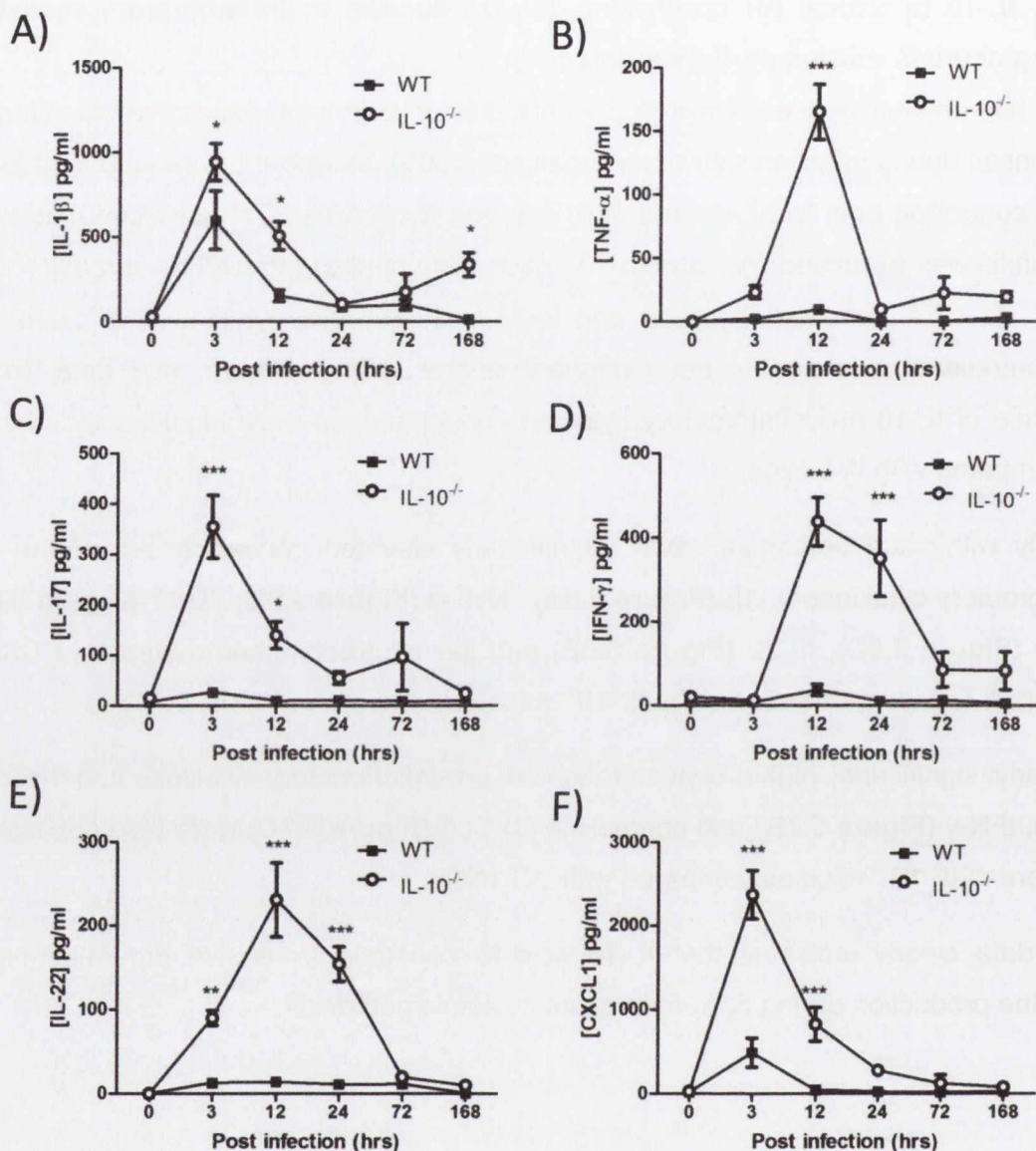


Figure 5.6. IL-10 deficiency results in excessive pro-inflammatory cytokine production locally in response to *S. aureus* induced acute peritoneal infection.

Groups of WT and IL-10^{-/-} mice received an intra-peritoneal injection of *S. aureus* (Newman, 1 x 10⁸ CFU). At the indicated time points post challenge, the peritoneal cavity was lavaged and the concentrations of IL-1β (A), TNF-α (B), IL-17 (C), IFN-γ (D), IL-22 (E), and CXCL1 (F) were measured by ELISA. Results are expressed as mean±SEM (n=4-5). Data is representative of 2 individual experiments. Statistical analysis was performed using two-way ANOVA with Bonferroni post-test. *P≤0.05 **P≤0.005 ***P≤0.001

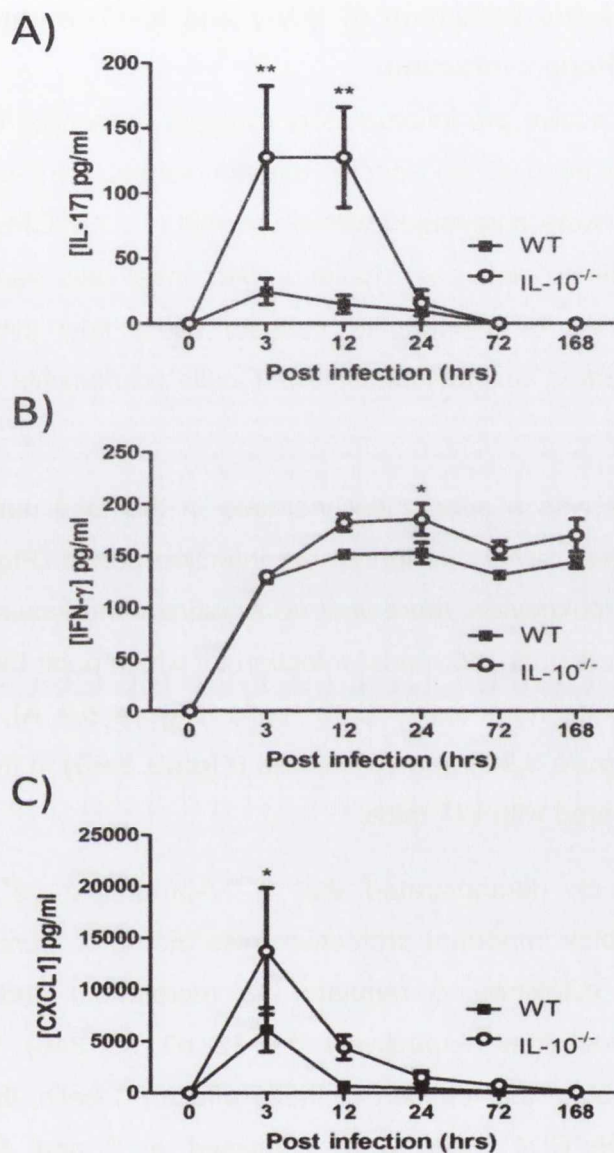


Figure 5.7. IL-10 deficiency results in excessive pro-inflammatory cytokine production systemically in response to *S. aureus* induced acute peritoneal infection.

Groups of WT and IL-10^{-/-} mice received an intra-peritoneal injection of *S. aureus* (Newman, 1 x 10⁸ CFU). At the indicated time points post challenge, the sera was isolated from the blood and the concentrations of IL-17 (A), IFN-γ (B), and CXCL1 (C) were measured by ELISA. Results are expressed as mean±SEM (n=4-5). Data is representative of 2 individual experiments. Statistical analysis was performed using two-way ANOVA with Bonferroni post-test. *P≤0.05
**P≤0.005

5.2.4. IL-10 controls the activation of IFN- γ and IL-17-producing T-cells during acute *S. aureus* peritoneal infection.

Having observed excessive pro-inflammatory cytokine production in the absence of IL-10, further characterisation of the specific effector subsets of T-cells was carried out. WT and IL-10^{-/-} mice were challenged with *S. aureus* (1 x 10⁸ CFU) via intra-peritoneal injection. At specific time points post challenge peritoneal 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 IFN- γ and IL-17 production.

In IL-10^{-/-} mice there was a substantial increase in the total numbers of leukocytes infiltrating the peritoneal cavity throughout the infection period (**Figure 5.8**). Examining different subtypes of leukocytes, there was no significant increases in the expansion of individual T-cell subsets until 168 h post-infection by which point there was a significant increase in the absolute numbers of CD3⁺ cells (**Figure 5.9 A**), CD4⁺ cells (**Figure 5.9B**), CD8⁺ cells (**Figure 5.9C**) and $\gamma\delta$ ⁺ T-cells (**Figure 5.9D**) in the peritoneal cavities of IL-10^{-/-} mice, compared with WT mice.

It has previously been demonstrated that IL-17-producing $\gamma\delta$ ⁺ T-cells and IFN- γ producing Th1 cells play important protective roles during *S. aureus* infection through the ability of these cytokines to regulate the recruitment and activation of both neutrophils and macrophages respectively (11, 12, 63, 69, 315). To ascertain if IL-10 acts upstream to regulate the function of these effector T-cells, the proportions of IL-17⁺ $\gamma\delta$ ⁺TCR⁺ and IFN γ ⁺CD4⁺ cells were assessed at 3 and 168 h post-infection respectively. It has previously been shown that IL-17-producing $\gamma\delta$ ⁺ T-cells peak at 3 h post-infection in WT mice (69). There was a significant increase in IL-17-producing $\gamma\delta$ ⁺ T-cells at this time point in the IL-10^{-/-} mice as compared with the WT mice (**Figure 5.10A**). As CD4⁺ T-cells were significantly expanded at 168 h post-infection, this time-point was chosen to examine their production of IFN- γ . The proportion of IFN- γ producing CD4⁺ T-cells (**Figure 5.10B**) was significantly increased at 168 h following *S. aureus* infection in IL-10^{-/-} mice as compared with WT mice.

Taken together, these data demonstrate an overall increase in the recruitment and expansion of total leukocytes in the peritoneal cavity of IL-10^{-/-} mice during the course of *S. aureus* infection compared with WT mice. More specifically, this data demonstrates that in the absence of IL-10, IL-17 production by $\gamma\delta^+$ T-cells is exacerbated initially during infection. Additionally, IFN- γ producing CD4⁺ T-cells, generally only significantly detected in the context of a memory response are prominent in the absence of IL-10 (4).

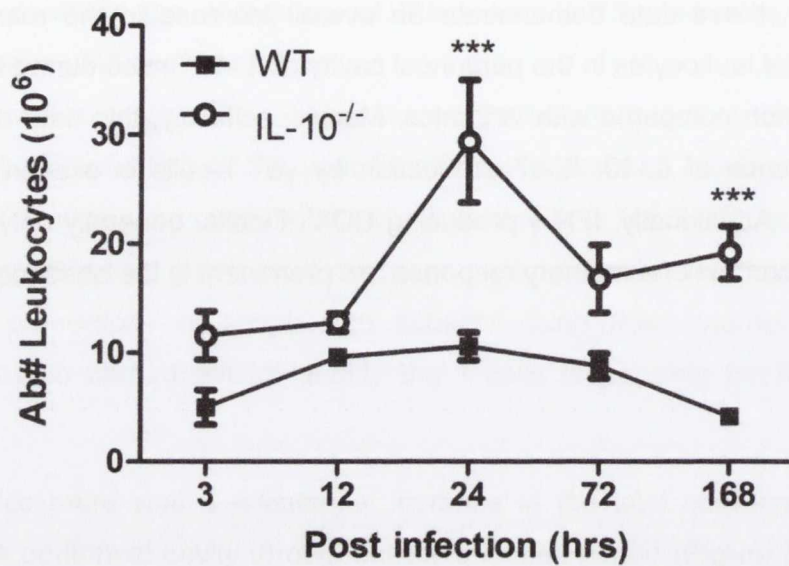


Figure 5.8. IL-10 deficiency results in the increased infiltration of leukocytes into the peritoneal cavity compared with WT mice in response to *S. aureus* induced acute peritoneal infection.

Groups of WT and IL-10^{-/-} mice received an intra-peritoneal injection of *S. aureus* (Newman, 1 x 10⁸ CFU). At indicated time points post challenge total leukocytes were evaluated in the peritoneal cavity. Results expressed as mean±SEM (n=5 per group). Data is representative of 2 individual experiments. Statistical analysis was performed using two-way ANOVA with Bonferroni post-test. ***P≤0.001

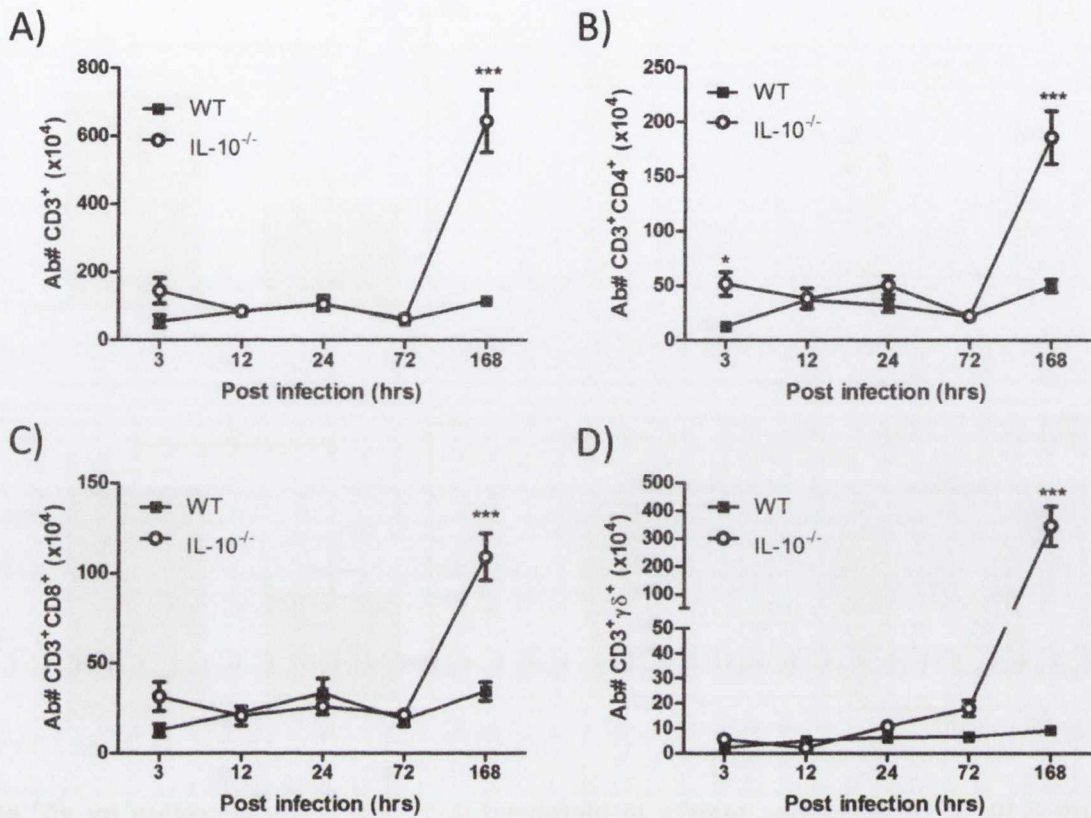


Figure 5.9. IL-10 deficiency results in the significant infiltration of T-cells into the peritoneal cavity compared with WT mice in response to *S. aureus* induced acute peritoneal infection.

Groups of WT and IL-10^{-/-} mice received an intra-peritoneal injection of *S. aureus* (Newman, 1 x 10⁸ CFU). At indicated time points post challenge peritoneal CD3⁺ (A), CD3⁺CD4⁺ (B), CD3⁺CD8⁺ (C) and CD3⁺γδ⁺ (D) T-cells were isolated to assess the proportions and absolute numbers of each cell population using flow cytometry. Results expressed as mean±SEM (n=5 per group). Data is representative of 2 individual experiments. Statistical analysis was performed using two-way ANOVA with Bonferroni post-test. *P≤0.05 **P≤0.005 ***P≤0.001

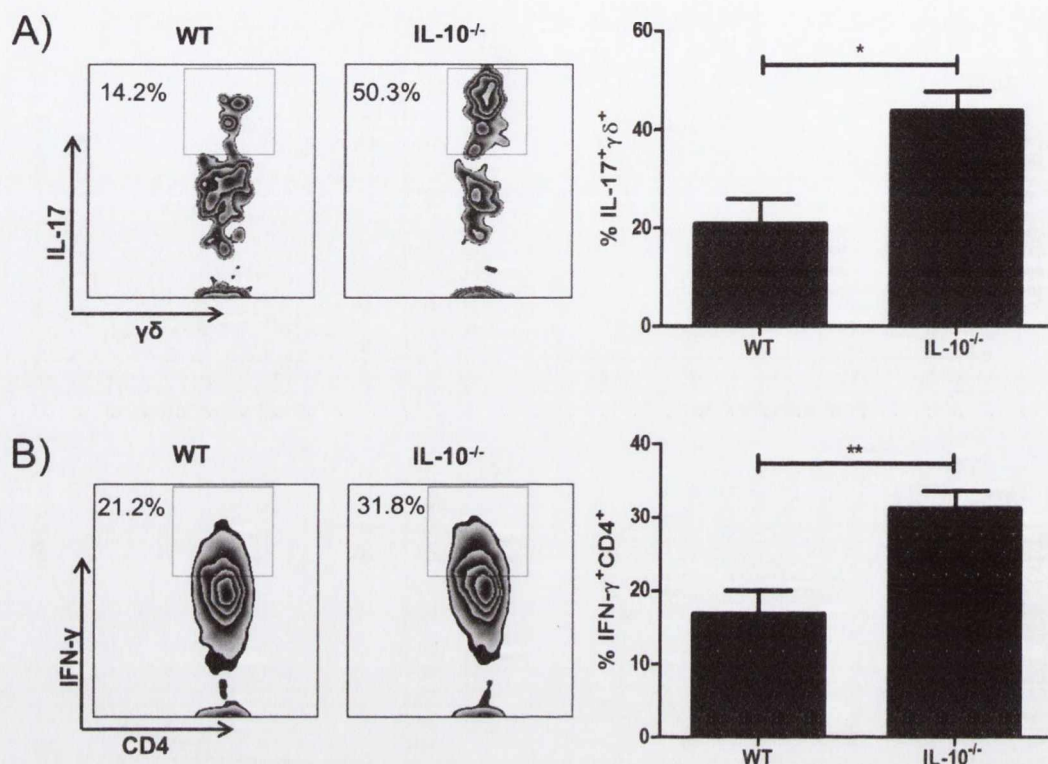


Figure 5.10. IL-10 deficiency results in increased IL-17 and IFN- γ secretion by $\gamma\delta^+$ and CD4 $^+$ T-cells respectively locally in response to *S. aureus* induced acute peritoneal infection.

Groups of WT and IL-10 $^{-/-}$ mice received an intra-peritoneal injection of *S. aureus* (Newman, 1×10^8 CFU). At 3 and 168 h post-infection leukocytes were isolated from the peritoneal cavity for FACS analysis to assess the proportions of IL-17-producing $\gamma\delta^+$ T-cells (**A**) and IFN- γ -producing CD4 $^+$ T-cells (**B**) respectively. Results are expressed as mean $\pm$ SEM (n=4-5) with representative FACS plots shown. Data are representative of 2 individual experiments. Statistical analysis was performed using a Mann-Whitney t-test. * $P \leq 0.05$ ** $P \leq 0.005$

5.2.5. IL-10 controls the recruitment and activation of neutrophils and macrophages locally in the peritoneal cavity during acute *S. aureus* peritoneal infection.

During *S. aureus* infection IFN- γ production by CD4⁺ T-cells has been shown to be important for macrophage recruitment and activation (12), while IL-17 produced primarily by $\gamma\delta$ ⁺ T-cells is required for the recruitment of neutrophils to sites of infection (316, 317). As IL-10 acted upstream of T-cells to regulate the production of these pro-inflammatory cytokines, the impact on phagocytic cell responses were assessed within the peritoneal cavity in WT and IL-10^{-/-} mice. WT and IL-10^{-/-} mice were challenged with *S. aureus* (1×10^8 CFU) via intra-peritoneal injection. At specific time points post challenge peritoneal cells were isolated and stained for phagocyte markers and analysed by flow cytometry (**Figure 5.11**).

There were significantly higher numbers of neutrophils (CD11b⁺F480⁺Ly6G⁺) recruited to the peritoneal cavity at 3 h post-infection following *S. aureus* infection in IL-10^{-/-} mice as compared with WT mice (**Figure 5.12A**). Secreted MPO concentrations which are indicative of local neutrophil activation were assessed by ELISA and were significantly increased at 72 and 168 h post-infection (**Figure 5.12B**). In addition, ROS activity by infiltrating neutrophils as assessed by 123-dihydrorhodamine staining was also significantly elevated at 3 h post-infection in the IL-10^{-/-} mice compared with WT mice (**Figure 5.12C**). At 24 h post-infection macrophage (CD11b⁺F480⁺Ly6G⁻) recruitment was also significantly increased in IL-10^{-/-} mice, compared with WT mice (**Figure 5.13A**). Moreover, these macrophages expressed significantly higher levels of the activation marker nitric oxide synthase 2 (NOS2) at this time point and 72 h post-infection (**Figure 5.13B**). Furthermore, macrophages from IL-10^{-/-} expressed significantly greater levels of MHCII at 12, 72 and 168 h post-infection compared with WT mice (**Figure 5.13C**). Taken together, it appears that in the absence of IL-10, over active $\gamma\delta$ ⁺ T-cell and Th1 responses lead to excessive recruitment and activation of phagocytes. Although these cells are normally required for the clearance of *S. aureus*, in this case they may be contributing to host pathology and subsequent bacterial dissemination.

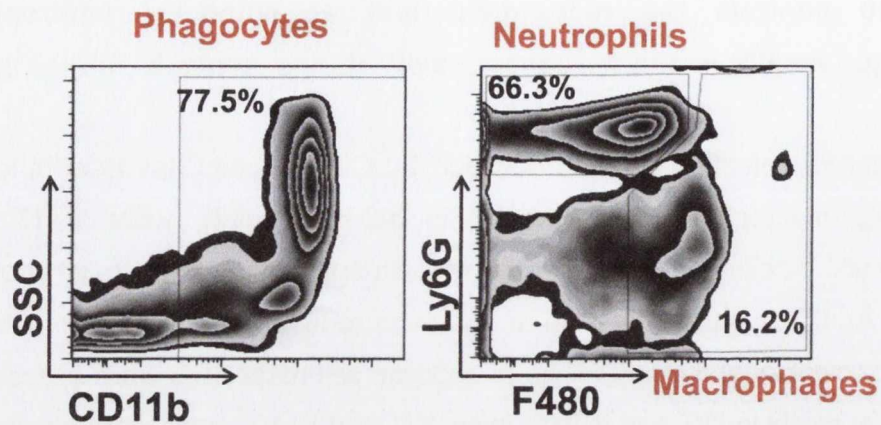


Figure 5.11. Peritoneal cavity phagocyte staining panel during *S. aureus* acute peritoneal infection.

Groups of WT and IL-10^{-/-} mice received an intra-peritoneal injection of *S. aureus* (Newman, 1 x 10⁸ CFU). The peritoneal cavity was lavaged and leukocytes were isolated for FACS analysis. Phagocyte populations were primarily detected by a population of CD11b⁺ cells comprising of CD11b⁺Ly6G⁺ Neutrophils and CD11b⁺F480⁺ Macrophages.

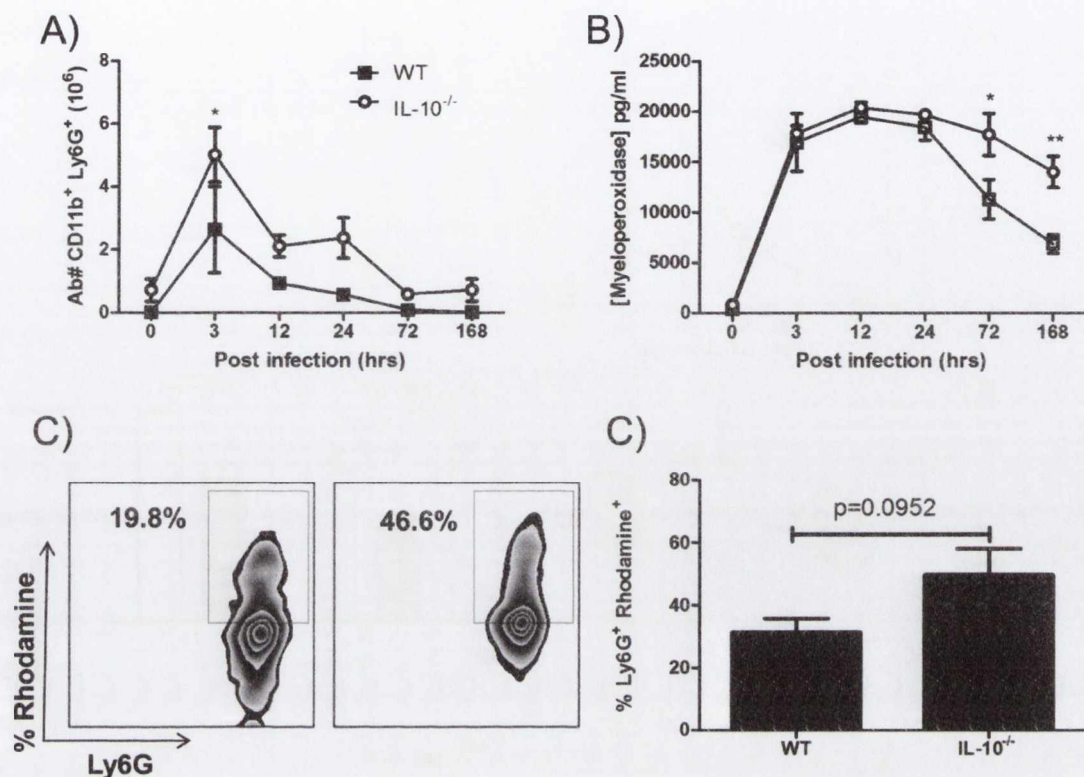


Figure 5.12. IL-10 deficiency results in increased recruitment to and activation of neutrophils locally in the peritoneal cavity in response to *S. aureus* induced acute peritoneal infection.

Groups of WT and IL-10^{-/-} mice received an intra-peritoneal injection of *S. aureus* (Newman, 1 x 10⁸ CFU). At indicated time-points post-infection leukocytes were isolated from the peritoneal cavity for FACS analysis to assess the absolute number of neutrophils (CD11b⁺Ly6G⁺) present (A). Secreted MPO concentrations were assessed by ELISA (B). At 3 h post-infection, neutrophils isolated from the peritoneal cavity were assessed for reactive oxygen species (ROS) activity, detected by rhodamine (Rho) positive cells (C). Results are expressed as mean±SEM (n=4-5). Representative FACS plots are shown for C. Data are representative of 2 individual experiments. Statistical analysis was performed using a Mann-Whitney t-test or two-way ANOVA with Bonferroni post-test. *P≤0.05 **P≤0.005

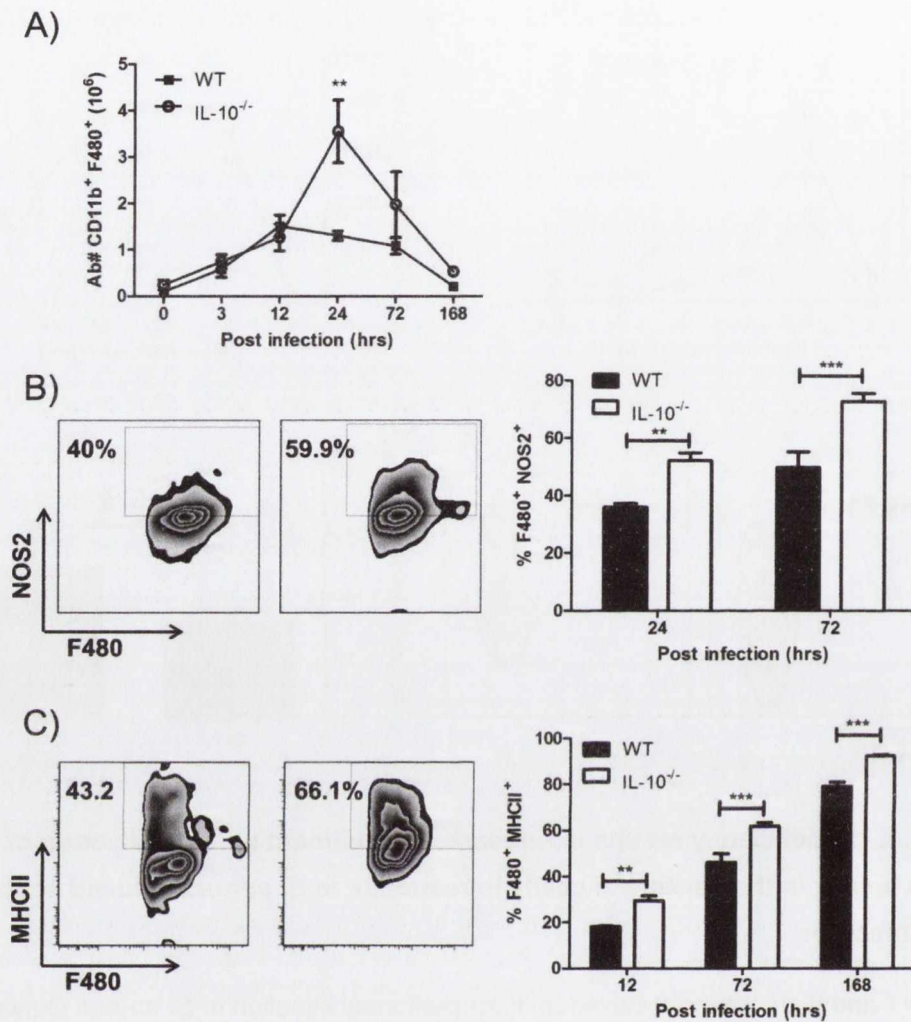


Figure 5.13. IL-10 deficiency results in increased migration and activation of macrophages locally in the peritoneal cavity in response to *S. aureus* induced acute peritoneal infection.

Groups of WT and IL-10^{-/-} mice received an intra-peritoneal injection of *S. aureus* (Newman, 1 x 10⁸ CFU). At indicated time-points post-infection leukocytes were isolated from the peritoneal cavity for FACS analysis to assess the absolute numbers of macrophages (CD11b⁺ F480⁺) present within the peritoneal cavity (**A**). The expression of Nitric oxide synthase 2 (NOS2) (**B**) and MHCII (**C**) in these cells was then assessed. Results are expressed as mean±SEM (n=4-5). A representative FACS plot is shown for B and D for 24 and 72 h respectively. Data is representative of 2 individual experiments. Statistical analysis was performed using a Mann-Whitney t-test or two-way ANOVA with Bonferroni post-test. *P≤0.05 **P≤0.005 ***P≤0.001

5.2.6. The protective role of IL-10 is non-redundant and cannot be compensated for by other immune-regulatory mechanisms.

In order to establish if IL-10 plays a non-redundant protective role during acute *S. aureus* peritonitis, WT and IL-10^{-/-} mice were challenged with *S. aureus* (1 x 10⁸ CFU) via intra-peritoneal injection. At specific time points post challenge the concentrations of TGF-β were measured in peritoneal fluid and peritoneal cells were isolated and stained for T-reg markers and analysed by flow cytometry.

In IL-10^{-/-} mice there was a slight increase in the concentrations of the anti-inflammatory cytokine TGF-β in the peritoneal fluid as compared with WT but this did not reach statistical significance (**Figure 5.14**). The absolute numbers of CD4⁺CD25⁺FoxP3⁺ T-reg cells were significantly higher in IL-10^{-/-} as compared with WT mice at 24 h post-infection (**Figure 5.15A**). In addition, T-reg cells from IL-10^{-/-} mice express significantly greater levels of the suppressive marker PD-1 at 72 and 168 h post-infection compared with WT mice (**Figure 5.15B**). This data demonstrates that although T-reg cells appear to be expanded locally in the peritoneal cavity, presumably as a compensatory mechanism in the absence of IL-10, this is not sufficient to protect the animals from the significant pathology associated with the IL-10 deficiency (see Figure 5.5).

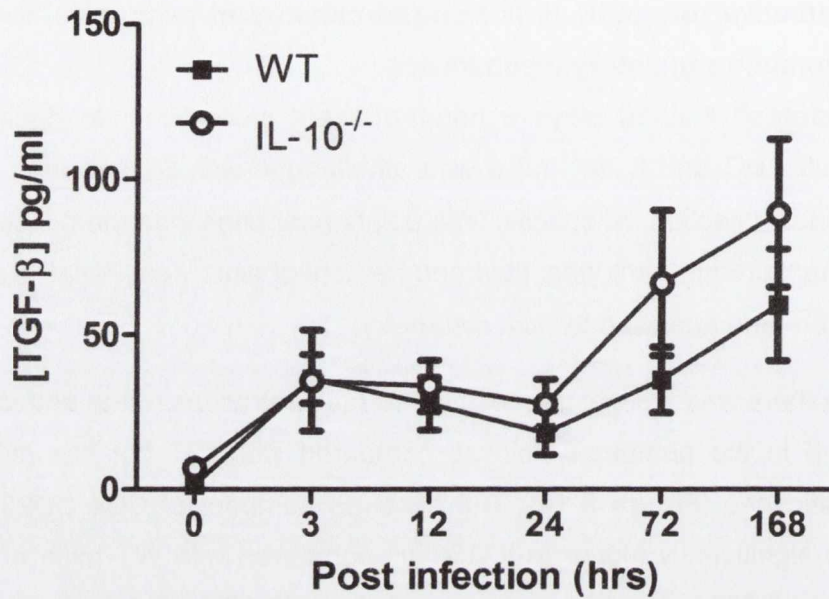


Figure 5.14. IL-10 deficiency results in only slightly increased TGF- β cytokine production locally in the peritoneal cavity in response to *S. aureus* induced acute peritoneal infection.

Groups of WT and IL-10^{-/-} mice received an intra-peritoneal injection of *S. aureus* (Newman, 1 x 10⁸ CFU). At the indicated time points post challenge, concentrations of TGF- β were measured locally in the peritoneal fluid by ELISA. Results are expressed as mean $\pm$ SEM (n=5). Data are representative of 2 individual experiments. Statistical analysis was performed using two-way ANOVA with Bonferroni post-test.

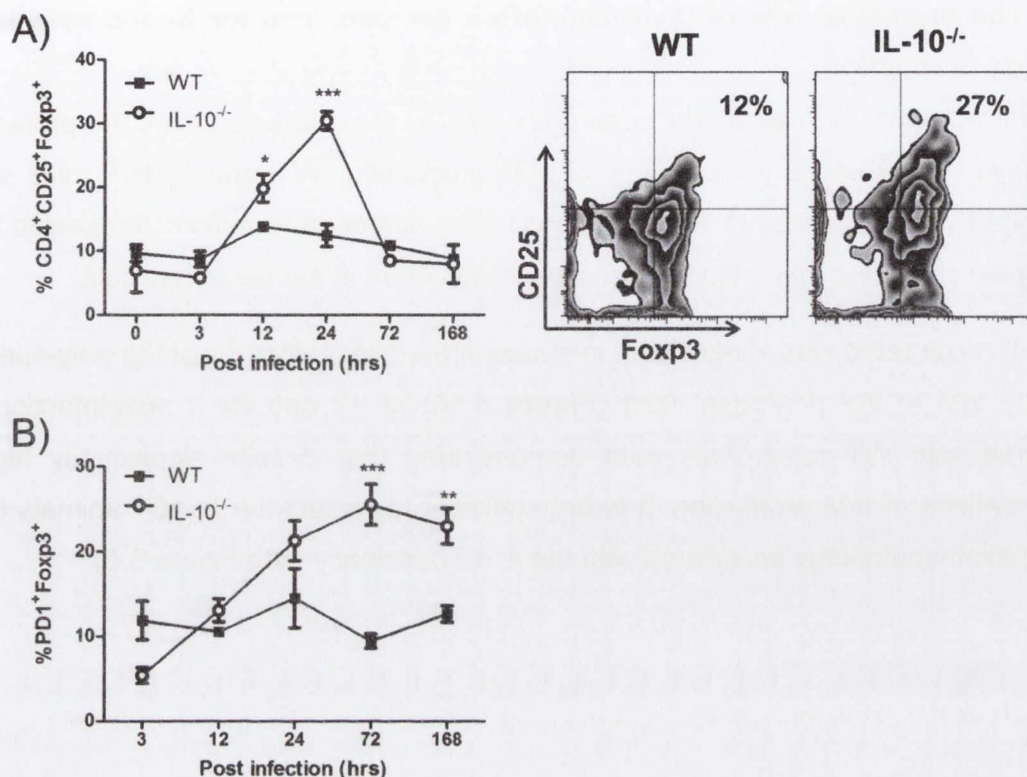


Figure 5.15. IL-10 deficiency results in increased levels of Tregs locally in the peritoneal cavity in response to *S. aureus* induced acute peritoneal infection.

Groups of WT and IL-10^{-/-} mice received an intra-peritoneal injection of *S. aureus* (Newman, 1 x 10⁸ CFU). At indicated time-points post-infection leukocytes were isolated from the peritoneal cavity for FACS analysis to assess the number of T-regulatory cells (CD4⁺CD25⁺Foxp3⁺) (**A**) and their expression of PD1 (**B**). Results are expressed as mean±SEM (n=4-5) with a representative FACS plot shown for T-reg cells at 24 h post-infection. Data are representative of at least 2 individual experiments. Statistical analysis was performed using two-way ANOVA with Bonferroni post-test. **P≤0.005 ***P≤0.001

5.2.7. The protective role of IL-10 cannot be compensated for by increased IgM antibody production.

B1 cell are known for their ability to produce IgM. In order to establish if the protective role played by IL-10 is independent of IgM production, WT and IL-10^{-/-} mice were challenged with *S. aureus* (1 x 10⁸ CFU) via intra-peritoneal injection. At specific time points post challenge IgM antibody titers were measured in the peritoneal fluid.

In IL-10^{-/-} mice there was a significant increase in the concentrations of the poly-specific antibody IgM in the peritoneal fluid (**Figure 5.16**) at 12 and 24 h post-infection as compared with WT mice. This data demonstrates that despite significantly higher concentrations of IgM production, it is not sufficient to protect the IL-10^{-/-} animals from the significant pathology associated with the IL-10 deficiency (see Figure 5.5).

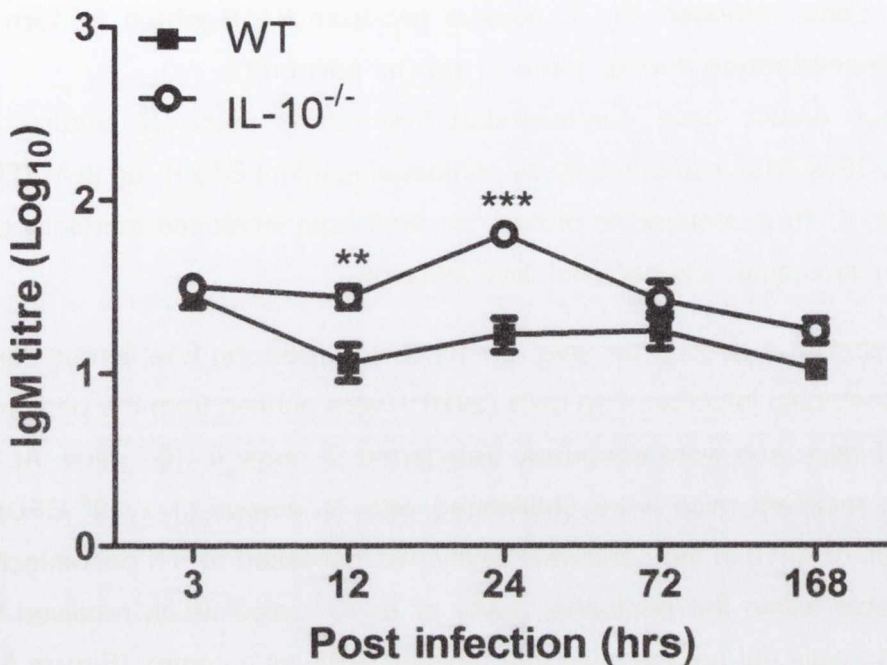


Figure 5.16. IL-10 deficiency results in increased IgM production in response to an acute *S. aureus* peritoneal infection.

Groups of WT and IL-10^{-/-} mice received an intra-peritoneal injection of *S. aureus* (Newman, 1 x 10⁸ CFU). At the indicated time points post challenge, the peritoneal cavity was lavaged and the concentrations of IgM were measured by ELISA. Results are expressed as mean±SEM (n=4-5). Data are representative of 2 individual experiments. Statistical analysis was performed using two-way ANOVA with Bonferroni post-test. **P≤0.005 ***P≤0.001

5.2.8. B1a cells activated by *S. aureus* produce IL-10 which in turn controls bacterial dissemination during acute *S. aureus* peritonitis.

The previous results have demonstrated that during acute *S. aureus* peritoneal infection, IL-10 is produced primarily by peritoneal resident B1a B-reg cells (**Figure 5.2**) and that this IL-10 is required to protect the host from increased morbidity caused by excessive inflammation and bacterial dissemination.

In order to confirm a direct protective role for IL-10-producing B1a B-reg cells during acute *S. aureus* peritoneal infection, B1a cells (2×10^5) were purified from the peritoneal cavity of naive WT mice and were adoptively transferred to naive IL-10^{-/-} mice. At 12 h post transfer the recipient mice were challenged with *S. aureus* (1×10^8 CFU) and the concentration of IL-10 in the peritoneal cavity was assessed at 3 h post-infection. IL-10 was detectable within the peritoneal cavity of IL-10^{-/-} mice which received WT IL-10 sufficient B1a cells but not the mice that received PBS as a control (**Figure 5.17**). This result demonstrates that adoptively transferred WT B1a cells were functional in their ability to produce IL-10 following transfer.

In order to show that these IL-10 producing B1a cells were protective, the bacterial burden in the peritoneal cavity and peripheral organs was assessed on day 5 post-infection. Although there was no reduction in the levels of *S. aureus* recovered from the site of infection (peritoneal cavity) in the IL-10^{-/-} mice that received WT B1a cells compared with the controls (**Figure 5.18A**), there was a significant reduction in dissemination of *S. aureus* to the kidney (**Figure 5.18B**) and the spleen (**Figure 5.18C**) in the mice that received the B1a cells as compared with the control mice. Importantly adoptive transfer of IL-10 deficient B1a cells to IL-10^{-/-} mice failed to control dissemination to the peripheral organs (**Figure 5.19**). This data confirms that IL-10-producing B1a B-reg cells are critically important for containing the bacteria locally in the peritoneal cavity preventing its dissemination to the peripheral organs during acute *S. aureus* peritoneal infection.

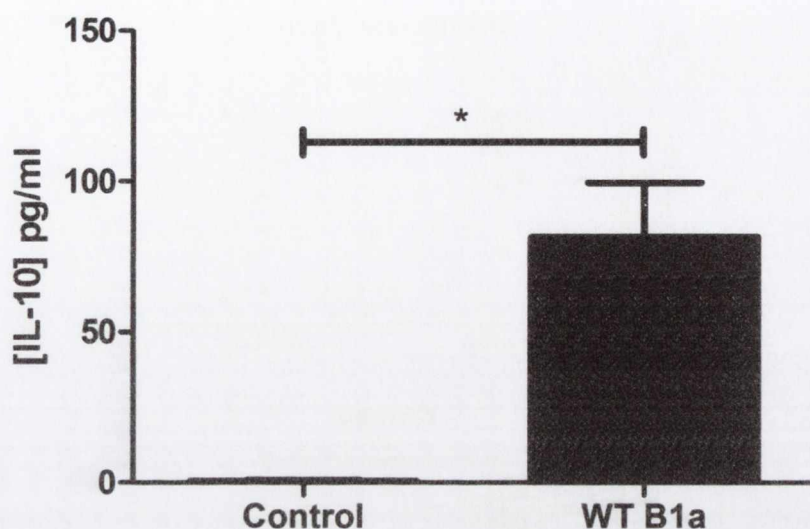


Figure 5.17. Adoptive transfer of WT B1a cells to IL-10^{-/-} hosts results in IL-10 production during acute *S. aureus* peritoneal infection.

Groups of IL-10^{-/-} mice received an adoptive transfer of naive IL-10 sufficient B1a (2x10⁵) cells originating from the peritoneal cavity of WT mice 12 h prior to infection. Control mice received a PBS injection. Mice then received an intra-peritoneal injection of *S. aureus* (Newman, 1 x 10⁸ CFU). At 3 h post-bacterial challenge, the peritoneal cavity was lavaged and the concentrations of IL-10 were measured by ELISA. Results are expressed as mean±SEM (n=3-4). Statistical analysis was performed using Mann-Whitney t-test. *P≤0.05.

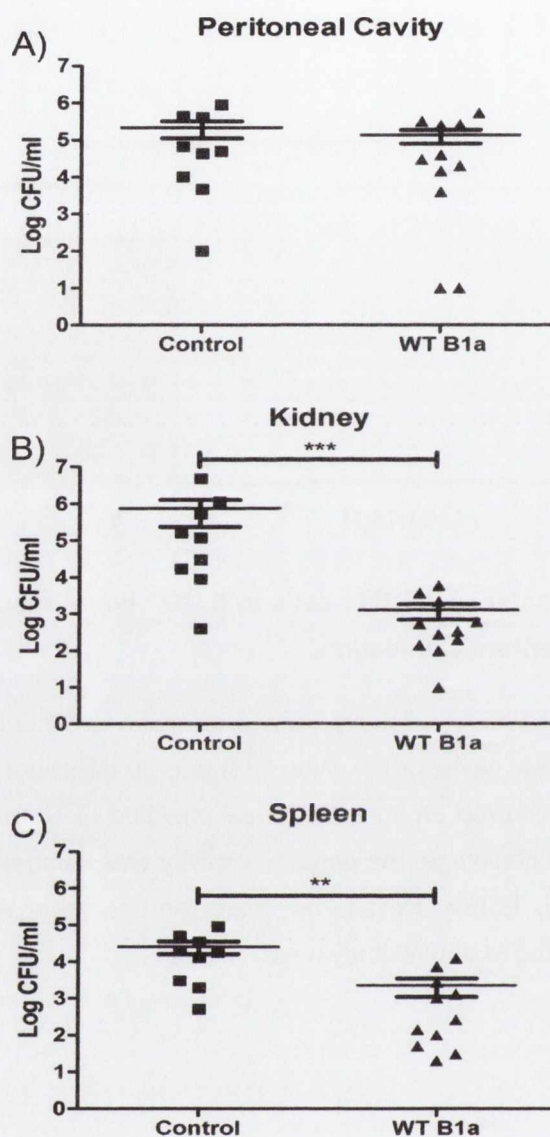


Figure 5.18. Adoptive transfer of WT B1a cells to IL-10^{-/-} hosts protect against bacterial dissemination during acute *S. aureus* peritoneal infection.

Groups of IL-10^{-/-} mice received an adoptive transfer of naive IL-10 sufficient B1a (2×10^5) cells originating from the peritoneal cavity of WT mice. Control mice received a PBS injection. At 12 h post transfer mice were challenged with an intra-peritoneal injection of *S. aureus* (Newman, 1×10^8 CFU). On day 5 post challenge the bacterial burden was assessed in the peritoneal cavity (A), kidneys (B) and spleen (C). Results are expressed as mean $\pm$ SEM (n=9-12). Data represents pooled data from 3 individual experiments. Statistical analysis was performed using a Mann-Whitney test. * $P \leq 0.05$ ** $P \leq 0.005$ *** $P \leq 0.001$

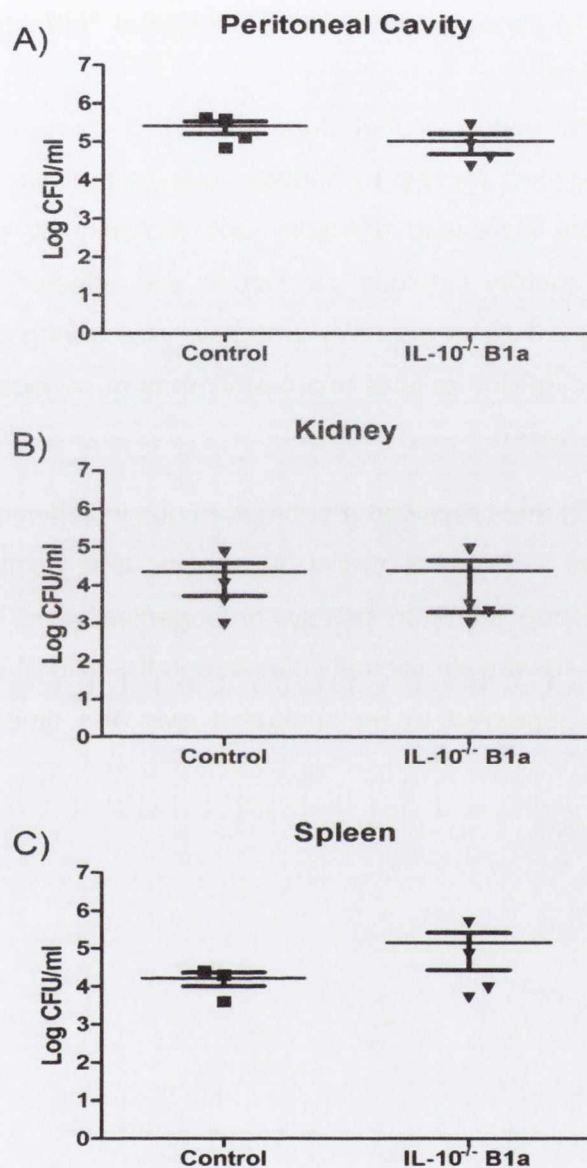


Figure 5.19. Adoptive transfer of IL-10^{-/-} B1a cells to IL-10^{-/-} hosts fails to protect against bacterial dissemination during acute *S. aureus* peritoneal infection.

Groups of IL-10^{-/-} mice received an adoptive transfer of naive IL-10 deficient B1a (2×10^5) cells originating from the peritoneal cavity of IL-10^{-/-} mice. At 12 h post transfer mice were challenged with an intra-peritoneal injection of *S. aureus* (Newman, 1×10^8 CFU). On day 5 post challenge the bacterial burden was assessed in the peritoneal cavity (A), kidneys (B) and spleen (C). Results are expressed as mean $\pm$ SEM ($n=3-4$). Statistical analysis was performed using a Mann-Whitney test.

5.2.10. IL-10 is induced locally within the skin tissue following acute *S. aureus* sub-cutaneous infection.

Data presented to date suggests that during acute *S. aureus* systemic peritoneal infection, IL-10 is produced rapidly by innate cells and plays an essential role in protecting the host from increased morbidity and mortality by controlling excessive activation of pro-inflammatory cytokine production and effector T-cell responses. In order to investigate if IL-10 plays a similar protective role during acute infection, at an alternative site with a different cellular micro-environment, a localised sub-cutaneous model of infection was chosen.

Groups of wild-type (WT) mice received a sub-cutaneous injection of *S. aureus* LAC::lux (2×10^7 CFU) or sterile PBS as a control. At specific time-points post-infection skin lesions were excised using an 8mm biopsy, homogenised and IL-10 concentrations quantified by ELISA. IL-10 was significantly elevated in the skin of WT mice following *S. aureus* challenge and appeared to be sustained over the time course of infection (Figure 5.20).



Figure 5.20. IL-10 is induced locally within the skin tissue following acute *S. aureus* sub-cutaneous infection. Groups of wild-type (WT) mice received a sub-cutaneous injection of *S. aureus* LAC::lux (2×10^7 CFU) or sterile PBS as a control. At specific time-points post-infection skin lesions were excised using an 8mm biopsy, homogenised and IL-10 concentrations quantified by ELISA. IL-10 was significantly elevated in the skin of WT mice following *S. aureus* challenge and appeared to be sustained over the time course of infection (Figure 5.20).

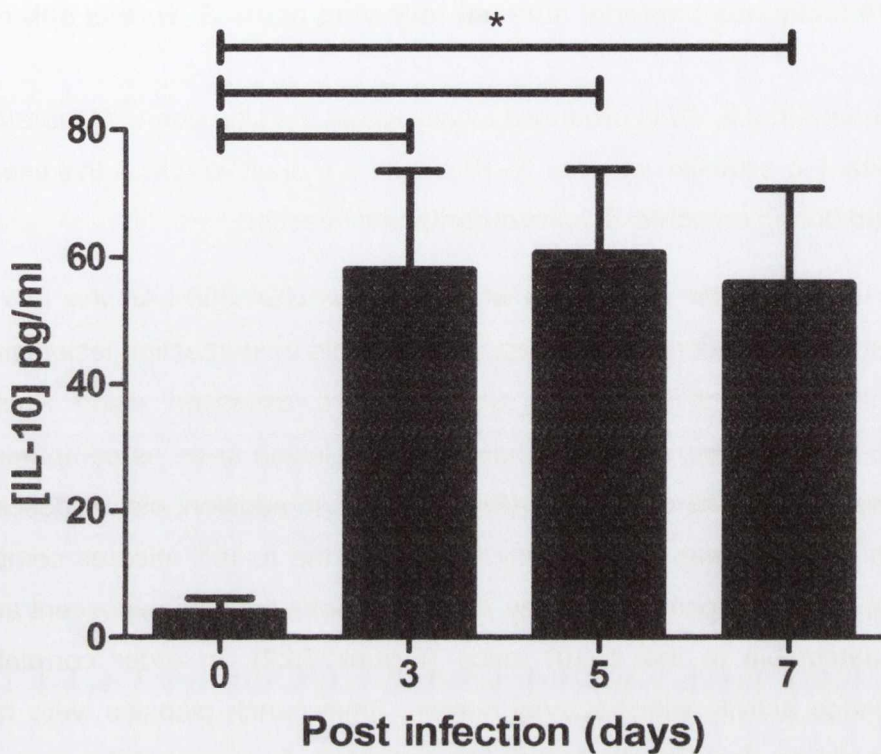


Figure 5.20. *S. aureus* sub-cutaneous infection induces local IL-10 production in the skin.

Groups of WT and IL-10^{-/-} mice received a sub-cutaneous infection of *S. aureus* (USA300 LAC::lux, 2 x 10⁷ CFU). At the indicated time points post challenge, skin biopsies were homogenised and the concentrations of IL-10 measured by ELISA. Results are expressed as mean±SEM (n=4-5). Data are representative of 2 individual experiments. Statistical analysis was performed using one-way ANOVA with Tukey's Multiple Comparisons test. *P≤0.05

5.2.11. IL-10 facilitates bacterial survival following acute *S. aureus* sub-cutaneous infection.

Having observed that IL-10 is produced locally within the skin during *S. aureus* infection it was important to establish whether IL-10 played a protective role at this site, similar to that observed during an acute *S. aureus* peritoneal infection.

WT and IL-10^{-/-} mice were challenged with *S. aureus* USA 300 LAC::*lux* (2×10^7 CFU) via sub-cutaneous injection and at specific time-points post-infection lesion sizes and *in vivo* bioluminescent activity of live bacteria were assessed using PhotonImager Software. IL-10^{-/-} mice were found to have reduced lesion sizes as compared with WT mice over the time course of infection (**Figure 5.21**). In addition, bioluminescent activity at the site of infection was substantially reduced in the IL-10^{-/-} mice as compared with the WT mice at all time points up to day 7 at which point the bioluminescent activity was no longer detectable in the IL-10^{-/-} mice (**Figure 5.22**). In order correlate *in vivo* bioluminescence activity with bacterial burden, 8mm-punch biopsies were taken from the infection site on day 3 post-infection. As expected, *S. aureus* CFUs recovered from the 8-mm punch biopsies were significantly reduced in the IL-10^{-/-} mice as compared with WT mice (**Figure 5.23**).

Importantly, it was confirmed that there was no bacterial dissemination to peripheral organs following sub-cutaneous challenge with *S. aureus* in either the WT or the IL-10^{-/-} mice. Levels of bacteria in the liver, kidney and spleen were below the limit of detection (≤ 1 Log of bacteria). To summarise, during acute localised skin infection, *S. aureus* induced IL-10 appears to facilitate bacterial persistence but does not lead to bacterial dissemination to peripheral organs.

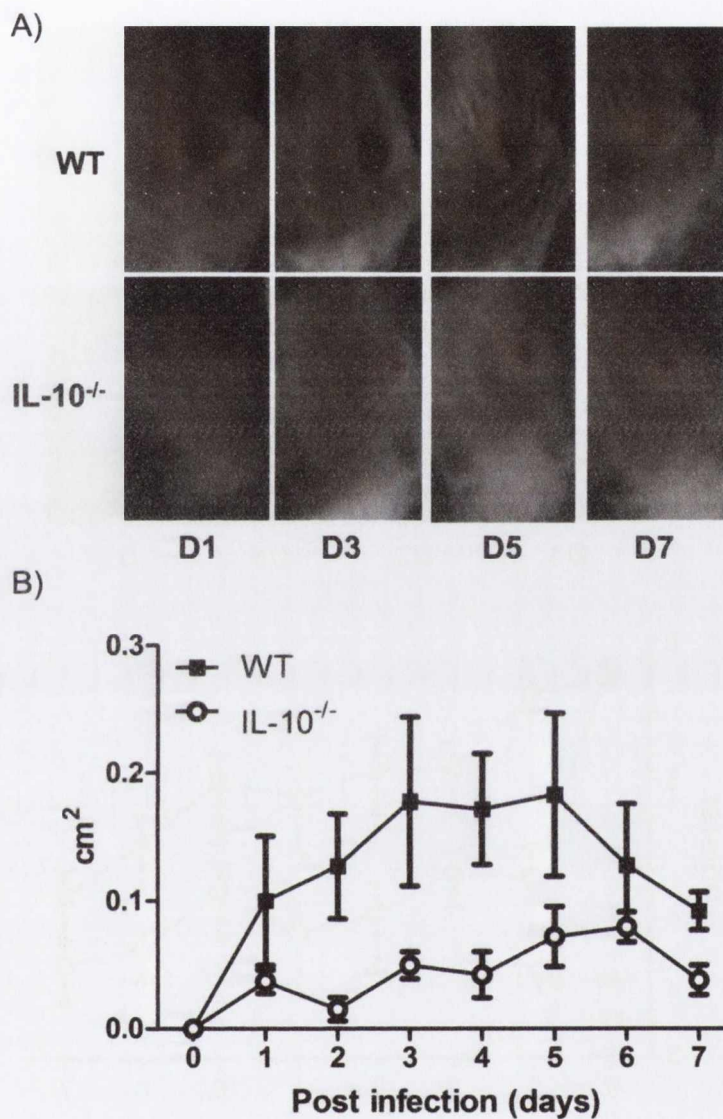


Figure 5.21. IL-10^{-/-} mice develop markedly smaller lesions compared with WT mice in response to sub-cutaneous infection with *S. aureus*.

Groups of WT and IL-10^{-/-} mice received a sub-cutaneous infection of *S. aureus* (USA300 LAC::lux, 2 x 10⁷ CFU). At indicated time points post-challenge the mean total lesion size (cm²) ± SEM was assessed using PhotonImager Software (A), representative lesions for each mouse strain at each time point are shown. Results are expressed as mean ± SEM (n=4-5) (B). Data is representative of 2 individual experiments. Statistical analysis was performed using a two-way ANOVA with Bonferroni post-test.

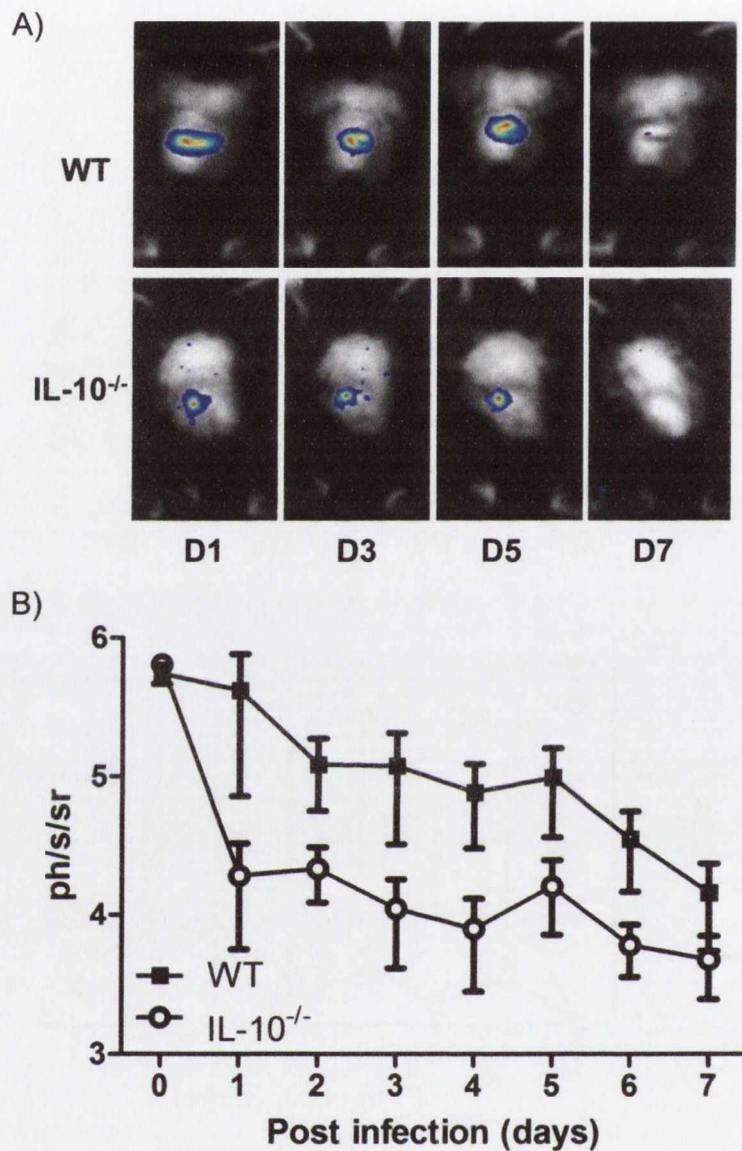


Figure 5.22. IL-10^{-/-} mice display markedly less bioluminescent activity compared with WT mice in response to sub-cutaneous infection with *S. aureus*.

Groups of WT and IL-10^{-/-} mice received a sub-cutaneous infection of *S. aureus* (USA300 LAC::lux, 2 × 10⁷ CFU). At indicated time points post-challenge the mean total flux (photons/s/sr) ± SEM was assessed using PhotonImager Software. Representative *in vivo* bioluminescence images for each mouse strain at each time point are shown (A). Results are expressed as mean ± SEM (n=4-5) (B). Data is representative of 2 individual experiments. Statistical analysis was performed using a two-way ANOVA with Bonferroni post-test.

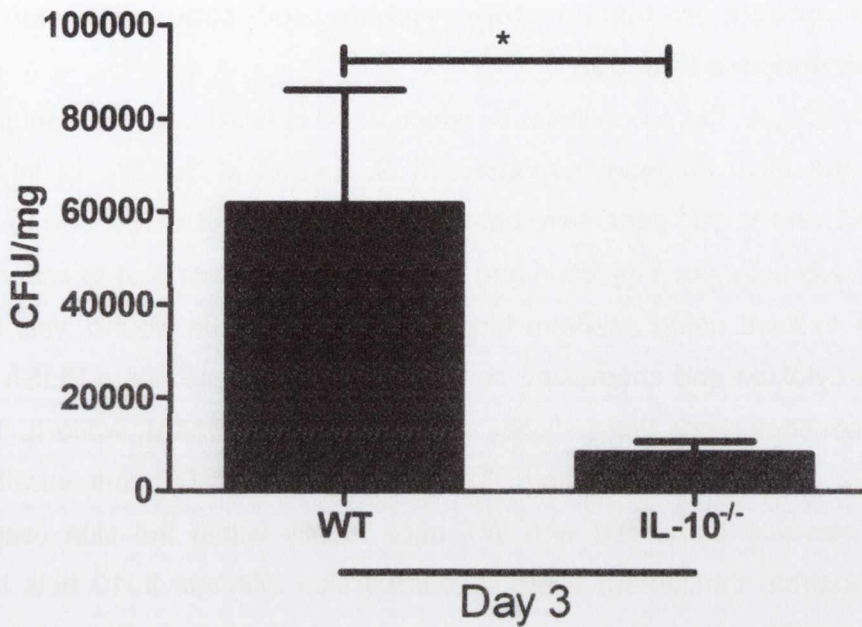


Figure 5.23. IL-10^{-/-} mice have significantly fewer bacteria at the site of infection compared with WT mice in response to sub-cutaneous infection with *S. aureus*.

Groups of WT and IL-10^{-/-} mice received a sub-cutaneous infection of *S. aureus* (USA300 LAC::*lux*, 2×10^7 CFU). The bacterial burden in the skin lesion was quantified on day 3 post-infection by 8mm biopsy punch. Results are expressed as mean $\pm$ SEM (n=4-5). Data is representative of 2 individual experiments. Statistical analysis was performed using Mann-Whitney test. * $P \leq 0.05$

5.2.12. IL-10 controls pro-inflammatory cytokine production in the skin during *S. aureus* sub-cutaneous infection

In order to investigate the mechanism by which IL-10 is facilitating bacterial persistence in the skin, the local immune responses to *S. aureus* at the site of infection was examined. WT and IL-10^{-/-} mice were challenged with *S. aureus* USA 300 LAC::lux (2 x 10⁷ CFU) via sub-cutaneous injection and at specific time points post-infection local skin lesions were excised using an 8mm biopsy, homogenised in wound lysis buffer and assessed for cytokine and chemokine concentrations by whole tissue ELISA. In IL-10^{-/-} mice, IL-1 β concentrations were slightly elevated (**Figure 5.24A**), while IL-17 (**Figure 5.24B**), IL-22 (**Figure 5.24C**) and CXCL1 (**Figure 5.24D**) concentrations were significantly elevated compared with WT mice locally within the skin tissue. These results demonstrate that during acute *S. aureus* skin infection IL-10 acts to dampen down local pro-inflammatory responses in the skin which likely facilitate persistence of the infection at this site.

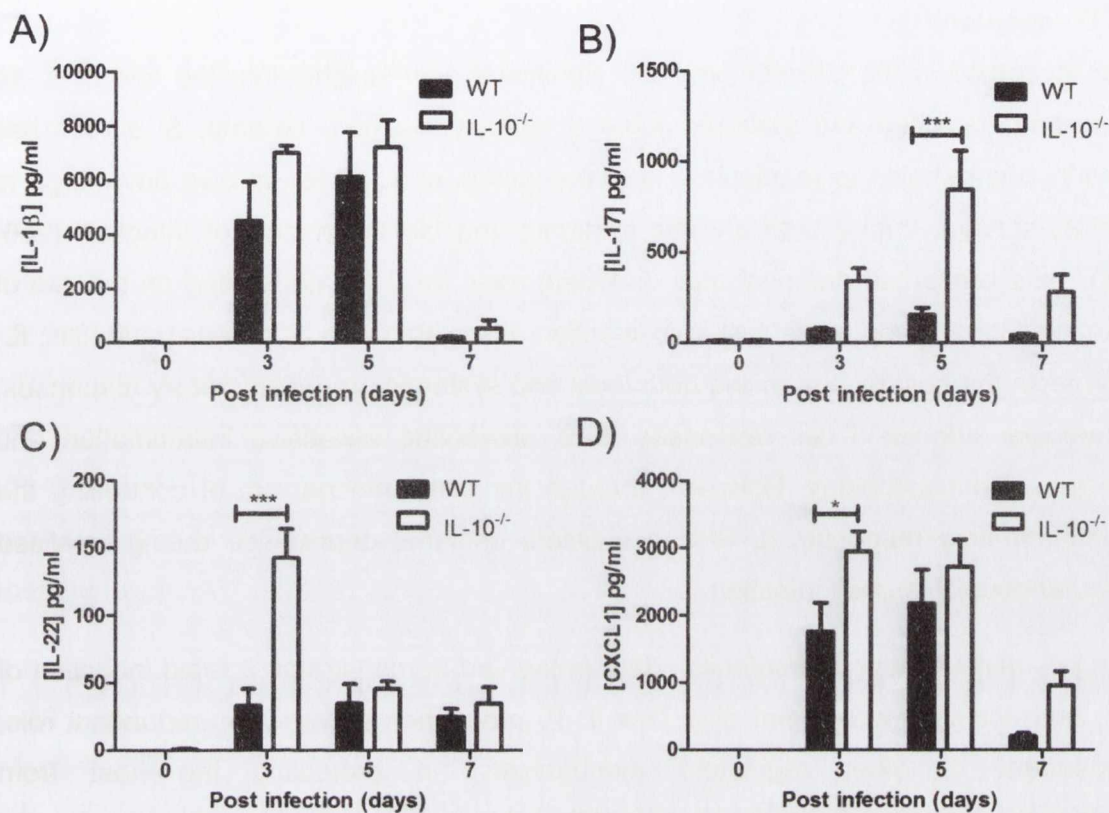


Figure 5.24. IL-10 deficiency results in increased pro-inflammatory mediators locally in the skin during *S. aureus* sub-cutaneous infection.

Groups of WT and IL-10^{-/-} mice received a sub-cutaneous infection of *S. aureus* (USA300 LAC::lux), 2×10^7 CFU). At the indicated time points post challenge, 8mm skin biopsies were homogenised in wound lysis buffer and the concentrations of IL-1β (A), IL-17 (B), IL-22 (C) and CXCL1 (D) were measured by ELISA. Results are expressed as mean±SEM (n=4-5). Data is representative of 2 individual experiments. Statistical analysis was performed using two-way ANOVA with Bonferroni post-test. * $P \leq 0.05$ *** $P \leq 0.001$

5.3. Discussion

Data presented in the chapter provides significant new insights into the role of IL-10 during both localised and systemic acute *S. aureus* infection. To date, *S. aureus* has primarily been shown to manipulate host production of IL-10 for its own advantage to facilitate survival during both chronic systemic and biofilm models of infection (256) (258). Data presented demonstrates divergent roles for IL-10 depending on the site of acute infection. During acute systemic infection associated with *S. aureus* peritonitis, IL-10 protects the host by controlling both local and systemic pro-inflammatory responses, in particular effector T-cell responses, thus preventing excessive inflammation and associated host pathology. However, through the same mechanism of controlling the pro-inflammatory response, IL-10 can facilitate bacterial persistence during localised sub-cutaneous *S. aureus* infection.

During *S. aureus* induced peritonitis, data presented demonstrates a rapid induction of IL-10 both locally and systemically. This IL-10 production plays a non-redundant role, independent of other regulatory mechanisms, in protecting the host from immunopathology and bacterial dissemination by controlling local and systemic pro-inflammatory responses. In particular, results clearly demonstrate that IL-10 acts upstream of effector T-cells, controlling their cytokine responses. It was previously shown that $\gamma\delta$ derived IL-17 and type I immune responses are required at different stages during *S. aureus* peritoneal infection to promote effective bacterial clearance through the ability of these cytokines to regulate downstream phagocyte responses (12, 69). However, it appears that these T-cell responses cannot be allowed to proceed unchecked and that effective resolution of infection requires the $\gamma\delta^+$ T-cell and $CD4^+$ T-cell responses to be tightly controlled so as to avoid host pathology that would ensue from excessive downstream phagocyte activation. In the absence of IL-10, there was a significant expansion of IL-17-producing $\gamma\delta^+$ T-cells locally at 3 h post-infection as compared with that observed in WT mice. IL-17 can directly control neutrophil activation (318) and consistent with this, neutrophils produced greater amounts of ROS in the absence of IL-10. In addition, IFN- γ -producing Th1 cells were also significantly elevated in IL-10 $^{-/-}$ mice on day 7 compared with WT mice. This was associated with significantly increased recruitment of macrophages and greater NOS2 production from the

macrophages in the absence of IL-10. It has previously been demonstrated that excessive neutrophil migration to the peritoneal cavity leads to increased bacterial burden and mortality as a consequence of *S. aureus*'s ability to survive intracellularly within neutrophils following intra-peritoneal challenge (44). Furthermore, it was previously shown that intracellular survival within phagocytes promotes systemic bacterial dissemination following intra-peritoneal challenge with *S. aureus* (301). In addition, it has been shown that excessive macrophage activation and pro-inflammatory cytokine production leads to host tissue injury following acute *Citrobacter rodentium* infection (319). Consequently, it is clear that phagocytic responses and by extension, effector T-cell responses controlling these phagocytes, must be tightly regulated during bacterial infection so as to constrain bacterial dissemination and prevent associated host immunopathology.

In contrast to the host protective role of IL-10 during acute systemic peritoneal infection, IL-10 appears to facilitate bacterial persistence during acute localised sub-cutaneous infection. IL-10 production was sustained locally within the skin tissue up to 7 days post sub-cutaneous infection with *S. aureus*. This local induction of IL-10 was associated with persistence of the bacteria within the skin. IL-10^{-/-} mice exhibited reduced lesion size and reduced bacterial burden as evidenced by bioluminescent activity at the site of infection compared with WT mice and had effectively cleared the infection by day 3 as quantified by CFUs. In WT mice appreciable levels of bacteria were still recoverable from the skin abscess site at this time point. Similar to its role during acute peritonitis, IL-10 appears to be controlling local effector T-cell responses in the skin. In the absence of IL-10, substantially greater amounts of pro-inflammatory cytokines, specifically IL-1 β , IL-17, IL-22 and the neutrophil chemo-attractant CXCL1 were found locally within the infected tissue compared with WT mice over the course of infection. IL-17-producing $\gamma\delta^+$ T-cells have previously been shown to be critical in the effective clearance of *S. aureus* from the skin through their ability to regulate CXCL1 driven neutrophil recruitment, and this activation of IL-17 production by $\gamma\delta^+$ T-cells in the skin is known to be critically IL-1 β -dependent (11). Recent data has shown that neutralisation of either IL-17A or IL-22 during a sub-cutaneous skin infection resulted in a decrease in T-cell and neutrophil infiltration to the site of infection. This resulted in an increase in abscess

severity and CFU in skin (77). Therefore, as IL-10 is inhibiting the production of these cytokines during this sub-cutaneous infection, it also acts to limit bacterial clearance. Future experiments are required to examine whether IL-10 is upstream of the activation of $\gamma\delta^+$ and $CD4^+$ T-cells that have infiltrated the skin in response to *S. aureus* infection in both WT and IL-10^{-/-} mice.

So it appears that in the skin once again IL-10 acts upstream to control effector T-cells, however in this case suppression of the T-cell response favors the bacterium by impairing the host's ability to clear the infection. Importantly, during localised sub-cutaneous infection, systemic dissemination of bacteria in the absence of IL-10 was not observed. This is similar to the local chronic biofilm infection model, where the absence of IL-10-producing MDSCs did not lead to bacterial dissemination (257, 258). Therefore, it appears that during localised *S. aureus* infection, IL-10 production facilitates bacterial persistence at the site of infection only but does not promote systemic dissemination.

Taken together, these results demonstrate that the host can tolerate the lack of an IL-10 anti-inflammatory response during local *S. aureus* infection and in fact removing the brake on the pro-inflammatory response actually benefits the host by facilitating a more rapid clearance of the bacteria. However, this response cannot be tolerated systemically where a lack of IL-10 leads to excessive pro-inflammatory responses that are actually detrimental to the host.

This study has uncovered a previously undocumented immune-regulatory role for regulatory B1-cells during acute systemic *S. aureus* peritoneal infection. Following intra-peritoneal challenge with *S. aureus*, IL-10 is rapidly produced locally by a population of B-reg cells comprising both B1a and B1b subsets. Consistent with previously published studies (151, 258), $CD11b^+ Ly6G^+ Ly6C^+$ MDSCs and $CD11b^+ F480^+$ macrophages were also capable of producing IL-10 at this site however the B-reg response significantly dominated. Interestingly, $CD4^+$ T-cells or T-reg cells did not appear to produce IL-10 at this site, although they have been shown to be an important source of IL-10 during pulmonary infections (314). Adoptive transfer of IL-10 sufficient B1a cells conferred protection in recipient IL-10^{-/-} mice and prevented bacterial dissemination suggesting that these IL-10 producing B-reg cells are critical for protecting the host from

increased morbidity and potentially mortality associated with acute systemic *S. aureus* infection, presumably as a consequence of these cells ability to constrain excessive pro-inflammatory responses. This is consistent with other studies showing peritoneal B1 cells capable of immunosuppression and host protection in response to systemic *Coxiella burnetii* infection (239).

When considering the role of B-cells during *S. aureus* infections, focus has almost exclusively been on their function as antibody producers (68). Here this study reports for the first time an immune-protective role for IL-10⁺B1 cells during *S. aureus* infection. B1 B-cells represent a regulatory innate-like population highly enriched in peritoneal and pleural cavities and more recently in the skin during inflammation and are capable of potent IL-10 production (320, 321). IL-10 producing B1a cells have previously been shown to be capable of limiting NK/NKT-cell inflammation and macrophage activation following a pulmonary challenge with *Francisella tularensis* (322) and Th1 responses during colitis (323). They have been shown to respond to TLR activation, and express B-cell receptors that often recognise conserved pathogen patterns (324). B1 cells bridge innate and adaptive immunity by efficiently mounting rapid T-cell-independent antibody responses, engaging in phagocytic and antimicrobial activity through the production of IgM (325). Data presented demonstrates that *S. aureus* can activate IL-10 production by B1a cell and that these cells play a protective role in systemic infection. Importantly, the protective effect of B1a cells is independent of IgM, as the concentrations of IgM were found to be significantly greater in the absence of IL-10, however this was not sufficient to prevent immunopathology. Interestingly, systemic *S. aureus* infection in TLR2^{-/-} mice also resulted in significant mortality caused by excessive pro-inflammatory response and increased bacterial dissemination which was associated with decreased IL-10 production by macrophages (326). This result mirrors that which was observed during systemic infection in IL-10^{-/-} mice, suggesting that TLR2-dependent IL-10 production from B1a cells is likely controlling pro-inflammatory T-effector cell responses in this setting.

Although the specific cellular source of IL-10 was not identified in the skin in this study, IL-10⁺B1a cells have recently been found to migrate to the skin during inflammation

(321). Therefore, these cells may represent a possible target for *S. aureus* inducing IL-10 via TLR2 signaling as a mechanism of immune evasion in this localised setting.

Overall this study provides significant new insight into the contrasting roles played by IL-10 in response to *S. aureus* at different infection sites. In addition, data presented identifies a novel subset of IL-10-producing B-cells that undergo rapid activation in response to *S. aureus* and are important for protecting the host against the significant morbidity and mortality associated with acute systemic *S. aureus* infection. Interestingly, clear contrasting roles for IL-10 during systemic and localised acute *S. aureus* infection were observed. This suggests that the cellular microenvironment at the site of infection can significantly impact on the ultimate disease outcome, potentially due to the presence of different cells being present at the infection site and/or the burden of bacteria present. Ultimately however, *S. aureus* maintains a symbiotic relationship with the host. During systemic infection IL-10 ensures host and by extension, bacterial survival, while during local infection, IL-10 forgoes host morbidity to facilitate bacterial survival.

Chapter 6

6. General Discussion

The report "Antimicrobial resistance: global report on surveillance", published by the WHO highlighted the epidemic of *S. aureus* antibiotic resistance as a particular threat to society (327). It identifies the need for alternative approaches to antibiotics in order to treat this type of infection. First described in the 1960s, by 2003 50% of all *S. aureus* isolates in US hospitals were resistant to methicillin (MRSA). Resistance has now been reported to every licensed anti-staphylococcal agent available. Better hygiene and infection control guidelines have reduced the rates of MRSA, but largely ignore the bulk of life-threatening *S. aureus* disease, ~80% of which is caused by methicillin-sensitive strains (MSSA) whose rates are rising (328). Therefore alternatives therapeutics to antibiotics such as vaccines and immunomodulatory therapies are urgently required.

The development of such therapies requires a much better understanding of the interaction between *S. aureus* and the host. Recently, it has shown that T-cells are an important facet of the hosts' protection against *S. aureus* infection, in addition to B-cells. Therefore, vaccines should be designed to elicit a response that encompasses both B-cells and the appropriate T-cell response, to promote an immune response towards efficient clearance of this pathogen. *S. aureus* colonisation is a significant risk factor for invasive disease. However, the vast majority of the population co-exist in harmony with *S. aureus* throughout their lives and rarely develop invasive infection. Colonisation represents a dynamic process with the host, in which *S. aureus* has to constantly evolve mechanisms to overcome multiple pressures, including host immunity, in order to survive within its niche. Exposure to *S. aureus* through colonisation may have important immunomodulatory effects on the cellular and/or humoral immune response that could potentially influence the host's ability to respond to invasive infection and also vaccine-induced immunity. However, as of yet these immunomodulatory mechanisms during colonisation have not been investigated. Understanding the consequence of this dynamic inter-play between the bug and the host will assist substantially in understanding how it impacts upon the host's ability to respond to both subsequent infection and to anti- *S. aureus* vaccination.

The overall goal of this thesis was to establish if the co-evolution of *S. aureus* with the human host has resulted in this organism being highly equipped at manipulating the immune system's regulatory responses for its own advantage. It was proposed that during nasal colonisation and infection, *S. aureus* can exert immuno-suppressive mechanisms on the host in order to control pro-inflammatory responses, and more precisely effector T-cell responses and the subsequent activation of phagocytic cells, in order to facilitate its persistence. This thesis is aimed specifically at unravelling the role of *S. aureus* induced IL-10 in the commensal setting of the nose and secondly at two contrasting infection sites.

IL-17 producing Th17 cells have recently been suggested to be responsible for the neutrophil-mediated clearance of *S. aureus* during nasal colonisation (123). This study validates that CD4⁺ T-cells are the dominant producers of IL-17 during *S. aureus* nasal colonisation, alongside $\gamma\delta$ ⁺ T-cells. In addition, this thesis observed that IL-22, another Th17 cytokine, was also produced by CD4⁺ T-cells, along with CD8⁺ T-cells and ILCs. IL-22 was shown to play an important role in the decolonisation process. While these mechanisms may be effective at clearing *S. aureus* from the nose, this data suggests that IL-10 acts upstream of IL-17 producing CD4⁺ and $\gamma\delta$ ⁺ T-cells, suppressing their cytokine production and the subsequent influx of neutrophils, thereby providing the bacterium with a survival advantage. IL-10^{-/-} mice were shown to have accelerated *S. aureus* clearance from the nose compared with WT mice. Interestingly, IL-10 did not impact upon IL-22 responses which are responsible for AMP production (288). Although previous studies have shown that *S. aureus* colonising strains have the capacity to induce IL-10 from phagocytes (152). No data was available on the actual effect of this IL-10 production during *S. aureus* nasal colonisation *in vivo*. The data generated in this thesis provides novel insight into the role of *S. aureus* induced IL-10 in facilitating bacterial persistence during colonisation, indicating that IL-10 is specifically controlling the IL-17 driven neutrophil-mediated aspect of the immune response during colonisation. IL-10 acts to suppress IL-17 production by CD4⁺ T-cells and controls the subsequent recruitment of neutrophils to the site of *S. aureus* colonisation.

This project also demonstrates that *S. aureus* is capable of inducing IL-10 production predominantly from macrophages. As previously shown (152), this study validates that both colonising and invasive *S. aureus* strains can induce simultaneous production of IL-10 and TNF- α from macrophages upon stimulation. Interestingly, this study discovered that colonising and invasive *S. aureus* isolates have differential patterns of IL-10 and TNF- α induction. Colonising strains favoured an early induction of these cytokines, whereas the invasive strain, USA300, preferentially induced cytokines at a later stage in murine BMMs and overall seemed to be more inclined to induce pro-inflammatory as opposed to anti-inflammatory responses. Therefore, this would help explain why during infection, USA300 would elicit a more robust immune response in certain invasive diseases including bacteraemia, endocarditis, severe necrotising pneumonia and osteomyelitis causing severe pathology. Interestingly, Newman was the only strain shown to induce IL-10 production by both murine and human macrophages, albeit at the later time point. In contrast to the invasive strain USA300, Newman was not a potent inducer of TNF- α in human macrophages. This consistent and prolonged ability to induce IL-10 production probably explains why Newman is the most efficient nasal coloniser *in vivo*, as demonstrated in Chapter 3. As macrophages have been suggested to represent the primary immune cell type in the nose (254), they could well represent a potential target for *S. aureus* during nasal colonisation due to their potent ability to induce IL-10 in response to this bacterium. As stated, the source of IL-10 was not identified *in vivo* during nasal colonisation. Isolating sufficient cell numbers for FACS from the nasal tissue was a major problem in not detecting an IL-10 GFP⁺ signal. Therefore, alternative approaches such as pre-treating mice with clodronate liposomes prior to *S. aureus* colonisation, in order to deplete the macrophage populations, would help determine if macrophages were the main producers of IL-10.

In addition to controlling pro-inflammatory cytokine production by macrophages, this study suggests that IL-10 can directly interfere with *S. aureus* killing by macrophages, which could provide an additional mechanism by which *S. aureus* could survive and maintain its nasal niche *in vivo*. During nasal colonisation there was an expansion of macrophages in both the nose and in the NT. The role of these macrophages was not

fully elucidated, however as macrophages generally represent a heterogeneous population of cells which can range from both M1 to M2 phenotypes, they more than likely have multiple roles during colonisation. During successful *S. pneumoniae* colonisation, CD206 expressing M2 macrophages were observed as the dominant macrophage population present in the nasopharyngeal tissue. However, at higher levels of bacterial burden these M2-type macrophages were replaced by a CD206 negative macrophage population (265). Thereby, supporting the argument that there are different macrophage populations with different functions present in the nose during colonisation.

In addition to IL-10, T-regs were also expanded during *S. aureus* nasal colonisation. However, data presented in this thesis would suggest that they may be playing a more complex role in potentially protecting the host by leveraging bacterial persistence against bacteria dissemination and associated host morbidity/mortality. In contrast to IL-10, which was shown to play a protective role for *S. aureus* during colonisation, preliminary data would suggest that T-regs play an important function in bacterial containment and maintaining host homeostasis. This type of role for TGF- β and/or T-regs was previously shown during models of *S. pneumoniae* colonisation (265) and *S. aureus* biofilm infection (241), where the inhibition or depletion of T-regs resulted in increased bacterial dissemination. This induction of T-regs by *S. aureus* is an example of how this bacteria promotes for the most part what is a symbiotic relationship, benefiting both the bacterium and the host.

Having identified that IL-10 facilitated the survival of *S. aureus* nasal colonisation, this project investigated the role of IL-10 during acute infection at two distinct sites. The role of IL-10 in determining disease outcome during acute *S. aureus* infection appears to be critically dependent upon the site of infection, most likely due to the differential cellular environments with which the bacterium engages. As the skin is the largest organ of the body and an important barrier against invading bacteria, it comprises a complex immune system involving dendritic cells, keratinocytes, T-cells, and regulatory B-cells that create a tolerant microenvironment, in order to prevent undesirable inflammation in response to commensal organisms (321, 329). Data presented suggests that *S. aureus*

can manipulate this cellular environment for its own advantage, inducing IL-10 to facilitate its persistence within this niche by dampening down pro-inflammatory T-cell mediators similar to the situation during nasal colonisation. In contrast, a systemic infection leads to a more hostile inflammatory host response as there is a greater impetus on host protection. In this setting, IL-10 functions to protect the host by controlling the excessive production of T-cell pro-inflammatory cytokines, in particular IL-17 and IFN- γ , which can subsequently hyper-activate the local phagocytic response which is potentially capable of causing host tissue damage. Unlike, the localised environment in the skin or in the nose during colonisation where immune cells can become tolerogenic due to constant bacterial exposure (330), this data suggests that during a systemic infection the cellular environment is primed for a more robust pro-inflammatory response to *S. aureus* in this setting.

It is also possible that the level of bacterial burden and duration of *S. aureus* being present during an infection can also impact upon the disease outcome. It could be proposed that systemic infections have two distinct phases, an acute phase and a chronic phase. During the acute phase with high bacterial burden, it appears IL-10 acts to protect the host, by regulating excessive effector T-cell responses which can lead to excessive inflammation and associated tissue damage and bacterial dissemination in mice (105, 240). However, as the *S. aureus* bacterial burden is reduced, persisting beyond a certain point, T-cell efficiency becomes impaired and the immune system is skewed towards an anti-inflammatory environment, where IL-10 facilitates *S. aureus* persistence (255, 256, 331).

This thesis identified a novel population of IL-10 producing CD11b⁺CD19⁺CD5⁺ B1 cells that are activated within the peritoneal cavity in response to acute *S. aureus* infection; which uncovers an entirely novel potential role for regulatory B-cells (324, 332) in the pathogenesis of *S. aureus* infection. IL-10 production by B1a cells was shown to be TLR2-dependent and during acute systemic infection IL-10 production by these B1 cell prevented excessive T-cell cytokine production and the subsequent over-activation of downstream phagocytic cells and therefore containing the bacteria at the site of

infection, preventing host morbidity and possible mortality. However, as this IL-10 production by B1a cells is *S. aureus* driven, this also means that B1a cells represent a potential immune evasion target for the bacterium, that at other localised sites such as the nose or skin, could be manipulated as a mechanism of immune evasion and facilitate its persistence. Future studies will determine if B1 cells migrate to the nasal cavity or the skin during *S. aureus* colonisation and infection respectively. Until recently B1 cells were believed to be confined to the peritoneal cavity and pleural cavities, however recent data has observed the IL-10⁺B1 cells can migrate from the peritoneal cavity to the skin during inflammation (321).

In summary, this thesis significantly advances the understanding of *S. aureus*' ability to manipulate the hosts' immuno-regulatory machinery, highlighting the important role that IL-10 can play in facilitating nasal colonisation and determining infection outcome. Similar to gut commensals, *S. aureus* manipulates host production of IL-10 during nasal colonisation. This is the first study demonstrating how *S. aureus* maintains its nasal niche *in vivo* by manipulation of the hosts' immuno-regulatory machinery and thereby limiting the activation of the hosts protective response. Another critical finding that emerged from this thesis is the opposing roles played by IL-10 in determining the outcome of a *S. aureus* infection at contrasting sites of infection. In both local and systemic models of infection, IL-10 was suggested to function upstream of the production of T-cell cytokine production and the subsequent activation of phagocytes. However, the site of this IL-10 induction is critically relevant. Similar to the nose, IL-10 induction at a localised skin infection site protects the niche of *S. aureus*, while in contrast, during an acute systemic infection, IL-10 is protective for the host by controlling the excessive production of same pro-inflammatory cytokines. Therefore during a systemic infection, IL-10 functions to protect the host. However, if the host does not survive, neither does the bug, therefore it appears IL-10 promotes a symbiotic relationship in which both *S. aureus* and the host can both survive (**See Figure 6.1**).

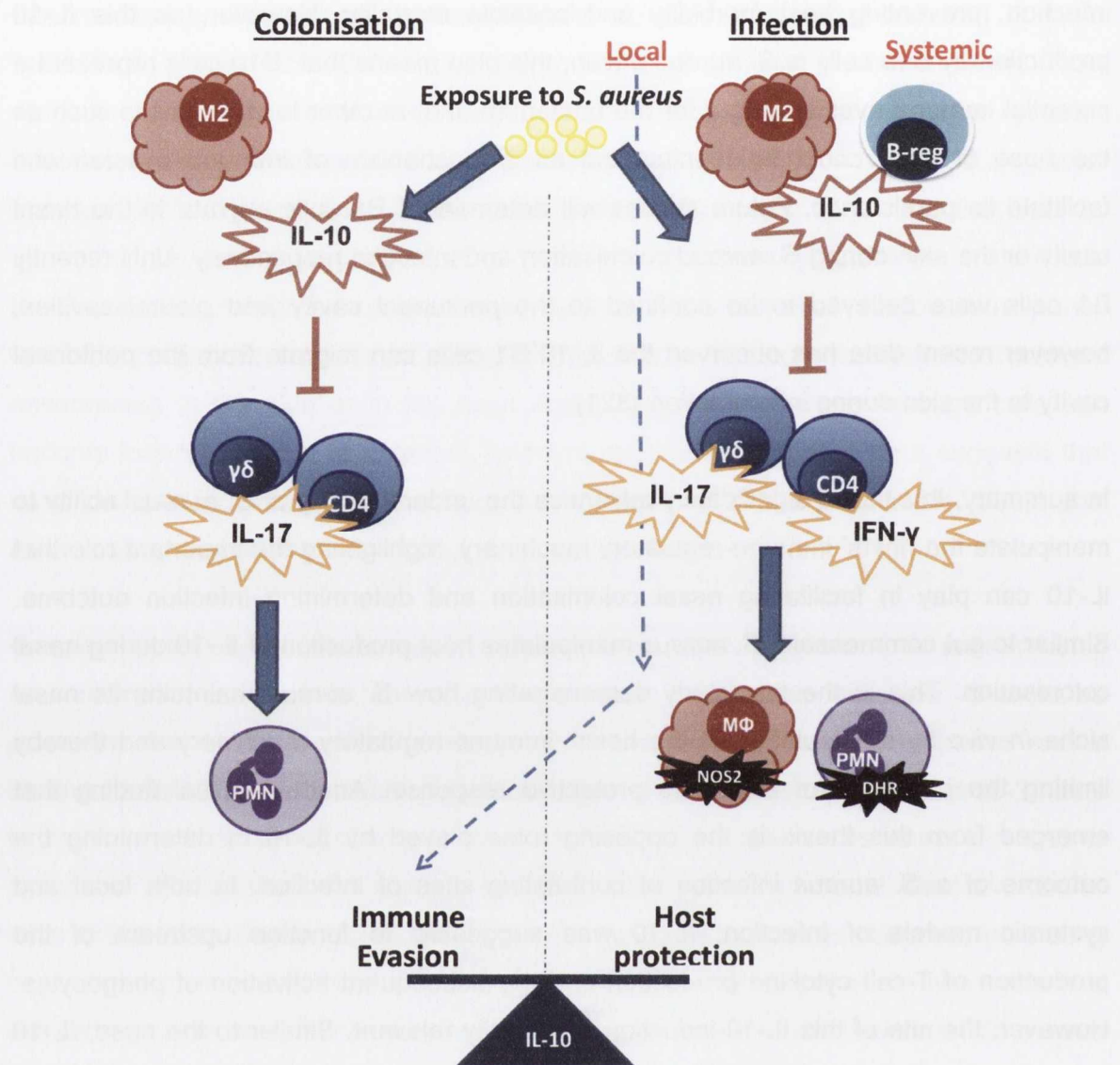


Figure 6.1. *S. aureus* manipulates the hosts production of IL-10 as a mechanism of immune evasion during local nasal colonisation and skin infection.

During **nasal colonisation**, *S. aureus* induces the production of IL-10 locally within the nose. This IL-10 functions to limit the activation of IL-17 producing CD4⁺ T-cells and the subsequent activation of neutrophils, which facilitates bacterial survival. During a **local skin infection**, *S. aureus* induced IL-10 limits the production of effector T-cell cytokines which results in bacterial persistence. In contrast, during a **systemic infection**, IL-10 functions to protect the host by controlling excessive production of pro-inflammatory T-cell cytokine and the hyper-activation of phagocytes, which can cause host pathology.

The development of strategies to neutralise *S. aureus* induced IL-10 responses may have the potential to improve bacterial clearance during colonisation and also in certain infection settings, in particular localised infection settings e.g. SSTIs. Previous studies have demonstrated that *S. aureus*-induced pro- and anti-inflammatory cytokine production can be uncoupled due to the organism's capacity to manipulate TLR2 plasticity and engage unique downstream signalling pathways (151, 152). Consequently, it may be possible to specifically disarm anti-inflammatory signalling induced by *S. aureus* while maintaining the pro-inflammatory effects, particularly during nasal colonisation and skin infection in order to skew the immune response towards bacterial clearance. Secondly, *S. aureus*' capacity for immuno-suppression during colonisation is potentially also a significant factor impeding vaccine efficacy. There is evidence that the induction of bacteria induced immunosuppression can contribute to vaccine failure with other organisms (333-335). Therefore, in the context of an anti- *S. aureus* vaccine, blocking IL-10 in a time and dose dependent manner, would promote better vaccine efficacy. However as highlight by this thesis, the complete inhibition of IL-10, while promoting a robust T-cell response during a vaccine could in fact result in an uncontrolled T-cell response which is detrimental to the host, especially during a systemic *S. aureus* infection. Overall, it is clear that a balance is required when considering inhibition of IL-10 as an anti- *S. aureus* therapeutic.

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7. Appendix

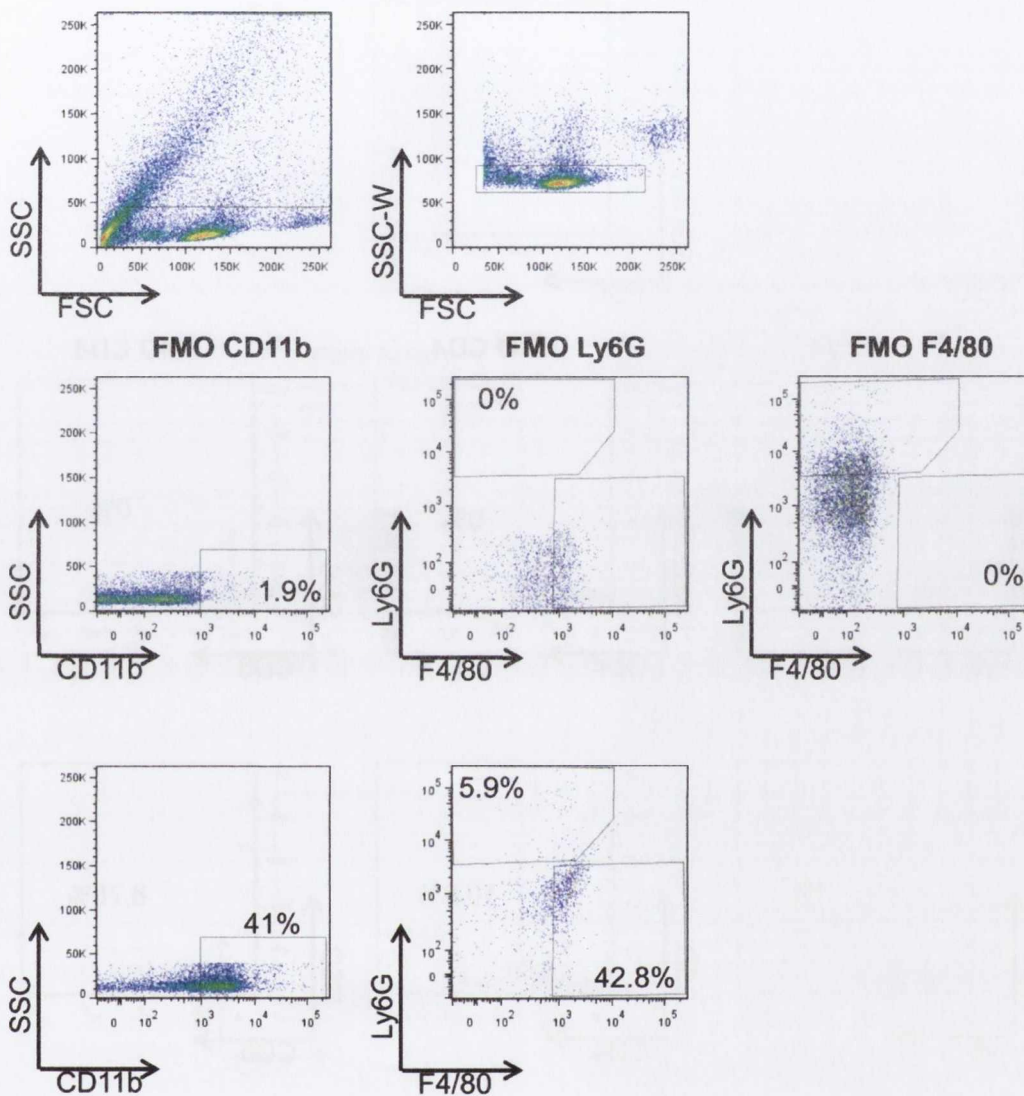


Figure 7.1 Nasal tissue phagocyte staining panel during *S. aureus* nasal colonisation.

Groups of WT mice were inoculated intra-nasally with *S. aureus* (2×10^8 CFU) or were administered with PBS. Cells were isolated from the nasal tissue, surface stained with fluorochrome-conjugated antibodies against CD11b, Ly6G and F480 and analysed by flow cytometry. Representative FACS plots are shown for FMOs and experimental samples.

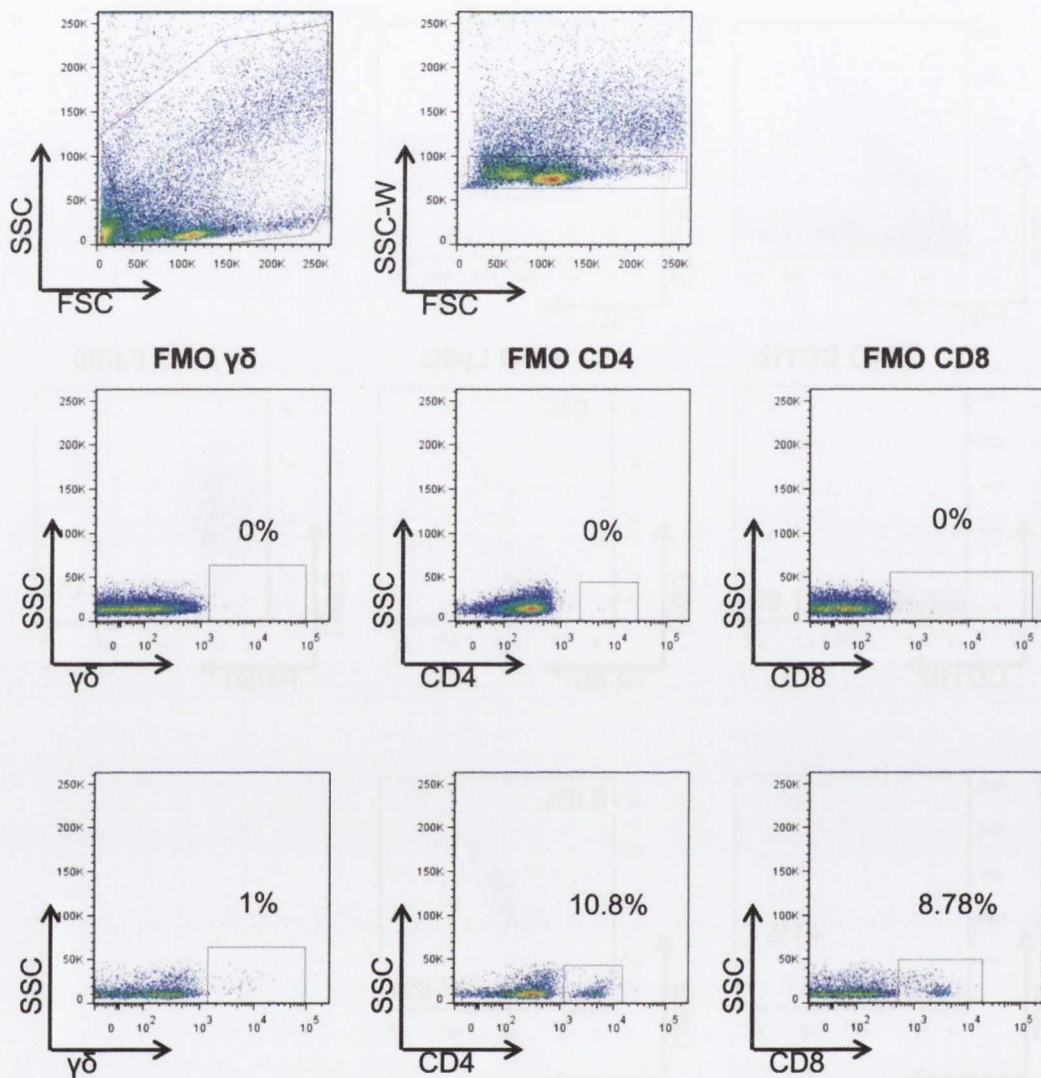


Figure 7.2 Nasal tissue T-cell staining panel during *S. aureus* nasal colonisation.

Groups of WT mice were inoculated intra-nasally with *S. aureus* (2×10^8 CFU) or were administered with PBS. Cells were isolated from the nasal tissue, surface stained with fluorochrome-conjugated antibodies against $\gamma\delta$, CD4 and CD8 and analysed by flow cytometry. Representative FACS plots are shown for FMOs and experimental samples.

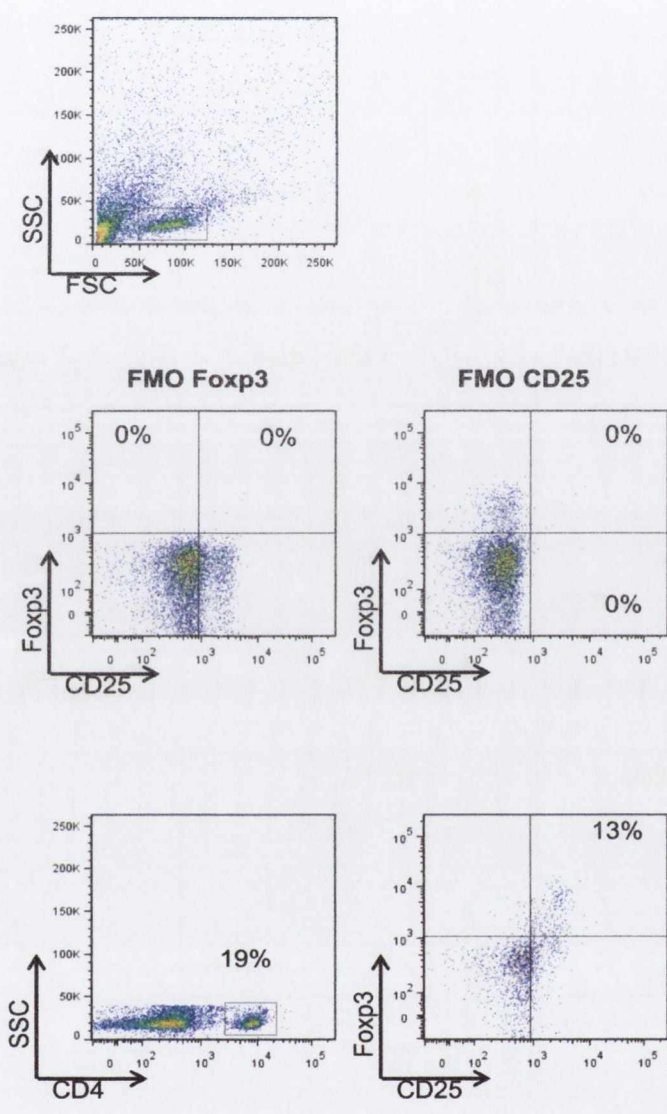


Figure 7.3 Nasal tissue T-reg staining panel during *S. aureus* nasal colonisation.

Groups of WT mice were inoculated intra-nasally with *S. aureus* (2×10^8 CFU) or were administered with PBS. Cells were isolated from the nasal tissue, surface stained with fluorochrome-conjugated antibodies against CD4, CD25 and Fxp3 and analysed by flow cytometry. Representative FACS plots are shown for FMOs and experimental samples.

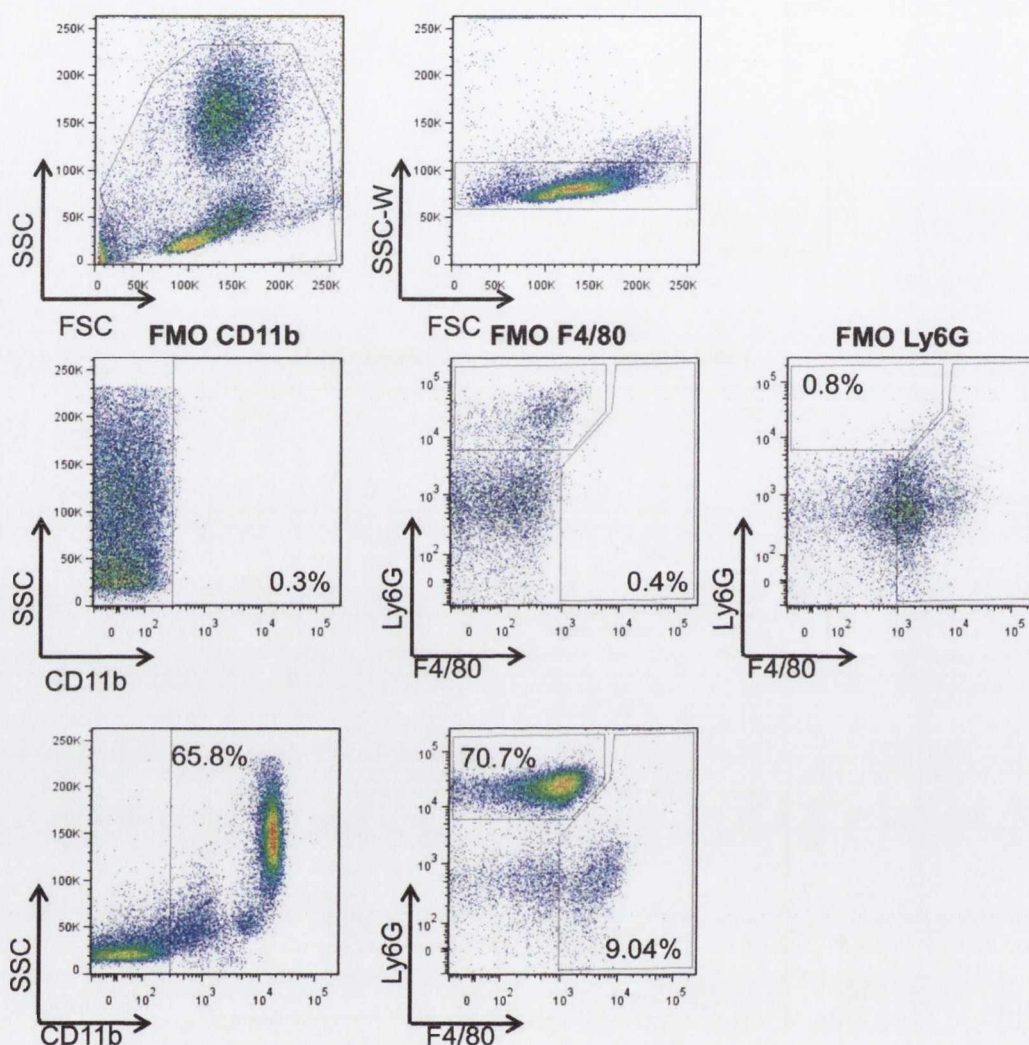


Figure 7.4 Peritoneal cavity phagocyte staining panel during *S. aureus* induced peritonitis.

Groups of WT mice received an intra-peritoneal injection of *S. aureus* (Newman, 1×10^8 CFU). Leukocytes were isolated from the peritoneal cavity for analysis, surface stained with fluorochrome-conjugated antibodies against CD11b, Ly6G and F480 and analysed by flow cytometry. Representative FACS plots are shown for FMO and experimental samples.

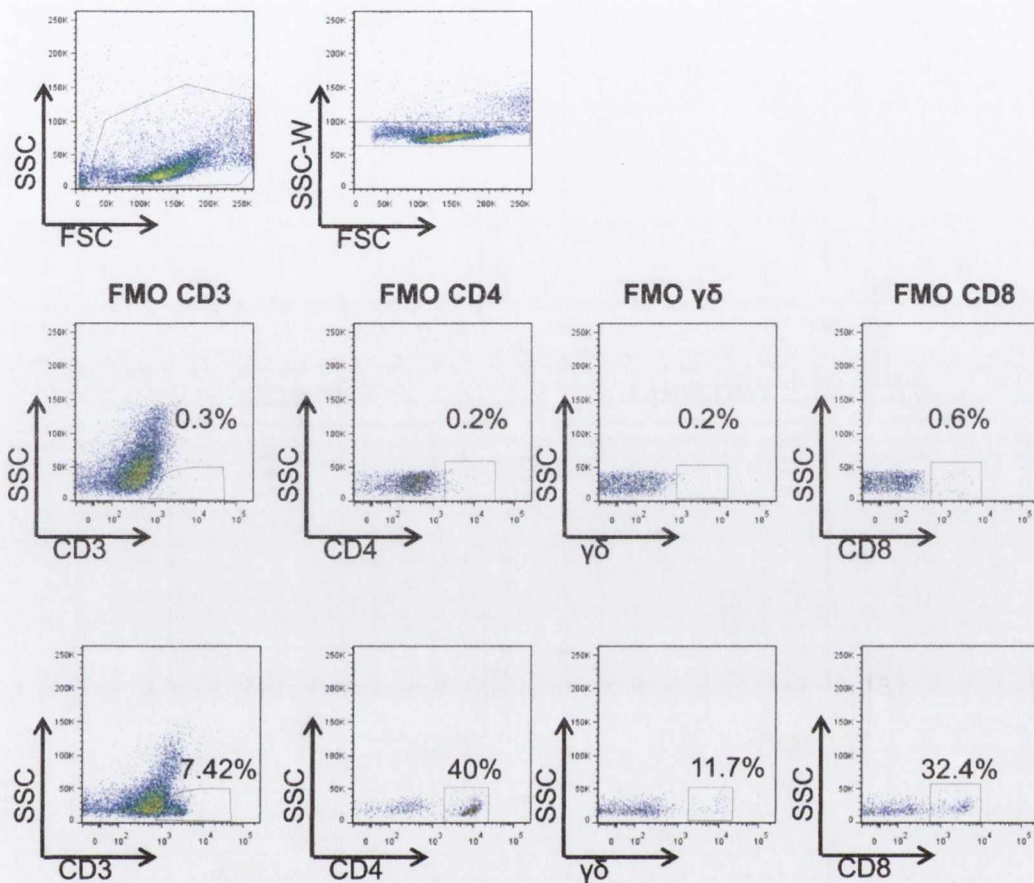


Figure 7.5 Peritoneal cavity T-cell staining panel during *S. aureus* induced peritonitis.

Groups of WT mice received an intra-peritoneal injection of *S. aureus* (Newman, 1×10^8 CFU). Leukocytes were isolated from the peritoneal cavity for analysis, surface stained with fluorochrome-conjugated antibodies against CD3, CD4, $\gamma\delta$ and CD8 and analysed by flow cytometry. Representative FACS plots are shown for FMO and experimental samples.

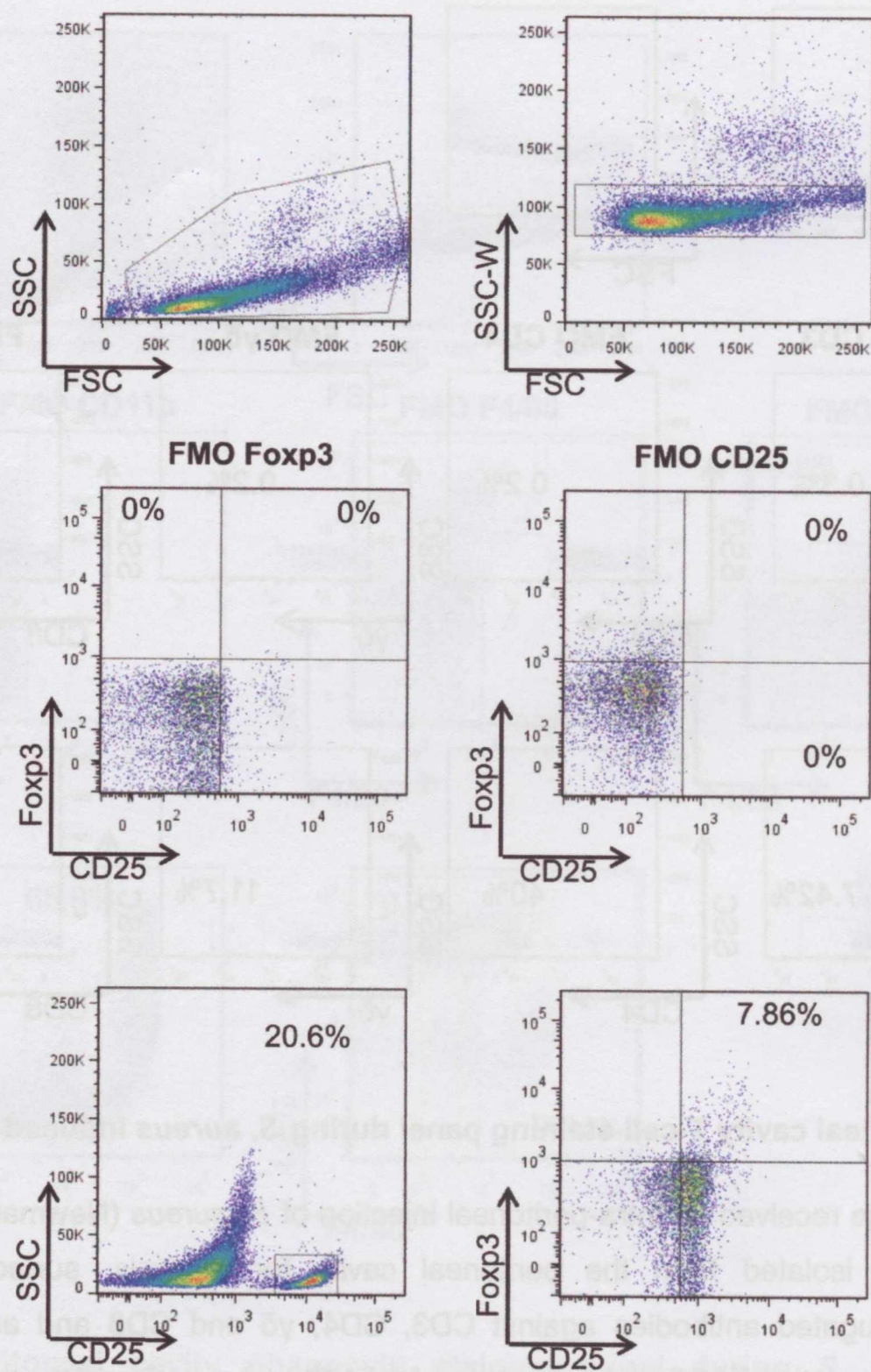


Figure 7.6. Peritoneal cavity T-cell staining panel during *S. aureus* induced peritonitis.

Groups of WT mice received an intra-peritoneal injection of *S. aureus* (Newman, 1×10^8 CFU). Leukocytes were isolated from the peritoneal cavity for analysis, surface stained with fluorochrome-conjugated antibodies against CD4, CD25 and Fxp3 and analysed by flow cytometry. Representative FACS plots are shown for FMO and experimental samples.