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The role of Mal in alveolar macrophage-mediated resistance to *Bordetella pertussis*

Thesis submitted to

the University of Dublin

for the

Degree of Doctor of Philosophy

by

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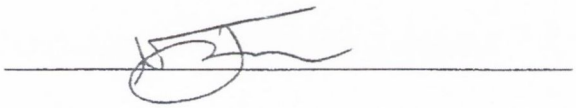

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Abstract

There is a global resurgence in pulmonary infection with *Bordetella pertussis*, the causative agent of whooping cough. *B. pertussis* is known to encode a number of virulence factors, some of which can function as pathogen-associated molecular patterns, including the Toll-like receptor 4 (TLR4) agonist, lipopolysaccharide, and lipopeptides that could be detected by TLR2. MyD88 adaptor-like protein (Mal; also known as Tirap) is an important TIR domain-containing TLR adaptor that connects TLR2 and TLR4 to the transcriptional activation of various antibacterial genes, including those encoding proinflammatory cytokines. TLR4-defective C3HeJ mice are more susceptible to infection with *B. pertussis*, with higher colonisation of bacteria in the lungs. Neither $Mal^{-/-}$ mice nor other TLR adaptor-defective mice have been infected with *B. pertussis*, and the pathogenesis of severe *B. pertussis* infection has not been completely characterised. Furthermore, Mal is now recognised to contribute to MyD88-independent and TLR4-independent signalling pathways.

In this study, Mal was found to be a vital host-protective signalling molecule that is required to prevent *B. pertussis* growth in the lungs, dissemination into the vasculature, and ultimate fatality. In $Mal^{-/-}$ mice there was a defect in a very early 'burst' of proinflammatory cytokines (including IL-1 , MIP-1 and TNF) in response to infection. Importantly, it was discovered that alveolar macrophages are vital to Mal-dependent protection against *B. pertussis* infection. With alveolar macrophages and bone marrow-derived macrophages *in vitro*, Mal was shown to be important for proinflammatory cytokine production, intracellular killing of *B. pertussis*, activation of the oxidative burst, and host cell apoptosis. *In vivo*, the severity of *B. pertussis* infection correlated with pathogen-induced depletion of alveolar macrophages. Although depletion of alveolar macrophages is associated with influenza infection and subsequent risk of pneumonia, bacterial infection of

the lungs alone has not previously been shown to cause depletion of alveolar macrophages. These data suggest that alveolar macrophages are vital for protection against *B. pertussis*, and that severity of lung bacterial infection (measured by fatality, bacterial growth and dissemination) correlates with alveolar macrophage numbers. Mal is a vital component of the initial alveolar macrophage response to *B. pertussis* infection and the signalling pathways mediated by Mal are important, not just for proinflammatory cytokine production, but also for activation of the oxidative burst and intracellular killing of *B. pertussis* by macrophages. These data also suggest that Mal is an important first line responder to invasive pulmonary bacteria, initiating the mobilisation of alveolar progenitors and at the same time activating programmed cell-death of *B. pertussis*-infected cells. In short, these data are evidence that Mal is a master regulator of lung bacterial infection and alveolar macrophage homeostasis.

Severely *B. pertussis*-infected $Mal^{-/-}$ mice were also found to have dissemination of bacteria into the vasculature and inflammatory foci in their livers. Only IFN-receptor knockout mice and NK-cell-depleted mice are known to be susceptible to disseminating *B. pertussis* infection. These data, therefore, indicate potential mechanisms of prevention of lung bacterial dissemination, contribute to our understanding of severe disseminating bacterial infection in general, and help to explain why certain individuals might be more or less susceptible to bacterial pneumonia. Understanding of these findings could, therefore, contribute to future attempts to make superior next-generation *B. pertussis* vaccines

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In this time of economic uncertainty, I heartily and humbly thank the Health Research Board of Ireland for giving me, a non-Irish non-EEA national, the funding that allowed me to complete these studies. I would also like to thank Paul Moynagh of the National University of Ireland for coordinating this cross-university HRB-funded PhD program, and Trinity College Dublin for hosting my studentship.

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... eat, poo, love.

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Contents

Abbreviations

ACT	adenylate cyclase toxin
AM	Alveolar macrophage
APC	antigen presenting cell
ATP	adenosine-5′-triphosphate
B. pertussis	*Bordetella pertussis*
BAL-F	broncho-alveolar lavage fluid
BBB	blood brain barrier
BCAP	B cell adaptor for PI3K
BCR	B cell receptor
BG	Bordet Gengou
BMDM	bone-marrow-derived macrophage
BrkA	*Bordetella* resistance to killing A
Btk	Bruton's tyrosine kinase
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
CFU	colony forming unit
CLR	C-type lectin receptor
CNS	central nervous system
COX2	cyclooxygenase 2
CRD	carbohydrate recognition domain
CREB	cAMP response element-binding protein
DAMP	damage associated molecular pattern
DC	dendritic cell
DTaP	diptheria, tetanus, acellular pertussis vaccine
E	glutamic acid

E. coli	*Escherichia coli*
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FasL	Fas ligand
FHA	filamentous haemagglutinin
FIM	fimbriae
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPCR	G protein-coupled receptor
GST	glutathione S transferase
H&E	haematoxylin and eosin
HMVEC	human lung microvascular endothelial cells
IFN-γ	interferon gamma
IgA	Immunoglobulin A
IL	interleukin
IL-1Ra	IL-1 receptor antagonist
IL-1RAPL	IL-1 receptor accessory protein-like 1
iNOS2	inducible nitric oxide synthase 2
IRAK	IL-1R-associated kinase
IRF	interferon regulatory factor
iT_{REG} cell	induced regulatory T cell
LOS	lipo-oligosaccharide
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MAC	membrane attack complex
Mal	MyD88 adaptor-like
Malp-2	macrophage-activating lipopeptide-2

MAPPIT	mammalian protein-protein interaction trap
MAVS	mitochondrial antiviral signalling protein
MHC	major histocompatibility complex
MyD88	myeloid differentiation primary response gene
NaCl	sodium chloride
NAD	nicotinamide adenine dinucleotide
NK	natural killer
NLRP3	NOD-like receptor pyrin domain-containing family 3
NO	nitric oxide
O_2^-	superoxide anion
OMV	outer membrane vesicle
P	proline
P_a	acellular pertussis vaccine
PAM_3Cys	Pam_3Cys-Ser-(Lys_4)
PAMP	pattern associated molecular pattern
PG	prostaglandin
PI3K	phosphatidylinositol-3-OH kinase
PIP2	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PRN	pertactin
PRR	pattern recognition receptor
PT	pertussis toxin
P_w	whole cell pertussis vaccine
RAGE	receptor for advanced glycation end products
RLR	RIG-I-like receptor
RORγt	RAR-related orphan receptor gamma t
ROS	reactive oxygen species

S	serine
SARM	sterile α- and armadillo-motif-containing protein
SFK	SRC family kinase
SIRS	systemic inflammatory response syndrome
SLE	systemic lupus erythematosus
SNP	short nucleotide polymorphism
SOCS	suppressor of cytokine signalling
STAT	signal transducer and activator of transcription
T	threonine
TA-1	transactivation-1
TAP	tracheal antimicrobial peptide
Tbx	T-box transcription factor
TCR	T cell receptor
TCS	two-component system
TCT	tracheal cytotoxin
T_{FH}	T follicular helper
TGF-β	transforming growth factor-beta
T_H	T helper
TIR	Toll/IL-1R/resistance
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TNF	tumour necrosis factor
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adaptor molecule
T_{REG} cells	regulatory T cells
TRIF	TIR domain-containing adaptor protein inducing IFN-β
VLA-5	very late antigen 5

Chapter One

General Introduction

1.1 *Bordetella pertussis*

In 1906 the Belgian scientists Octave Gengou and Jules Bordet isolated bacteria that they believed to be the causative agent of whooping cough. Jules Bordet, a disciple of the now famous cellular immunologist Elie Metchnikoff, gave his name to the bacterial genus, *Bordetella*, while Octave Gengou would have to be content with shared naming rights on the culture base used to grow it, *Bordet-Gengou* agar.

The *Bordetella* species most commonly isolated from patients with whooping cough, and thereby the best studied in humans, is *Bordetella pertussis*, although other *Bordetella* species such as *B. parapertussis* are also common. *B. bronchiseptica* can be pathogenic in immuno-compromised individuals,[1] but more commonly affects dogs, cats, pigs and other animals. Others that can infect humans, in rare cases, include *B. hinzii*, *B. trematum*, and *B. holmseii.* The bird-specific *B. avium* and the environmental *B. pertrii* have not been cultured from humans.

The bacteria that Bordet and Gengou isolated can cause a potentially fatal respiratory disease that has been called the '100-day cough' but is more commonly referred to as 'whooping cough' owing to the characteristic high pitch *whoop* sound at inhalation. The *whoop*, of unknown aetiology, is often followed by a paroxysmal bout of coughing. These contractions can be severe enough to induce post-tussive vomiting, asphyxia/hypoxia, and even the fracturing of bones of the rib cage.

Although vaccines are commonly used in industrialised nations to prevent *B. pertussis* infection, large areas of sub-Saharan Africa remain under-vaccinated, and there is a resurgence of fatal cases in highly vaccinated regions, including the USA, Canada, Australia and Western Europe.[2,3] Because of this resurgence, basic microbiological and immunological research are required to discover new ways to treat infection, design better vaccines, and prevent fatalities.

1.1.1 *B. pertussis* epidemiology

B. pertussis was once one of the most common childhood killers. Following widespread vaccination in the 1950s and 1960s, the World Health Organisation (WHO) January 4th 2013 Weekly Advisory Meeting report calculates that 83% of the global infant population was triple immunised, leading to an estimated reduction of the incidence and fatality of more than 90% relative to the pre-vaccination era. Despite this success, the WHO estimates that in 2008 there were still 16 million cases of *B. pertussis* infection, causing as many as 195,000 infant fatalities,[4] making pertussis one of the world's most fatal vaccine-preventable diseases.

The resurgence in *B. pertussis*-related illness and fatality is because of better reporting and diagnoses, poor vaccine practice in some sectors of society, and more worryingly, it might also be related to antigenic variation between vaccine strains and differences in chemical components of vaccine compositions.

Replacement of the whole cell *B. pertussis* vaccine (P_w) with a 'safer' acellular (P_a) form, consisting of inactivated pertussis toxin and other bacterial components, now a component of the tripartite DT_aP (diptheria, tetanus, acellular pertussis) vaccine, is widely suspected to be responsible for the emergence of long-term failure of immunity in vaccinated adults in industrialised nations, and for recent *B. pertussis* outbreaks.[5,6]

Evidence indicates that an important cause of *B. pertussis* resurgence is that the quality of the host immune response generated by newer vaccines leads to non-sterilising immunity and consequent transmission from infected immunised individuals.[6] As such, more accurate comprehension of *B. pertussis*-related pathogenesis, and a better understanding of the immune response to *B. pertussis* infection, is again of importance to global healthcare and basic research initiatives.

1.1.2 Clinical pathology of *B. pertussis* infection

B. pertussis does not ordinarily disseminate beyond the site of colonisation in the airways, therefore, most recognised pathology of severe infection is lung-specific. *B. pertussis* pathology includes tracheitis, bronchitis, necrotising bronchiolitis, pleural oedema, intractable pulmonary hypertension, and various types of alveolar and blood vessel damage.[7]

As early as 1925 it was recognised that, along with severe lung inflammation characterised by the accumulation of leukocytes, *B. pertussis* infection can lead to coincident features of systemic toxicity and pathology.[8] One type of agent of systemic damage are the bacterial toxins, some of which are known to enter the bloodstream and can affect the blood brain barrier (BBB), resulting in cerebral inflammation, encephalopathy, and consequent coma, seizures and death. Also, intractable pulmonary hypertension, observed in as many 75% of fatal cases, a consequence of bronchiolar and vascular blockage by immune cells, is known to be responsible for *B. pertussis*-induced cases of shock and cardiac failure.[9,10,11]

1.1.3 *B. pertussis* microbiology

The most commonly used experimental laboratory strain of *B. pertussis* is the Japanese patient isolate, *Tohama 1*. Microbiological culture of *B. pertussis* is slower than many other bacterial species. Cultured virulent bacteria are small, dome-shaped, shiny, white colonies that only become visible after approximately three days of culture on blood-enriched Bordet Gengou (BG) agar. BG agar includes glycerol as a carbon source, NaCl for osmotic balance, and potato infusion for essential nutrients.[12] Diagnostic laboratories involved in the primary isolation of suspected *Bordetella spp*. instead use Regan–Lowe charcoal blood agar as routine.

B. pertussis is an aerobic Gram negative coccobacillus arranged in singlets or as diplococci with an approximate length of 0.5 µm. Although various animals, including mice, can be infected with the bacterium, humans are the only natural reservoir of infection.

B. pertussis is non-motile due to the lack of flagellae, the locomotive whips present on many bacterial strains. Infectious particles have long been suspected to be transmitted as an inhaled aerosol created by the cough. Surprisingly, however, it was as recently as 2012, in a *Papio anubis* (baboon) model, that aerosol, as opposed to direct contact, was experimentally demonstrated to be an effective means of *B. pertussis* transmission.[13]

B. pertussis has an array of toxins and virulence factors that are vital for colonisation and persistence in the host, including lipooligosaccharide (LOS, labelled LPS in this study), pertussis toxin (PT), filamentous haemagglutinin (FHA), tracheal cytotoxin (TCT), adenylate cyclase toxin (ACT), pertactin (PRN), and fimbriae (FIM).[14] Expression and phenotypic modulation of these virulence factors is controlled by a BvgA-BvgS two-component system (TCS).

1.1.3.1 BvgA-BvgS Two-Component System

Expression of prokaryotic virulence factors is often controlled by a two-component system (TCS). TCSs are widespread signalling systems for bacterial interaction with the environment. The 'two components' ordinarily refer to a surface-expressed sensor of environmental inputs and a connected intracellular signal transduction component that controls gene expression. In the case of *B. pertussis,* the predominant TCS for control of virulence is the *bvg* locus encoded BvgA-BvgS, a member of the phosphorelay family of TCSs.[15] Signalling by this TCS requires

phosphotransfer between numerous BvgS histidine kinase environmental sensors before signal transduction to the intracellular BvgA regulator.

The phenotypic modulation of *B. pertussis* virulence factors by BvgA-BvgS is not as simple as an 'on-off switch', there is also an intermediate phase.[16] ACT and PT are known as 'late factors', whereas FIM and FHA are 'early factors'. Subtle changes in environmental stimuli such as temperature can regulate activation and the most efficient use of virulence factor expression.[17] Although the role of phenotypic modulation in infection is still unclear, one explanation for the role of temperature in diverging TCS function is that different virulence factors might be required for early stages of infection where the bacterium is located in the upper airways, compared to later stages in the lower airways where the temperature is higher.

High temperatures are known to counteract *B. pertussis* growth *in vitro*. Unlike *B. pertussis* grown on BG blood agar plates at 37°C, colonies grown at 39°C or 40°C are non-haemolytic. This is relevant for primate models of infection. Rhesus macaques, which have naturally high body temperatures, are less likely to get sick, relative to *Papio anubis*, which have natural body temperatures more similar to humans.[18] In the context of human infection the immune response to live *B. pertussis* does not include fever,[3] and yet heat-killed *B. pertussis* can cause fever, indicating that *B. pertussis* actively depresses host temperature control, possibly through BvgA-BvgS TCS-controlled ACT activity. It could be argued that the *B. pertussis* TCS not only responds to, but also actively 'terraforms', the environment.

Other environmental stimuli known to affect BvgA-BvgS include the concentration of important growth factors such as nicotinic acid or magnesium sulphate. Because of extreme sensitivity to these factors the growth conditions for *B. pertussis* in the laboratory are particular. Incorrect

dosage of components can lead to the growth of non-haemolytic avirulent cultures, and thereby misinterpretation of host-pathogen interactions.

1.1.3.2 Filamentous haemagglutinin

FHA is a secreted adhesin, which is also present on the bacterial cell surface. It includes three separate binding activities mediated by RGD, carbohydrate, and heparin-binding domains, allowing efficient adherence to ciliated epithelial cells via lactosylceramides,[19,20] and also to phagocytic cells by the complement receptor 3 (CD11b).[21] FHA has various immunosuppressive roles, predominantly via the induction of interleukin-10 (IL-10)-producing regulatory T (T_{REG}) cells, monocytes and dendritic cells, and the inhibition of IL-12-induced T helper type 1 (T_H1) cell responses.[22,23,24]

1.1.3.3 Pertactin

The outer membrane protein pertactin is an RGD-binding domain-containing protein with a role in adherence to epithelial cells and monocytes.[25] Although PRN-deleted mutant bacteria are able to colonise the lungs,[26] indicating some redundancy of adhesion mechanisms, one study identified that PRN is a protective antigenic target for immunisation,[27] leading to the inclusion of PRN in P_a vaccine formulations. There is some controversy as to whether or not PRN should be included in pertussis vaccine formulations.

1.1.3.4 Fimbriae

Although *B. pertussis* lacks flagella it does have the smaller and finer flagella-like structures known as fimbriae (FIM). FIM are vital for

bacteria-bacteria adhesion and bacteria–host cell adhesion. *B. pertussis* FIM include a minor subunit designated FimD, which binds very late antigen 5 (VLA-5), an integrin that is surface-expressed by monocytes.[28] The major fimbrial subunit is reported to act as a molecular mimic of the extracellular matrix (ECM) protein fibronectin, thereby allowing FIM to bind to sulphated sugars.[29]

1.1.3.5 Adenylate cyclase toxin

ACT, encoded by the *cyaA* gene, is an important AB-type toxin that has multiple roles in adhesion, cell death, and immuno-modulation. ACT specifically binds the integrin CD11b via glycosyl residues,[30] which enhance intracellular translocation of the toxin. ACT also activates a pore-forming function that is coincidentally responsible for the haemolysis of erythrocytes (although erythrocytes do not express CD11b) commonly seen on BG agar plates as a measure of virulence.

Following a poorly understood mechanism of membrane translocation into the cytoplasm of target cells, the adenylate cyclase domain of ACT interacts with mammalian calmodulin and increases the conversion of cyclic adenosine monophosphate (cAMP) from adenosine-5'-triphosphate (ATP), and in so doing inhibits phagocytosis and the oxidative burst in neutrophils and alveolar macrophages.[31] This adenylate cyclase function also induces cell-cycle arrest,[32] cell death, and apoptosis of macrophages;[33,34,35] although the mechanism remains unclear and is more complicated than the simple induction of toxic concentrations of cytoplasmic cAMP.[36] ACT has also been demonstrated to increase the concentration of intracellular Ca^{2+} thereby affecting expression of inflammatory mediators and cytoskeletal re-arrangement.[37]

Research on ACT has revealed that it has substantial immunomodulatory potential, including the inhibition of proinflammatory tumour necrosis factor (TNF) and IL-12, coincident with the promotion of anti-inflammatory IL-10

production and T_H2 responses,[38,39] which suppress intracellular antibacterial adaptive immunity. Converse to its anti-inflammatory role, ACT is also known to affect K^+ efflux as a result of its pore-forming function,[40] itself an initiator of NOD-like receptor, pyrin domain-containing family 3 (NLRP3) inflammasome activation and proinflammatory IL-1β release.[41]

1.1.3.6 Tracheal cytotoxin

Little is known about the function of the diaminoimelic acid (DAP)-type peptidoglycan muramyl peptide, TCT. Early studies *in vitro* with hamster tracheal cells identified that it can disrupt tight junctions.[42,43] To date, its major virulence function appears to be the damage of ciliated cells,[44] presumably to prevent the function of the 'mucociliary elevator' and consequent expectoration of inhaled bacteria. However, few studies have been carried out and early isolates of TCT could have been contaminated by significant quantities of *B. pertussis* LPS, which is thought to act synergistically with TCT.[45] LPS is well characterised to affect tight junction permeability as well as other effects attributed to TCT toxicity. A clear picture of TCT toxicity, thereby, remains to be defined.

The process of innate recognition of TCT and the consequent immune response is better understood. TCT can be sensed by the murine pattern recognition receptor (PRR) NOD-like receptor (NLR), NOD-1,[46] which leads to activation of antibacterial proinflammatory gene activation.

1.1.3.7 Pertussis toxin

PT, encoded by *ptx* is arguably the best known *B. pertussis*-specific virulence factor because: of its use as an enhancer of pathogenicity of the mouse model of multiple sclerosis (experimental autoimmune

encephalomyelitis); its importance for *B. pertussis* virulence; it's a basic component of most *B. pertussis* vaccines; and principally, because it is used *in vitro* as a classical activator of intracellular cAMP by preventing G_i proteins from interacting with membrane-bound G protein-coupled receptors (GPCR).[350]

In the 1970s the incorrect attribution of the P_w vaccine as the cause of encephalopathy[47] led to a general belief that PT permeabilises the BBB, and that this is the mechanism by which PT promotes EAE.[48,49] In fact, the function of PT in EAE may be more complicated, involving systemic effects on the activation and transmigration of autoreactive T cells into the brain.[50,51,52] It remains to be discovered if *B. pertussis* infection has any effect on the emergence of autoimmune disease.

PT is an exotoxin that requires the activity of a *ptl*-encoded type IV secretion system for its export across the bacterial outer membrane.[53,54] The toxin structure is AB5, indicative of an active subunit and a pentameric ring of five mammalian cell-binding subunits[55] that interact with glycosylated molecules allowing the docking and subsequent endocytosis of the toxin into target cells, including the lung-resident alveolar macrophage population.[56] *In vivo*, the primary toxic function of PT in these target macrophages, and other cells, is to inhibit G_i protein-coupled signalling pathways by nicotinamide adenine dinucleotide (NAD)-dependent adenosine diphosphate (ADP) ribosylation.[57] Numerous anti-microbial signalling pathways exist downstream of GPCRs. GPCR function in macrophages is known to include control of chemotaxis, adhesion, survival, activation, and inflammation,[58] indicating that PT could potentially interfere with all of these processes.

PT is important in the early stages of infection of the lungs because PT-deficient bacteria do not colonise the lungs as efficiently in the first 24 hours of infection.[59] PT targeting of alveolar macrophages is also thought to affect the recruitment of neutrophils by inhibiting the production

of neutrophil-attracting chemokines such as (C-X-C-motif) ligand 2 (CXCL2; also known as MIP-2), CXCL1 (KC), and the murine homolog of CXCL5, LIX.[60]

1.1.3.8 *B. pertussis* LPS

B. pertussis displays a type of *rough* lipopolysaccharide (LPS) coating where the O-side chain has been replaced with a non-repeating trisaccharide of α-*N*-acetylglucosamine, β-2-acetamido-3-acetamido-2,3-dideoxy-mannuronic acid, and β-l-2-acetamido-4-methylamino-fucose.[61] For this reason *B. pertussis* LPS is sometimes referred to as lipooligosaccharide (LOS) (Here the term '*B. pertussis* LPS' is used for simplicity). Although LPS is commonly considered to be a specific target for immune responses, this unique *B. pertussis* LPS functions as a significant virulence factor that aids lung colonisation by resisting antimicrobial binding by surfactant protein A.[62] It has also been shown that residual *B. pertussis* LPS is partially responsible for the incidence of convulsion in some P_w-vaccinated individuals.[63] LPS is the best studied pathogen-related molecule and has been implicated in an array of immunological processes. The most important functions relate to its immunogenicity; LPS is detected by Toll-like receptor 4 (TLR4).

1.1.4 *B. pertussis* infection and colonisation of the lungs

Infection begins with inhalation of aerosolised bacteria.[13] During this catarrhal stage, TCT mediates *B. pertussis* attachment to the tracheal epithelial cells. It also affects damage to the cilia, leading to ciliostasis, and allowing *B. pertussis* to move deeper into the lungs where bacteria can attach to epithelial cells in the bronchioles and alveoli via the adhesive function of FHA, FIM, PRN and ACT. This second stage, characterised by

the whooping cough, is referred to as the paroxysmal stage. It can last weeks or even months before convalescence. The unique *Bordetella* inspiratory *whoop* sound is aetiologically undefined, although there have been suggestions that bacterial bio-films or the inflammatory peptide bradykinin are involved in the cough mechanism.[64]

In the alveoli, various *B. pertussis* virulence factors combine to allow attachment and invasion of phagocytes.[21,65] Much of the knowledge surrounding these processes has been assumed from studies of non-pulmonary macrophage and monocyte populations. A complicated picture of *B. pertussis* adhesion and invasion has emerged. Binding by individual adhesins regulates the binding of others. For example, it is known that when FimD binds VLA-5, and also when PT binds macrophages via selectin-mimicry,[66] there is up-regulation of CD11b, which can itself serve as an attachment point for invasion via FHA.[28,67] Given that ACT is able to lyse $CD11b^-$ erythrocytes via its pore forming function, a similar undefined mechanism of pore forming might occur with resident alveolar macrophages, which are constitutively $CD11b^{lo}$ or $CD11b^-$. ACT entry in the absence of CD11b could be mediated by ACT-containing outer membrane vesicles (ACT-OMVs),[68] although they would not provide a means for whole bacteria to enter cells.

Invasion of phagocytic cells might seem to be an odd marker of virulence, but in fact, the CD11b-dependent method of invasion has been shown to bypass the oxidative burst ordinarily associated with phagocytosis, thus allowing intracellular survival and even proliferation of bacteria.[69] In this context, the lack of CD11b expression by resident alveolar macrophage populations might be interpreted as a host protective mechanism.

Human macrophages *in vitro*,[70] and mouse alveolar macrophages *in vivo*,[71] have been shown to house viable *B. pertussis*, providing evidence of intracellular escape of macrophage killing mechanisms, and possible evidence that *B. pertussis* can invade cells via anchors other than

CD11b.[72] Sequestration to intracellular niches could function as a means of avoiding extracellular innate anti-microbial host immune responses, as well as intracellular killing mechanisms that occur in certain compartments, such as lysosomes. Neutrophils, which are generally short-lived cells, have previously been suggested to be an unlikely medium-to-long term reservoir for *B. pertussis* persistence, but unpublished evidence suggests that ACT can inhibit neutrophil apoptosis.[73] Although $CD11b^{-}$, resident alveolar macrophages are a more obvious source of bacterial persistence, because they are lung resident and long-lasting. If *B. pertussis* does persist in these cells it could contribute to the long paroxysmal stage that is characteristic of severe infection.

1.2 THE IMMUNE RESPONSE TO INFECTION

Lasting protective immunity to *B. pertussis* and other infectious pathogens requires interplay between innate and adaptive immune responses, and the efficient activation of humoral and cell-mediated processes. Traditional antibody-mediated humoral responses such as neutralisation and opsonisation are considered to be specific for extracellular pathogens, such as parasitic worms, whereas cell-mediated adaptive immune responses, including $CD8^{+}$ T cell-mediated cytotoxicity and $CD4^{+}$ T helper cell activity, are thought to be more focused on intracellular pathogens such as invasive bacteria. In reality, both arms of the adaptive immune system are functional in response to infectious disease; viruses invade cells but can also be neutralised extracellularly, and bacteria are initially extracellular and will often invade cells or are phagocytosed. Furthermore, parasites such as plasmodium and trypanosome species often have multiple stages in their life cycles that require both intracellular and extracellular immune responses for protective immunity.

1.2.1 CD8$^+$ cytotoxic T cell-mediated immunity

CD8$^+$ T cells have MHC I-restricted αβ-TCRs and have cytotoxic function, principally targeting tumour cells and virally infected cells, but also cells with intra-cytoplasmic bacteria. Cytotoxicity is mediated by the cytolytic and apoptogenic activity of granzyme,[74] perforin and granulysin, and also via Fas ligand (FasL),[75] all of which induce caspase-3/7-mediated cell death mechanisms to prevent further pathogen or tumour spread. CD8$^+$ T cells also produce immuno-modulatory cytokines such as IFN-γ and TNF, which boost the antimicrobial function of phagocytic cells.

1.2.1.1 CD8$^+$ T cells and *B. pertussis*

The role of CD8$^+$ cytotoxic T cells in protection from *B. pertussis* remains to be identified. Few studies have identified a protective role for CD8$^+$ T cells. In one study, the P_a booster vaccine induced T_H1 and memory phenotype CD69$^+$CD8$^+$ T-cell responses in adolescents.[76] In another study, antigen-specific IFN-γ production by CD8$^+$ T cells was found in infected individuals, but this was CD4$^+$ T cell-dependent,[77] and in both studies the activation of CD8$^+$ T cells could be a consequence of *B. pertussis* infection and not necessarily protective.

In other research that has directly tested the protective effect of T cells, there appears to be little role for the CD8$^+$ subset. Passive transfer of CD8$^+$ T cells from donor mice immunised with a live attenuated *B. pertussis* vaccine, BPZE1, did not protect SCID mice from *B. pertussis* infection,[78] whereas the passive transfer of either CD4$^+$ T cells or antibodies did provide protection. In another study, transfer of purified CD4$^+$ T cells from immune mice limited *B. pertussis* growth in the airways, whereas transfer of CD8$^+$ T cells from the same immune mice actually exacerbated the subsequent infection.[79]

1.2.2 $CD4^+$ T_H cell-mediated immunity

$CD4^+$ T_H cells have MHC II-restricted αβ-TCRs and secrete cytokines that activate and direct other immune cells. In a seminal paper published in 1986, Mosmann *et al.* identified two classes of clonally selected and differentiated $CD4^+$ T_H cells,[80] which were subsequently identified as the intracellular pathogen-specialist T_H1 cells and the extracellular pathogen-specialist T_H2 cells. With the 'dichotomy-breaking' discovery in 2008 of a third group (T_H17 cells),[81,82,83] the theory of Mosmann *et al.* has since expanded into an ever more complex system of T-cell classification and nomenclature. It is now recognised that these T_H cell classes play multifactorial roles in diverse processes beyond anti-pathogen responses, including autoimmunity and tumour surveillance.

Following differentiation and activation, antigen-specific T_H effector cells can migrate to sites of infection and inflammation to deliver specific 'helping' effector functions. T_H1 cells are masters of helping macrophages to deal with intracellular pathogens, T_H2 cells help B-cell antibody-production specific for extracellular pathogens, and T_H17 cells help to recruit neutrophils and activate antimicrobial peptide production, providing protection from extracellular pathogens. There are, of course, many other functions attributed to $CD4^+$ T cells.

1.2.2.1 Differentiation of T_H cells

Naïve T cells can differentiate into effector T_H cells in response to T-cell receptor (TCR) ligation of antigen-presenting cell (APC) MHC II-restricted antigens, in combination with a co-stimulatory second signal for quantitative activation, and a cytokine milieu that directs proliferation and qualitative differentiation. As such, the differentiation of $CD4^+$ T cells is directed by innate immune recognition of pathogens.

In vivo, T_H cells are known to be poly-functional, able to produce mixed cytokine sets, making classification difficult and even unnecessary. *In vitro*, with the optimal stimuli listed below, the distinct T_H cell subsets can be highly polarised by particular differentiation stimuli.

In short summary: IL-12p70,[84] and innate sources of IFN-γ,[85] drive the expression of T-box transcription factor (Tbx21) and signal transducer and activator of transcription 4 (STAT4), which promote the production of the key effector cytokines IFN-γ, TNF, and lymphotoxin, and differentiate naïve T cells into T_H1 cells. IL-4 and IL-2 drive the expression of GATA3 and STAT6, which induce production of the key effector cytokines IL-4, IL-5 and IL-13 and differentiate naïve T cells into T_H2 cells.[86,87,88] IL-6 and transforming growth factor-beta (TGF-β)[89] drive the expression of RAR-related orphan receptor gamma 2 (RORγt) and STAT3, which induce the production of the key cytokines IL-17A, IL-17F and IL-22,[89,90,91] as well as IL-21, granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNF, and differentiate naïve T cells into T_H17 cells. IL-1 and IL-23 also promote the expansion of murine T_H17 cells and boost IL-17A production.[92]

1.2.2.2 Regulatory T cells

Early reports of 'suppressor T cells' went out of favour with the scientific community until they were rebranded as various subsets of regulatory T cells. The most common variants *in vivo* in mice are the thymic-differentiated $CD4^+CD25^+FoxP3^+$ 'natural T_{REG} cells, and the peripherally 'induced T_{REG} (iT_{REG}) cells, which include subtypes, such as the so-called Tr1 and T_H3 cells. iT_{REG} cells are differentiated in the periphery, or in the 'extreme periphery', *in vitro*. T_{REG} cells are thought to induce suppressive immuno-regulatory control, in part through the production of the anti-inflammatory cytokines TGF-β and IL-10.

The latest theory of T_{REG} cell differentiation and function suggests that rather than being an independent lineage, T_{REG} cells mirror and suppress the function of T_H1, T_H2 and T_H17 cells.[93] In support of this hypothesis is the evidence that T_{REG} cells variably require the same transcription factors required by those T_H effector cells for their differentiation. This work shows that IRF4-expressing T_{REG} cells are the counter-balance to IRF4-dependent T_H2 cells, because conditionally deleting IRF4 in T_{REG} cells leads to uncontrolled T_H2 development. It is possible that T_H2 cells are differentiated to deal with a threat, then once that threat is dealt with, a negative feedback loop inhibits their function by skewing development into the antithetical inhibitory IRF4-dependent T_{REG} cells. According to this theory T_H1-dominant and T_H17-dominant cells would also have their own 'mirror' T_{REG}-cell counterparts.

1.2.2.3 Other T_H cells

The classification of T_H cells is a work in progress. The strict definitions of the triad of proinflammatory T_H1, T_H2 and T_H17 cells have done a great service to those researchers able to culture and differentiate them *in vitro* with the aim of studying the function of T_H1, T_H2 and T_H17 immune responses. Less obvious, however, is that as a result, some disservice to our understanding of *in vivo* T_H cell function has occurred.

Poly-functional T cells including T_H1/T_H17 cells are important for antiviral[94] and antibacterial[95] long-term immunity in humans. Therefore, *in vivo* it sometimes makes sense to understand T_H1 or T_H17 'responses' as those that are dominated by IFN-γ or IL-17-producing T_H cells respectively. It makes less sense to speak directly of individual cells as T_H1 or T_H17 cells. But without multi-panel intracellular FACS analysis of multiple cytokines in single cells, such nomenclature persists and is generally accepted.

Although progress has been made toward identification and function, poly-functional T cells remain largely ignored.

The identification of IL-17-producing $CD4^+$ T_H cells as 'T_H17 cells' opened the door for further 'iconoclastic' nomenclature. IL-9-producing T_H cells, which are T_H2 cells that have been reprogrammed with TGF-β,[96,97,98] are sometimes labelled as 'T_H9' cells.[99] IL-6-driven, aryl hydrocarbon receptor (AHR) and T-bet-dependent, IL-22-producing $CD4^+$ T_H cells[100,101,102] have also been labelled as T_H22 cells.[103] This nomenclature exists despite the fact that T_H1 cells do not particularly produce IL-1 any more than T_H2 cells characteristically produce IL-2. In fact, IL-2 is often listed as a characteristic T_H1 cytokine.

Another T_H cell variant are the specialised B-cell-helpers required for germinal centre formation and maintenance, known as T follicular helper (T_{FH}) cells.[104,105] T_{FH} cells are either a separate differentiated lineage or, parallel with the previously mentioned T_{REG} cell theory on differentiation, they might be a heterogeneous population derived individually from T_H1, T_H2 or T_H17 cells. At least in this case the nomenclature does nothing to exacerbate confusion as to the nature of differentiation and lineage, because the title reflects the tissue location of T_{FH} cells, and not their function or cytokine production profile.

1.2.2.4 T_H responses and *B. pertussis*

In the context of *B. pertussis* infection, convalescent children are known to have T cell responses that are dominated by T_H1 cells.[106] Vaccination with P_w is reported to induce a similar degree of T_H1 response but is mixed with T_H17 responses. An important variance in T_H responses is seen in those individuals vaccinated with P_a vaccines, which are dominated by T_H2 responses, leading many to suspect that the T_H type of response is vital to vaccine efficacy or the protection afforded by natural infection. Vaccine

adjuvants have also been shown to skew T_H responses and the consequent resistance to *B. pertussis*.

Like natural immunity, the P_w vaccine induces T_H1 and T_H17 cells, whereas most alum-containing P_a vaccines induce T_H2 and T_H17 in mice. Vitally, if alum is replaced with CpG in those P_a vaccines, then T_H1 and T_H17 responses are induced, and superior protection, equivalent to P_w, is generated.[107] These data indicate that rather than wasting resources on the development of new vaccines, a potentially more efficacious option could be to replace the adjuvant component of already commercialised vaccine preparations.

1.2.2.5 T_H1 and *B. pertussis*

Studies have identified that T_H1 responses, and not T_H2 responses, are associated with *B. pertussis* convalescence in infants.[106,108,109] Also, IFN-γ receptor (IFN-γR) knockout mice develop a lethal disseminating *B. pertussis* infection and fail to clear bacteria from the lungs,[110] although these phenomena may relate to innate sources of IFN-γ rather than a T_H1-mediated protective function. A more convincing role for IFN-γ in the P_w vaccine was evident; P_w vaccination of IFN-$\gamma^{-/-}$ mice was not efficacious but the same vaccination of IL-17A$^{-/-}$ mice was efficacious.[107]

1.2.2.6 T_H2 and *B. pertussis*

T_H2 responses are important for the production of high anti-*B. pertussis* antibody titres. However, IL-4$^{-/-}$ mice have no defect in their ability to clear *B. pertussis* infection of the lungs, and importantly there is no differentiation or recruitment of T_H2 cells following infection of WT mice.[111] Furthermore, while the P_a vaccine does induce T_H2 responses, these were found to be less important than the T_H17 responses that are also induced

by P_a vaccination; IL-17A has an essential role, whereas IL-4 is redundant for immunity to *B. pertussis*.[107]

1.2.2.7 T_H17 and *B. pertussis*

There is no doubt that T_H17 cells are differentiated in response to *B. pertussis* infection, and that they play a role in the specific immune response. ACT has been shown to promote dendritic cell (DC)-mediated differentiation of T_H17 cells.[112] However, there is some controversy as to the role that T_H17 cells play in protective immunity to *B. pertussis*.

Given that immunopathology can be attributed to T_H17 cells in the context of certain autoimmune diseases, it has been suggested that anti-*B. pertussis* T_H17 responses might be responsible for the lung damage associated with severe *B. pertussis* infection.[5] However, more recent findings clearly show that innate sources of IL-17A production by lung resident innate T cells, such as $\gamma\delta$ T cells, are important for protective chemotactic signalling.[113] More importantly, murine studies have definitively identified a protective role for T_H17 responses[107,114,115] in the clearance of the bacteria.[41,116] However, the combination of T_H1/T_H17 is superior to T_H2/T_H17 indicating a vital role for T_H1 responses, and vaccination of IL-17A$^{-/-}$ mice was still efficacious, indicating that the protective role of T_H17 cells may not be entirely IL-17A-mediated.

1.2.2.8 T_{REG} cells and *B. pertussis*

B. pertussis can induce TLR4-dependent IL-10 secretion by DC, and that innate IL-10 can expand the pool of IL-10-secreting T_{REG} cells. Evidence indicates that these cells play a role in prevention of the lung immunopathology that is often associated with severe infection.[117]

1.3 ANTIBODY-MEDIATED IMMUNE RESPONSES

The term 'humoral immunity' is often used interchangeably with 'antibody-mediated immunity'; however it describes a wider network of non-cell-mediated immune responses. These include a diverse array of antibody functions, as well as the innate immune system's related complement system, and the unrelated activity of antimicrobial peptides, bacteriolysins, antitoxins, haemolysins, and generally any antimicrobial activity of the 'humours', i.e., the body fluids.

Typical antibody-mediated immune functions are actually cell-dependent. Such functions include the opsonisation and agglutination of pathogens so that effector B cells, macrophages, neutrophils, NK cells, and mast cells, can recognise them as foreign and either phagocytose them, or release lytic granules and immunomodulatory cytokines.

1.3.1 Antibody-mediated immunity to *B. pertussis*

Although antibody-driving cell-mediated T_H2 immune responses to *B. pertussis* infection may not be as vital as T_H1 and T_H17 responses, it has been shown in a limited number of studies that high anti-*B. pertussis* antibody titres correlate with immunity.[118,119,120] However, data indicates that in the same way that autoantibodies are coincident with autoimmune diseases, but are not always predictive of them, the presence of anti-*B. pertussis* antibodies in serum, and their correlation with immunity might be a simple coincident association with cell-mediated protection. This is supported by the observation that significant antibody titres are not even detectable until convalescent stages of *B. pertussis* infection in mice.[79]

Either infection or immunisation with the P_a vaccine induces high serum anti-*B. pertussis* antibody titres. Long-lasting protective immunity is only induced by immunisation with the P_w vaccine or previous infection

exposure, indicating that serum antibody titres are mostly a product of infection that play a role in secondary immunity, but not in protection from initial exposure. Memory T cells appear to be a closer determinant of long-lasting immunity.

1.4 THE INNATE IMMUNE SYSTEM

The innate immune system is the first line of defence against invading micro-organisms. It is also vital for guiding subsequent adaptive immune responses quantitatively and qualitatively. It consists mostly of mucosal immune and barrier-type cells such as epithelial cells, phagocytic cells, the antimicrobial factors secreted by those cells, and the receptor-driven signalling pathways that are responsible for sensing and responding to pathogens.

1.4.1 Antimicrobial peptides

Antimicrobial peptides were first identified more than 100 years ago, predating knowledge of cell-mediated immunity, and coincident with the first identification of antibodies by Paul Ehrlich in the late 20th century. Their antibacterial importance to human health was established with a number of discoveries made more than 50 years ago. The first antibiotics to be made commercially available were the protozoan gramacidins and tyrocidine after the microbiologist Rene Dubos first isolated them from tyrothricin in 1939.[121] Then in 1956 James Hirsch isolated phagocytin from the phagocytic granules of polymorphonuclear leukocytes,[122] which would have consisted predominantly of neutrophils.

Gramacidins and other antimicrobial peptides were later shown to exert their antimicrobial function by interfering with vital ionic gradients by inserting into the membrane of bacteria such as *E. coli,* causing permeability.[123] Although the hundreds of antimicrobial peptides that have

since been discovered have different specificity and mechanisms of action, in essence their membrane inserting function defines the eclectic group.

More recently it has been recognised that the *modus operandus* of some antimicrobial peptides is more complex than simple permeability. Once membrane inserted, some antimicrobial peptides target specific intracellular molecules involved in essential microbial processes such as metabolism, or nucleic acid and protein synthesis.[124]

1.4.1.1 Antimicrobial peptides and *B. pertussis*

An early systematic comparison of the concentration of antimicrobial peptides required to kill *B. pertussis* revealed that most tested peptides were efficacious, and more importantly that mutant strains of *B. pertussis* had altered resistance.[125] Fernandez *et al.* reported differential resistance by *bvg* and *BrkA* mutant *B. pertussis* strains to cecropin P1, indicating that *in vivo* there is likely a role for antimicrobial peptide resistance in *B. pertussis*-induced pathogenesis.

Various antimicrobial peptides, including defensins and cathelicidins, are produced in response to bacterial infection. In bovines the tracheal antimicrobial peptide (TAP) is secreted by tracheal epithelial cells[126] in response to LPS stimulation and NF-κB activation.[127] *B. pertussis* can counteract this β-defensin via virulence factor expression controlled by the type III secretion system.[128] In a pig model of *B. pertussis* infection, piglets are susceptible to *B. pertussis* infection, whereas older pigs are protected by a porcine homolog of β-defensin-1.[129] Interestingly, this molecule provides no such protection against the *Bordetella* strain that commonly infects pigs, *B. bronchiseptica*, perhaps indicating a role for β-defensin in the lack of non-human *B. pertussis* infection.

1.4.2 The complement system

The complement system is an array of serum proteins that either directly lyse pathogens or that 'complement' the antimicrobial function of antibody-mediated and cell-mediated immune processes by labelling, or opsonising, pathogens for immune recognition. Complement proteins ordinarily circulate in an inactive form and are activated by cytokines that can propagate protease-dependent cascades that culminate in the activation of the membrane attack complex (MAC) needed for lysis of target cells.

There are three known pathways of complement activation. Activation of the classical pathway requires the specificity of antibody—antigen complexes, whereas the alternative pathway and the lectin pathways can be activated directly, without specific antibodies.

1.4.2.1 Complement and *B. pertussis*

Jules Bordet, who discovered *B. pertussis,* first identified the complement system, which he called alexine, a heat-sensitive component of serum with antimicrobial function. Like other pathogens, *B. pertussis* has evolved mechanisms to prevent complement activation and the generation of the MAC. *B. pertussis* binds the complement inhibitor factor H, maintains its normal function, and in so doing inhibits complement-mediated killing via the alternative pathway.[130]

The classical pathway of complement activation is also inhibited by *B. pertussis,* via a protein called *Bordetella* resistance to killing A (BrkA),[131] and by inhibition of C1 via the C1 esterase inhibitor[132] and inhibition of C4b by the binding of C4b-binding protein to *B. pertussis* FHA.[133]

1.4.3 Resident lung cells and innate responses

Like other mucosal surfaces the lungs are a prime entry point for pathogenic invaders to enter the body. As such, the resident cells in the lungs that comprise the epithelial barrier and the other immune cells must be highly specialised and differentiated cells able to cope broadly with infections, but also with a degree of specificity for individual threats. The primary function of specialist immune cells in the lungs is to respond immediately to infection with antimicrobial measures to neutralise any threat. If the invading pathogen is able to resist these measures then the principal function of resident lung cells becomes immunomodulation and directing the recruitment of inflammatory cells and, if required, subsequently determining the nature of adaptive immune responses.

1.4.3.1 Epithelial cells

Arguably the most important cells in prevention of infection are the barrier cells, including skin cells, endothelial cells and the epithelial cells that line mucosal surfaces. In the lungs, epithelial cells are morphologically and functionally distinct, depending on the compartment. Upper airway epithelial cells consist mainly of ciliated stratified columnar cells interspersed with mucous-producing goblet cells and basal cells. This epithelial layer is specialised to capture and expectorate inhaled particles including pathogens. Deeper in the alveoli the epithelial cells consist of squamous epithelium interspersed by surfactant-secreting cells and macrophages. Surfactant functions principally to increase surface tension and to assist in the lung structural integrity during the mechanical perturbation of respiration. Surfactant A and surfactant D have also been implicated in innate immune function by opsonising bacteria for phagocytic removal.[134] In this context, surfactant has been shown to protect against various bacteria, including *Staphylococcus aureus.*[135]

In response to pathogen exposure, epithelial cells can perform various antimicrobial functions, including the secretion of antimicrobial peptides, cytokines and chemokines, and the tightening of intercellular bonds such as the tight junctions, thereby inhibiting translocation and dissemination of pathogens through the epithelial barrier.

1.4.3.2 Epithelial cells and *B. pertussis*

Given that conditional deletion of epithelium is not experimentally possible, *a posteriori* knowledge of the vitality of epithelial cells to anti-*B. pertussis* responses is difficult to determine. *B. pertussis* is a host-adapted pathogen; therefore, the protective mechanisms of the airway epithelial cells are mostly counteracted by *B. pertussis* virulence factors, with the exception of two vital epithelial functions. Firstly, *B. pertussis* does not readily disseminate out of the respiratory system into the lymphatic or circulatory vasculature, indicating that the barrier function of the epithelial layer is able to cope with *B. pertussis* infection. Secondly, the cytokine and chemokine production by epithelial cells is thought to be vital for the recruitment of protective inflammatory cells.

1.4.3.3 Natural killer cells

In 1983 Kumar *et al.* identified and characterised a resident population of natural killer (NK) cells in the lungs of mice.[136] NK cells generally possess a granzyme-mediated cytotoxic killing ability, typified by the inhibitory receptor-based recognition of low MHC I expression on tumour and infected cells, known as 'missing self'. In response to bacterial presence in the airways, NK cells also produce innate IFN-γ that is vital for the activation of macrophage killing ability, and they can skew T_H cell responses by producing IL-17 or IFN-γ.

1.4.3.4 NK cells and *B. pertussis*

Infection of NK-cell-depleted mice with *B. pertussis* results in a disseminating infection; viable bacteria can be cultured from the liver.[137] The only other example of *B. pertussis* dissemination is with IFN-$\gamma R^{-/-}$ mice.[110] NK cells are a major source of early innate IFN-γ in response to *B. pertussis* infection;[137] therefore, it is clear that NK cells play a vital role in containment as well as subsequent priming of IFN-γ-dependent T_H1 responses and macrophage activation.[138]

1.4.3.5 $\gamma\delta$ T cells

$\gamma\delta$ T cells are innate unconventional T cells with an invariant T-cell receptor (TCR), consisting of a gamma and a delta chain, believed to be specific for pathogen-produced lipid antigens and stress-related factors. Human $\gamma\delta$ T cells are known to possess some phagocytic potential,[139] but their cytokine producing and T_H-cell-skewing activity, mediated by the production of innate IFN-γ and IL-17, is much better characterised.

1.4.3.6 $\gamma\delta$ T cells and *B. pertussis*

$\gamma\delta$ T cells, mainly $V\gamma2^+$ (Garman's system) cells, are a major resident population in the lungs that would be expected to respond to *B. pertussis* infection. Following infection there is also substantial recruitment of $\gamma\delta$ T cells into the lungs,[140] although these cells might be phenotypically and functionally distinct. Further evidence for a role for $\gamma\delta$ T cells, and possibly also for a distinction between the resident $\gamma\delta$ T cells and the recruited cells, comes from another study that found that $\gamma\delta$ $TCR^{-/-}$ mice had less severe *B. pertussis* infection early, but greater pathology at later stages of infection.[113]

1.4.3.7 Pulmonary dendritic cells

Pulmonary DCs function in the lungs similarly to DC in other peripheral compartments. Their principal function is to sample antigen by phagocytosis, macropinocytosis or endocytosis; migrate to the draining lymph nodes, and 'professionally' present antigens on MHC I or MHC II molecules to activate T-cell responses. In the context of influenza infection, pulmonary DC have also been shown to be important for IL-15-dependent maintenance of anti-viral $CD8^+$ effector T cells in the lungs, independent from their role in the lymph nodes.[141] Pulmonary DC are also a major source of IL-12 and IL-18, which drive T_H1 responses, and IL-23,[142] which promotes T_H17 responses, indicating an important role in polarising T-cell subtypes. Like other lung cells, pulmonary DC have an immature phenotype in their basal state and lack co-stimulatory molecule expression.[143] The anti-inflammatory environment in the lungs, which is characterised by expression of IL-10 and prostaglandin E2 (PGE_2),[351-353] is suggested to maintain these DC in an immature state until pathogenic insult leads to rapid reversal and induction of proinflammatory responses. One of the most important cells responsible for the maintenance of such anti-inflammatory processes has been hypothesised to be the resident alveolar macrophage population.[354]

1.4.3.8 Pulmonary DC and *B. pertussis*

Pulmonary DC were once considered the sole presenter of *B. pertussis* antigens to T cells in the lung-draining mediastinal lymph nodes. Evidence from studies of other lung bacterial infections now indicates that alveolar macrophages are also capable of this function.[144] Also, like resident alveolar macrophages, pulmonary DC are involved in the initial response to *B. pertussis* infection, secreting proinflammatory cytokines, such as IFN-γ and IL-12p70. Non-resident $CD8\alpha^+$ DC are recruited to the lungs in response to *B. pertussis* infection, and function to induce protective T_H1

cells.[145] Pulmonary DC can also produce IL-1β, TNF, IL-6, IL-23 and IL-10 in response to *B. pertussis* infection, thereby modulating subsequent immune responses.

1.4.3.9 Alveolar macrophages

Alveolar macrophages are specialised cells that constitute approximately 95% of the cells that can be isolated from healthy mouse broncho-alveolar lavage fluid (BAL-F). This numerical predominance is indicative of a vital role in innate immune protection and their basal maintenance of the alveoli.

There is substantial confusion regarding the ontogeny of alveolar macrophages. Alveolar macrophages are no longer thought to be derived from circulating blood monocytes.[146,147] Following localised inflammatory activation in the tissues, $Ly6C^{hi}$ monocytes from the blood immigrate and can convert into macrophages, but steady-state repopulation and turnover of alveolar macrophages and other tissue-resident macrophages is most likely independent of blood monocytes, occurs locally, and is self-maintained.[146,147] If this understanding of the resident alveolar macrophage population is true, it indicates that studies of tissue macrophage populations in the context of inflammation, where there has been no distinction between the resident and monocyte-derived cells, are flawed in their interpretation of tissue macrophage function. Moreover, it is clear that the simplistic characterisation of all tissue-resident macrophages as M1 or M2 is of little benefit to our understanding of highly-specialised and distinctive tissue-specific macrophage populations, such as resident alveolar macrophages.

1.4.3.10 Alveolar macrophages and *B. pertussis*

Although alveolar macrophages are hypothesised to be a niche in which *B. pertussis* can hide from the immune system, clodronate depletion of alveolar macrophages enhances *B. pertussis* colonisation of the airways.[148] This suggests that alveolar macrophages are involved in long-term maintenance of bacterial colonies and dissemination, but that they are also involved in anti-bacterial killing or cytokine production to promote protective T_H1 responses.

1.5 PATTERN RECOGNITION RECEPTORS

PRRs are the key innate mediators of interaction between immune cells and pathogens. These receptors are highly concentrated on mucosal innate cells, such as epithelial cells, DC and alveolar macrophages. However, they are also known to be variably expressed by lymphoid cells, such as NK cells and conventional T cells and B cells.

Although PRRs have evolved to be far more complicated and varied, ironically they are functionally similar to the bacterial kinase-dependent environmental sensor-responder TCS, such as the *B. pertussis* BvgA-BvgS. PRRs respond to pathogen-related and damage-related molecular patterns in the environment, and affect the environment through transcriptional gene regulation via kinase-dependent signal transduction.[355]

In 1989, such simplistic understanding of receptor-signalling biology led Charlie Janeway to publish his now widely-accepted 'pattern recognition theory'.[149] This revolutionary theory was based on an understanding that innate microbe-sensing receptors could tailor immune responses to particular pathogen classes, or even particular bacterial species.[150] With typical Hegelian dialectical progression of theory, it was after a period of antithesis to Janeway's thesis, by a parallel concept of 'danger'

championed by Polly Matzinger,[151] that the synthesis co-evolved into our current understanding, that PRRs ligate highly conserved prototypic structures known as pathogen associated molecular patterns (PAMPs), or so-called damage associated molecular patterns (DAMPs), the products of damaged or infected tissues. It appears that PRRs sense 'danger', whether it is a microbial product or a component of damaged tissue, triggering inflammation to promote host defence and repair of injured tissue, respectively. The synthesis of these ideas, along with the recognition that each PRR can variably sense PAMPs from different species for alternative activation of the adaptive immune system is now the new thesis, a foundation for a 'grand theory' of microbial infection and immunity, and the basis for on-going developments in our understanding of cell stress, sterile inflammation and damage repair mechanisms.

Mammalian PRR signalling pathways interact and integrate, but also utilize independent signalling intermediates to affect a fine regulation that amounts to a specific response to a particular set of pathogens. Many of the signalling pathways converge on activation of transcriptional networks that control the production of proinflammatory cytokines and chemokines. PRRs are currently classified according to their ligand specificity and include C-type lectin receptors (CLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), and Toll-like receptors (TLRs).

1.5.1 C-type lectin receptors

CLRs are PRRs with extracellular carbohydrate recognition domains (CRDs), which ligate mannose (viral, fungal, mycobacterial), fucose (bacterial and helminth), and glucan (mycobacterial and fungal) carbohydrates present on diverse pathogenic species.[356]

CLRs are highly expressed on DC, and include receptors such as the mannose receptor, langerin, DEC-205, DC-SIGN, and dectin-1. Dectin-1 provides an important model for activation of ROS and phagocytic mechanisms. Dectin-1 was shown to become a phagocytic PRR only when bacteria were sensed directly, compared to the sensing of bacterial products from a distance, which requires the activation of proinflammatory cytokines, but not phagocytic and killing mechanisms.[152] These and other PRRs are not always simple sensors of the environment, they can be directly involved in antimicrobial activity. Mannose receptor and macrophage galactose-type lectin have been implicated in the proinflammatory response to heat-inactivated *B. pertussis* and *B. pertussis* LPS.[153]

1.5.2 NOD-like receptors

NLRs are expressed widely by innate immune cells including macrophages, DC, lymphocytes, and epithelial cells. The best-studied NLRs are NOD-1 and NOD-2. NOD-1 ligates the Gram negative bacterial peptidoglycan meso-diaminopimelic acid (mesoDAP), and NOD-2 detects bacterial muramyl dipeptide.

Another important group of NODs are the NALPs and NLRC4, which are involved in formation of the inflammasome, required for maturation of IL-1β and IL-18.[154] NALP3 constitutively active mutations lead to familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome, and other rare inflammatory disorders. In relation to *B. pertussis*, murine NOD-1, but not its human homolog, can detect the *B. pertussis* adhesin TCT.[46]

1.5.3 RIG-I-like receptors

RLRs, typified by RIG-I and MDA-5, are intracellular receptors for intracellular dsRNA,[155] indicative of the proliferation of dsRNA viruses. There is no evidence of a role for these receptors in *B. pertussis* infection.

1.6 TOLL-LIKE RECEPTORS

TLRs are frontline sensors of microbial infection and the best characterised PRR family. They trigger signalling cascades that culminate in increased expression of immune and inflammatory genes. TLRs have been identified in species as distinct from humans as the earthworm *Caenorhabditis elegans,* the plant *Arabidopsis thaliana* and the sea urchin, which is predicted to encode as many as 253 TLR sequences. These receptors are also ubiquitous within a given immune system. In humans and mice they have been identified on all known immune cells and also on cells sometimes considered to be 'non-immune' barrier-type cells such as epithelial cells.

1.6.1 Early history of TLR research

Before TLRs were discovered in vertebrates, it was known that bacterial LPS could bind to cell surfaces. The initial observation that LPS could cause endotoxic shock led to the discovery of a critical serum factor that bound LPS, LPS-binding protein (LBP). In 1980 Wright *et al.* described a white blood cell-surface molecule called CD14 that could act as a receptor for complexes of LPS—LBP.[156] The realisation that CD14 lacks a cytoplasmic tail necessary for signalling then began the hunt for further LPS-binding receptor molecules that might account for the observed induction of TNF and other LPS-inducible proteins. Although Toll had first been identified in 1988, as a transmembrane protein required for dorso-ventral polarity during development of *Drosophila melanogaster*,[157] it was

not until 1996 that Toll was shown to have antimicrobial function in that species.[158] Despite the identification of a number of 'Toll-like' receptors in humans, it was not until 1998 that Bruce Beutler's group first identified TLR4 as the LPS receptor.[159] The following year Shizuo Akira's TLR4$^{-/-}$ mice provided definitive evidence of the link.[160]

1.6.2 TLR signalling

TLRs have a base protein structure of an N-terminal extracellular leucine-rich repeat (LRR) domain connected to a transmembrane domain and the key signalling C-terminal cytoplasmic domain known as a Toll/IL-1R/resistance (TIR) domain.

The most important TLRs for recognition of bacteria and their products are homodimers of TLR4, specific for LPS;[159] TLR5 which recognises flagellin, the protein subunit component of bacterial motility known as flagella;[161] TLR9 which detects unmethylated CpG oligodeoxynucleotide DNA of bacterial and viral origin;[162] TLR1/2 heterodimers, specialised for triacylated lipopeptides of Gram positive bacteria;[163] and TLR2/6 heterodimers, which detect diacylated lipopeptides, also from Gram positive bacteria.[164] In mice, TLR11 can sense uropathogenic bacteria[165] and Salmonella,[357] and TLR13 recognises bacterial 23S ribosomal RNA.[166] Preliminary evidence indicates that TLR12 also detects bacterial products. The other TLRs have viral specificities: TLR3 binds double-stranded RNA,[167] and TLR7 and TLR8 ligate variations of viral single-stranded RNA.[168]

It should be noted that TLR-ligand 'interaction lists', such as the above, mask the actual complexity that occurs at the TLR-ligand interface. In the case of TLR4, for which the signalling mechanism is best understood, dimerization of TLR4 requires not only that LPS is bound by LBP, and detected by the cofactor CD14, but also the linking function of another molecule called MD-2,[169,170] which binds the lipid A moiety of LPS.[171] This complex interaction presumably leads to an activating rearrangement of

the TLR–TIR domains such that they are then able to interact with the cytosolic TIR domain-containing adaptors.

1.6.3 Proximal TLR signalling

Fine regulation of TLR signalling is known to occur at various stages of the TLR signalling cascade. Variation at the proximal end of the TLR4 signalling machinery is necessary as different bacteria produce various structural arrangements of the outer membrane glycolipid LPS. There are more than 150 variants in the O-chain of *E. coli* LPS alone.[172] The presence of full length O-chain, so-called 'smooth LPS', or reduction or absence of O-chain, so called 'rough LPS', differentially require the cofactor function of CD14 for effective TLR4 signalling.[173] Also the lipid A moiety of LPS varies greatly between species and has been suggested to confer a differential degree of endotoxicity, with *E. coli* LPS generally considered to be highly bioactive relative to other species, such as *B. pertussis* LPS.[174]

1.6.4 TLRs and *B. pertussis*

All of the cells that come into contact with *B. pertussis* in the early stages of airway infection, including the epithelial cells, pulmonary DC and alveolar macrophages, express surface TLRs (including TLR4) that could potentially respond to extracellular infection. Many of these cells also express intracellular TLRs for potential detection of intracellular infection or phagocytosed *B. pertussis*.

Although a number of *B. pertussis* PAMPs synergise with TLR activation, only *B. pertussis* LPS has been adequately proven to be TLR-specific. *B. pertussis*-infected TLR4-defective C3HeJ mice have more severe

infection with higher lung bacterial numbers and lung pathology, and TLR4 is involved in vaccine-induced protective immunity.[115,117] The evidence for other TLR–*B. pertussis* interactions is poor, either because of a lack of research in the area or because the data do not always support the claims.

There are a number of reports that PT can bind and activate TLR4,[175,176,177] but contamination of PT preparations with LPS is a confounding variable in the interpretation of some data. The effect of PT on TLR4-mediated immunomodulatory pathways *in vivo* remains questionable. Unsurprisingly, there is little evidence that mutant forms of PT are completely inhibited in TLR4 activation pathways. In any case, it would be unusual if TLR4, the highly-specific endotoxin receptor for all Gram-negative bacteria, were to have evolved an alteration to its binding specificity, at the risk of vital LPS specificity, that allowed it to bind a toxin from one relatively obscure species. PT might have evolved to bind TLR4, but for what purpose? TLR4 is known to have extracellular glycosylated regions, and PT is known to bind glycosylated regions, so it is possible that PT binds TLR4 without activating the receptor. Binding might simply assist PT in *B. pertussis* invasion.

The simple explanation for the reported TLR4—PT interaction is that PT is very difficult to purify or manufacture without contamination. PT could enhance LPS—TLR4 signalling, as has been reported,[175,176,177] but not necessarily by binding and activating TLR4 itself, rather it could do so via its well-documented intracellular enhancement of cAMP concentrations.[31,36] It might be expected that PT binding to TLR4 would only inhibit LPS binding by masking TLR4 glycosylation sites.

Owing to a lack of flagella, there is no flagellin-specific TLR5 activity in response to *B. pertussis*. However, it is thought that other *B. pertussis* components are sensed by TLRs. TLR1/2 and TLR2/6 heterodimers are candidates for recognition of *B. pertussis* lipoproteins. Although these receptors might have evolved to detect Gram-positive bacteria, which

contain large amounts of lipopeptide and lipoteichoic acid in their outer membrane structures, *B. pertussis* also includes many lipopeptide components. One such candidate is a characterised ornithine-containing lipid[178] with haemagglutinating activity.[179]

1.7 The TIR DOMAIN-CONTAINING SIGNALLING ADAPTORS

Ligand-induced TLR dimerisation leads to the recruitment of cytosolic Toll/IL-1R/resistance (TIR) domain-containing adaptors. These adaptors activate specific sets of signalling molecules, which in turn activate transcription of particular genes that are fine-tuned to counteract particular microbial infections.[355]

There are currently four well-characterised TLR adaptors: Myeloid differentiation primary response gene 88 (MyD88), which signals for all TLRs except TLR3; MyD88 adaptor-like (Mal), the focus of this thesis, which only signals downstream of TLR4 and TLR2; TIR domain-containing adaptor protein-inducing IFN-β (TRIF), which is specific for TLR3; and TRIF-related adaptor molecule (TRAM).[355] There are also two less-well studied cytoplasmic TIR domain-containing molecules that are proposed to be inhibitory TLR adaptors: Sterile α- and armadillo-motif-containing protein (SARM), which is a negative regulator of TRIF in humans; and B cell adaptor for PI3K (BCAP), a newly identified inhibitory adaptor.[180,181] Knowledge of BCAP is still lacking and the vast majority of function attributed to SARM is TLR-independent, especially in non-humans. Although they are clearly TIR domain-containing adaptors of a sort, future work will determine if these molecules continue to be labelled as TLR adaptors.

1.7.1 MyD88

MyD88 was the first, and is now the best, characterised cytoplasmic TLR adaptor, yet it is not dedicated to TLR signalling. A series of articles published between 1994 and 1997 described the function of MyD88 as part of the IL-1R1 signalling machinery.[182,183,184,185] It was not until 1998 that MyD88 was shown to play a similar role in the newly discovered TLR signalling.[186] More recent evidence indicates that it might also be a component of the IFN-γ signalling complex.[187]

For signalling to occur, MyD88 is recruited to interact with TLRs (except TLR3) at the cell or endosomal membrane. In the context of TLR4, and to a lesser extent TLR2, MyD88 requires Mal to act as a bridge for membrane localisation, whereas TLR5, TLR7 and TLR9 use the MyD88 pathway, but do so independent of Mal. Following interaction between the TLR–TIRs and MyD88–TIR the formation of a signalling complex, termed the 'Myddosome', that has been shown *in vitro*, is hypothesized to propagate signal transduction.[358]

According to crystal structure analysis the Myddosome is a hetero-complex of six MyD88 death domains (DDs) interacting with the DDs of four IL-1R-associated kinase 4 (IRAK-4) and four IRAK-2 or IRAK-1 molecules.[188] Phosphorylation of IRAK-1 by IRAK-4 then propagates signalling via an interaction with TNF receptor-associated factor 6 (TRAF6),[189] resulting in activation of the MAP kinase pathways and NF-κB-mediated induction of proinflammatory and antimicrobial gene expression, including IL-6, IL-1β and TNF.

Evidence now indicates that the function of MyD88 is more complex than previously appreciated. MyD88$^{-/-}$ mice, which lack TLR2/6-mediated sensing of diacyl lipopeptides, are susceptible to *Streptococcus pyogenes,* whereas mice with a mutation in the so-called *poc* site of MyD88 have a functional TLR2/6, but other MyD88-dependent TLR signalling is not functional.[190] Unlike MyD88$^{-/-}$ mice, *poc* mutant mice are resistant to

S. pyogenes infection. Clearly, there are different interactive surfaces of MyD88 that give it a true adaptor function, the ability to differentiate between different TLRs and activate alternative signalling pathways in response to specific PAMPs and DAMPs. Thereby, TLRs may be the sensors of specific pathogens, but the adaptors are the determinants of the immune response.

1.7.2 TRIF

After the discovery of MyD88 it was realised that LPS could drive delayed activation of NF-κB and MAP kinase pathways, even in MyD88$^{-/-}$ mice,[191] and that the same pathway could maturate MyD88$^{-/-}$ DCs[192] and induce the production of interferon regulatory factor 3 (IRF3) and a specific panel of interferon-inducible genes.[193] Clearly a TLR4-specific MyD88-independent pathway existed. After falsely identifying this pathway to be controlled by Mal, it was discovered that TIR domain-containing adaptor protein inducing IFN-β (TRIF) was the mystery adaptor for the MyD88-independent pathway.[194,195] It is currently known to signal in response to TLR4 and TLR3.

TRIF signals at endosomes where TLR3 is co-localised to detect viral double-stranded RNA in infected cells.[194,195] TRIF-dependent TLR4 signalling is more complicated because TLR4 senses extracellular LPS. TRIF is thought to function in this context only after LPS—TLR4 complex internalisation, in a process mediated by the bridging adaptor TRAM.

1.7.3 TRAM

TRIF-related adaptor molecule (TRAM) is so named because it bridges TRIF with TLR4 to induce IRF3 and IRF7, and IFN-β and RANTES production.[196,197,198] There is, therefore, no LPS-induced MyD88-

independent signalling pathway without TRAM. In bone marrow-derived DC (BMDC) the switch from MyD88—Mal-dependent to TRIF—TRAM-dependent, i.e., MyD88-independent, signalling is a sequential process controlled by the p110δ subunit of phosphatidylinositol-3-OH kinase (PI3K).[199]

1.7.4 SARM

In humans, SARM is a negative regulator of TRIF signalling in myeloid cells.[200] The evidence that SARM is a TLR adaptor in mice is unclear as most functions of SARM are now TLR-independent functions relating to apoptosis responses to viral infection, or brain cell homeostatic mechanisms for axonal maintenance.

SARM is expressed mainly in the central nervous system (CNS). The axons of $SARM^{-/-}$ mice are protected from Wallerian degeneration,[201] an active cytokine-dependent process for the removal of injured axons, prior to the regeneration of new ones. West Nile virus-infected $SARM^{-/-}$ mice are defective in TNF production, indicating a positive role for SARM in proinflammatory antimicrobial responses, countering evidence that it is an inhibitory adaptor.[202] Mukherjee *et al.* recently discovered that murine SARM is an RLR signalling adaptor as Bunyavirus infection activates mitochondrial antiviral signalling protein (MAVS) and up-regulates SARM.[203] This study provides further evidence that SARM plays a key role in axonal death regulation, and that SARMs principal role might not be as a TLR adaptor. The literature relating to SARM is rapidly evolving. Hopefully these most recent studies will soon contribute to a resolution in our understanding of this TIR domain-containing adaptor.

1.7.5 BCAP

B cell adaptor for PI3K (BCAP) was first identified as an adaptor linking the B cell receptor (BCR) to PI3K–Akt activation.[204,205] BCAP was also identified as the 'missing link' TIR domain-containing adaptor between TLRs and PI3K–Akt.[180,181] BCAP negatively regulates TLR-dependent cytokine production.

1.8 Mal (TIRAP)

Mal was the second discovered TIR domain-containing TLR adaptor. It was discovered independently by Horng *et al.*[206] and Fitzgerald *et al.*[207] and named TIR domain-containing adaptor protein (TIRAP) or MyD88 adaptor-like (Mal), respectively. Although the human gene name is *TIRAP*, the murine protein is still referred to interchangeably as either Tirap or Mal.

The discovery of Mal was a result of the search for the mystery MyD88-independent pathway adaptor, which turned out to be TRIF. Following this a series of publications shed light on the function of Mal, indicating that its principal function is to serve as a bridge between MyD88 and TLR4.[208,209]

It is now thought that Mal can also activate signal transduction pathways that are independent of, and thereby 'unlike', MyD88. Furthermore, the most recent finding is that, contrary to established dogma, Mal might also be involved in endosomal TLR signalling.[359]

1.8.1 Structural Biology of Mal

In 2011 Valkov *et al.* published the first crystal structure of Mal–TIR at a resolution of 3.0 Å,[210] and suggested dissimilarities between Mal–TIR and the previously solved TLR1, TLR2, TLR10,[211] MyD88,[212] and IL-1 receptor accessory protein-like 1 (IL-1RAPL)[213] TIR domains. This study revealed

that Mal–TIR has an extra-long AB loop, rather than the expected BB loop of other TIR domains. Subsequently, residues 79–221 of Mal–TIR were identified by Lin *et al.* at a superior resolution of 2.4 Å,[214] indicating that the protruding AB loop can bind both TLR4–TIR and MyD88–TIR.

High resolution crystal structures also provide definitive evidence that the homo-dimerization of Mal, previously demonstrated by yeast 2-hybrid analyses, glutathione S transferase (GST) fusion and coimmunoprecipitation assays,[207] is mediated by the Mal–TIR domains. These back-to-back homodimeric Mal–TIR domain crystal structures demonstrate how vital the TIR domains are for proper assembly not only of the Mal—MyD88—TLR4 signalling complex, but also of the quaternary structure of Mal itself.

1.8.2 The role of Mal in TLR2 signalling

TLR2 functions as a heterodimer with TLR1 or TLR6 to sense bacterial acylated lipopeptides and lipoteichoic acid. Early studies identified Mal as an adaptor critical for TLR4-mediated and TLR2-mediated activation of NF-κB and MAP kinase pathways.[206,207,208,209] The dependence of TLR4 signalling on Mal is now established. More recently however, the role of Mal in TLR2 signalling has been questioned.

Kenny *et al.* found that Mal is only necessary for IL-6 or NF-κB induction from TLR2 signalling at low ligand concentration.[215] At high dose of the TLR1/2 ligand Pam_3Cys-Ser-(Lys_4) (Pam_3Cys) or the TLR2/6 ligand macrophage-activating lipopeptide-2 (Malp-2), and also with high multiplicity of infection (MOI) of macrophages with *Salmonella enterica*, Mal deficiency has no effect, indicating that Mal might sensitise TLR2 signalling when the degree of activation is low. A study by Cole *et al.* supported these findings by demonstrating TLR2-dependent, but Mal-

independent, signalling in response to *Francisella tularaemia* through retention of bacteria within phagosomes, also highlighting the importance of localisation of TLR2 PAMPs.[216]

Further study in this area may yet reveal important Mal-dependent TLR2 signalling in the context of human infection *in vivo,* because colonisation usually begins with relatively low bacterial numbers and thereby with a relatively low concentration of TLR2 ligands. The identification of the S180L MAL variant, which attenuates TLR2 signalling and protects from bacterial infection when expressed heterozygous, highlights that the 'strength' of this initial Mal-mediated response to infection might be vital for subsequent control of certain pathogens. Initial exposure *in vivo* to low dose TLR2 bacterial ligands might be necessary to catalyse protective immune responses that could prevent bacterial growth and subsequent immunopathology.

In a model of gastritis and gastric cancer in $gp130^{F/F}$ mice that is dependent on TLR2 signalling, Kennedy *et al.* found that MyD88 was essential but there was no role for Mal.[217] It remains to be determined if Mal-dependent TLR2 signalling is important for aspects of antimicrobial immunity that are unrelated to tumour growth biology.

It should be noted that almost all published studies on the role of Mal in TLR2 signalling *in vitro* have experimentally used Malp-2 or Pam_3Cys, both of which are highly specific and bioactive molecules. It is possible that different bacteria produce lipopeptide variants that are far less bioactive. Consistent with this hypothesis is that there are significantly different bioactivities of LPS from different bacterial species, partly attributable to the different lipid A structures.[174] Therefore, it is possible that TLR2-mediated detection of certain bacterial strains is more reliant on Mal than is currently understood. An examination of a wider variety of bacterial lipopeptides with Mal-defective cells could clarify our understanding of the role of Mal in TLR2 signalling.

1.8.3 The bridge to MyD88

Mal is often described as a bridging adaptor due to its primary function of recruiting cytosolic MyD88 to interact with the activated TIR domains of TLR4 dimers at the cell membrane, thus catalysing formation of the Myddosome.[218] The ability of Mal to recruit and catalyse the formation of the Myddosome is dependent on unique structural elements. Crystal structure determinations of Mal identified that unlike the BB loop-containing TIR domains of other TLR signalling molecules, the unique extra-long protruding AB loop of Mal–TIR[210] appears to be essential for binding of Mal with MyD88–TIR and TLR4–TIR.[214] In essence, the AB loop is a component of the 'bridge'.

Another vital bridging feature of Mal is the presence of an N-terminal phosphatidylinositol 4,5-bisphosphate (PIP2) binding domain,[219] which specifically binds TLR4-rich regions of the plasma membrane. Once membrane-anchored via PIP2, spatial localisation facilitates interaction between Mal–TIR and the TIR domains of MyD88, TLR2[209] and TLR4.[207,214]

These membrane interactions play a vital role as important regulatory components that can skew immune responses either into MyD88–Mal-dependent surface membrane signalling pathways, or TRIF–TRAM-dependent endocytic signalling pathways. In this context, Mal interacts with PI3Ks, including the class 1A p110δ isoform, which controls the cellular compartmentalisation of TLR4 by converting PIP2 to PIP3, thus masking plasma membrane anchor sites for Mal,[199] and thereby propagating the MyD88-independent endocytic TRIF pathway of TLR4 signal transduction.

1.8.4 MyD88 bridging-independent functions of Mal

It is now generally accepted that Mal is more than just a bridge to MyD88. Studies have identified 'MyD88 bridging-independent' (not to be confused with the MyD88-independent TRIF pathway) functions of Mal (Figure 1.1).

1.8.4.1 NF-κB nuclear translocation and transactivation

TLR signalling activates NF-κB by interfering with IκB inhibition of translocation to the nucleus. However, PI3K subunit p85α-deficient B cells do not respond to LPS,[220,221] indicating that there is a parallel TLR–PI3K-dependent pathway of NF-κB activation. Transactivation of the NF-κB subunit p65 involves the serine phosphorylation of its transactivation 1 (TA-1) domain by PI3K–Akt kinase. The combination of these parallel pathways, nuclear translocation and transactivation, are thought to be essential for optimal activation of NF-κB.

In 2000 Arbibe *et al.* showed transactivation of the nuclear p65 subunit by identifying TLR2–Rac1 as an activator of PI3K–Akt.[222] The small GTP-binding protein, Rac1, activates NF-κB activity, partly through phosphorylation of IκBα,[223] allowing nuclear translocation, but also through PI3K-mediated p65 transactivation.[224] For inflammatory gene expression to occur, NF-κB must first be released by IκB for translocation to the nucleus where it can localise to κB-responsive elements present on relevant genes for regulation.

In the context of TLR2 signalling, Santos Sierra *et al.* found that Mal connects TLR2/6 to PI3K activation, yet TLR1/2 mediated Akt phosphorylation is Mal-independent.[225] Diacylated lipopeptide was found to induce PIP3 formation at the leading edge of macrophages. This is a MyD88-independent Mal function, distinct from NF-κB activation.

Sanlioglu *et al.* also reported LPS-induced Rac1-dependent NF-κB activation, thus implicating TLR4 in propagating the Rac1–PI3K–Akt

pathway.[226] This evidence is supported by other data showing that LPS stimulation of human microvessel endothelial cells also induces Rac1.[227] In that study Equils *et al.* also found that dominant negative expression of Mal, but not dominant negative expression of MyD88, blocked Rac1-induced regulation of the HIV-long terminal repeat, thus not only implicating Mal in MyD88-independent Rac1–PI3K–Akt NF-κB transactivating pathway, but also in modulation of HIV replication (Figure 1.2).

Coincident with the identification of a role for Mal in the NF-κB transactivating pathway was the discovery of a putative TRAF6 binding site on Mal–TIR at amino acid position 188–193.[228] The Pro–Pro–Glu–Leu–Arg–Phe was similar to previously characterised TRAF6 binding sequences on IRAK and TRIF, but importantly it was not present on MyD88. Mansell *et al.* validated the interaction by coimmunoprecipitation of TRAF6 with Mal. In this study over-expression with Mal E190A, a mutation of the TRAF6 binding site, inhibited TLR2 or TLR4 induction of an NF-κB reporter gene relative to WT Mal. Verstak *et al.* further characterised this observation in 2009, confirming that Mal binds TRAF6 and recruits it to the plasma membrane.[229] Importantly this study identified that interaction between Mal and TRAF6 is required for the serine phosphorylation of p65, thereby determining a mechanism of Mal-dependent NF-κB transactivation.

Although Mal can bind TRAF6 directly, independent of MyD88, in a network sense this function is obviously integrated with MyD88 signalling, as both NF-κB translocation and transactivating p65 phosphorylation are necessary for optimal inflammatory cytokine gene expression. These two pathways might operate in parallel or sequentially.

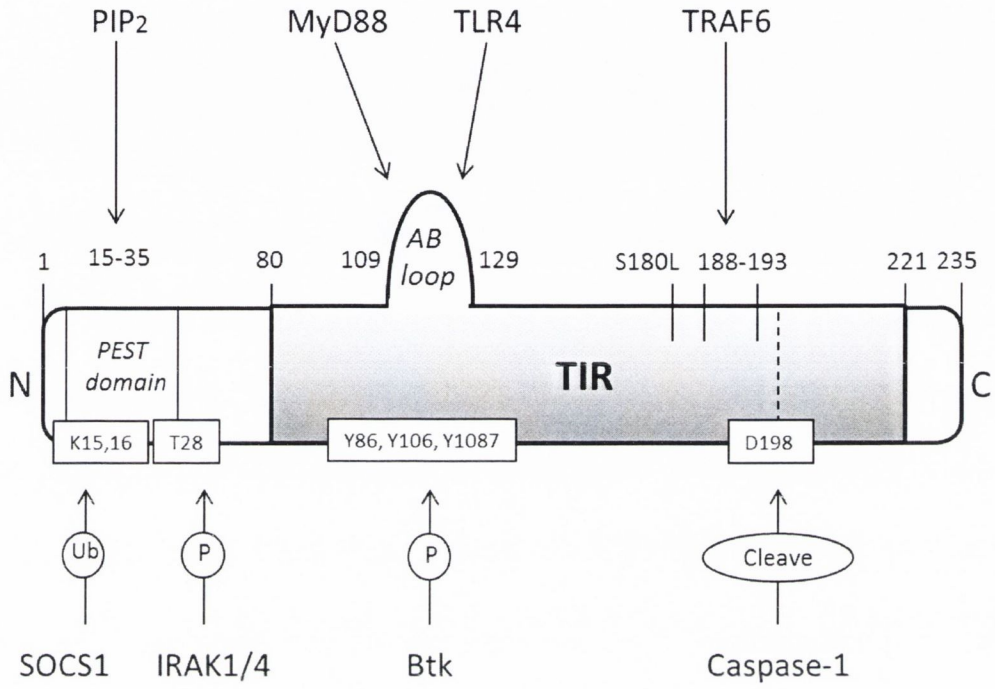


Figure 1.1 Mal

Mal binding sites (above) and post-translational modifications (below) and domains. Human and mouse MAL sites are presented together. P = phosphorylation. Ub = polyubiquitination.

1.8.4.2 Btk and SOCS-1

In parallel with the serine/threonine kinases that post-translationally modify TLR4 signal transduction are a group of LPS-activated tyrosine kinases with a less-well defined role in TLR signalling. In 2002 Mukhopadhyay *et al.* realised that one of these, a member of the TEC family of protein tyrosine kinases, known as Bruton's tyrosine kinase (Btk), is important for antimicrobial activity including nitric oxide (NO) and proinflammatory cytokine production.[230] Subsequently, Jefferies *et al.* discovered that Btk is involved in TLR4 signalling and that it interacts with Mal.[231] This pathway was further clarified in 2006 when Gray *et al.* showed that Btk phosphorylates the TIR domain of Mal at tyrosine residues 86, 106, and 187.[232] When these Mal tyrosine residues were replaced with phenylalanine, LPS-induced and Malp-2-induced signalling were inhibited, indicating that Btk phosphorylation of Mal activates, and is necessary for, TLR2-induced and TLR4-induced NF-κB activation.

Subsequently both p65 and Mal can be polyubiquitinated for rapid proteasomal degradation, in the case of Mal as a result of binding between its PEST domain, a peptide sequence rich in proline (P), glutamic acid (E), serine (S), and threonine (T), and the SH2 domain of suppressor of cytokine signalling 1 (SOCS-1), thus forming a negative regulation on Mal-dependent p65 phosphorylation.[233]

1.8.4.3 CREB and IL-10

A further TRAF6-dependent role for Mal, distinct from NF-κB activation, was revealed in 2011. The transcription factor cAMP response element-binding protein (CREB) was first shown to regulate expression of the somatostatin gene,[234] and has since been recognised to transactivate a variety of immune-related genes including TNF,[235] cyclooxygenase 2 highlighting the fact that Mal can modulate both proinflammatory and anti-inflammatory responses via different interactive surfaces of the protein, (COX2),[236] and IL-10.[237] Mellett *et al.* identified that in response to LPS

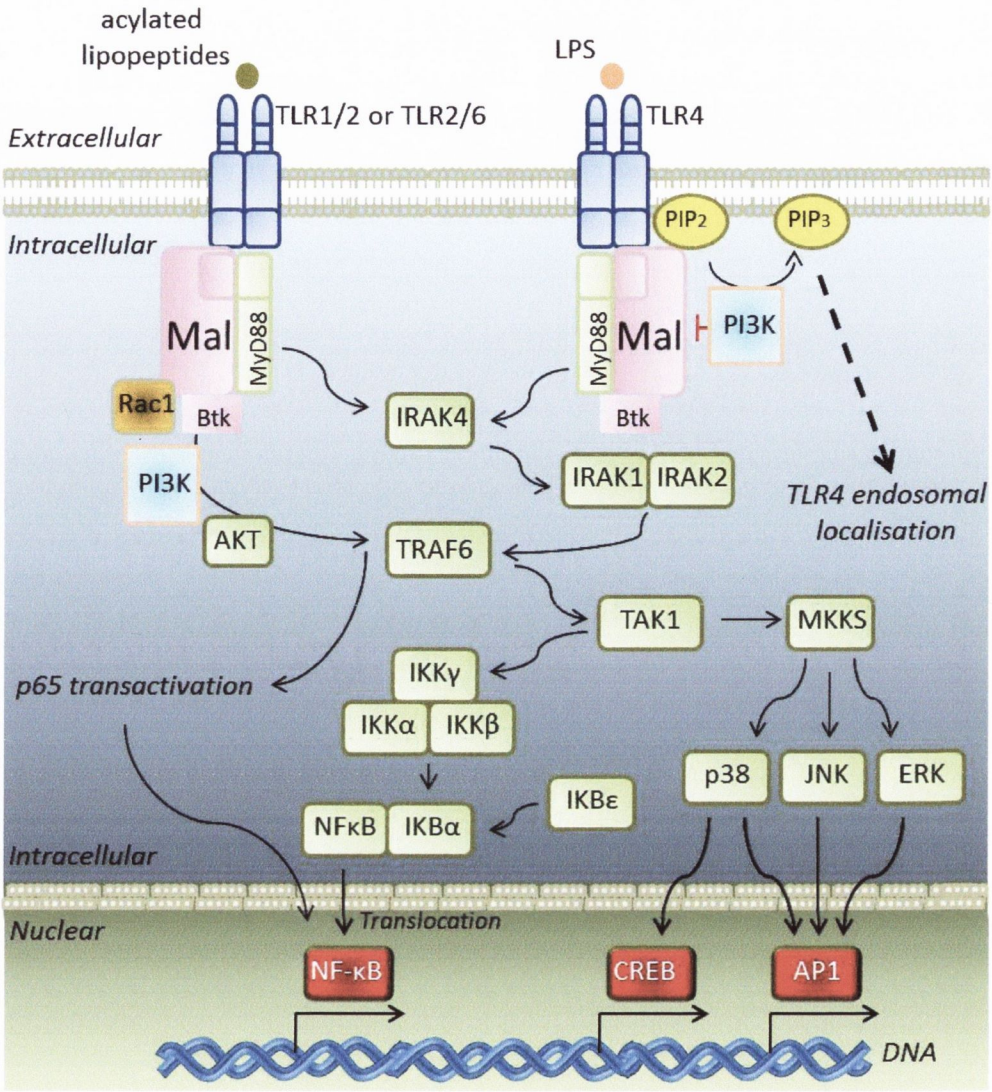


Figure 1.2 Mal-dependent TLR signalling

Mal only signals downstream of TLR2 and TLR4. TLR4 is activated by LPS. TLR1/2 heterodimers are ligated by triacylated lipopeptides. TLR2/6 heterodimers ligate diacylated lipopeptides. Mal recruits MyD88 to the plasma membrane leading to NF-κB nuclear translocation via IRAK4, IRAK1/2, TRAF6, TAK1, and release of NF-κB from IκB. Mal transactivates p65 NF-κB via a MyD88-independent Rac1, PI3K, Akt, TRAF6 pathway. Mal can also activate CREB independent of MyD88 via p38 Map kinase kinase (MKK) pathways. The PI3K p110δ isoform can negatively regulate Mal signalling by converting PIP2 to PIP3 thus removing the anchor point for Mal and pushing TLR4 into endosomes for TRIF/TRAM dependent signalling.

stimulation, Mal can enhance CREB activation by signalling through TRAF6, Pellino3, p38 MAPK and MK2.[238] Another interesting discovery from this study was that Mal E190A mutants had no defect in their ability to activate CREB despite a known inability to activate NF-κB, thus leaving open the possibility that Mal could be therapeutically targeted to modulate this balance in a clinical setting.

1.8.4.4 Caspase-1 and the inflammasome

In 2007 a yeast 2-hybrid screen picked out a unique Mal interacting clone encoding the catalytic domain of caspase-1.[239] The cysteine protease caspase-1 is known to cleave pro-IL-1β and pro-IL-18 into biologically active cytokines for secretion. In this context caspase-1 is requisite for the complex known as the 'inflammasome'.

Because the production and secretion of mature IL-1β require both TLR and inflammasome activation,[154] it is possible that TLR stimulation might directly regulate inflammasomes. However, there has been little evidence of signalling interaction. In the first piece of evidence that there is direct physical interaction, Miggin *et al.* found that caspase-1 does not cleave MyD88 but does cleave Mal at position D198, releasing a 4 kDa C-terminal fragment. This event is required for Mal-dependent TLR4 signalling, but not for IL-1 or TLR7, which do not signal via Mal. Subsequently a molecular mechanism for this was hypothesised in which an E-helix is removed from Mal by caspase-cleavage, thus revealing a MyD88 binding groove.[240]

Another study, in 2010, used a two-hybrid technique called a mammalian protein-protein interaction trap (MAPPIT) to contradict some of these findings. Ulrichts *et al.* confirm that caspase-1 and Mal do interact, but conclude that cleavage inhibits Mal function, and is not required for transactivation of NF-κB.[241] Inhibitory caspase cleavage of other PRR

pathway adaptors, TRIF and Cardif, has also been shown,[242] generally supporting such an inhibitory role for caspases in PRR pathways.

Equivalent amounts of the caspase-1 p10 subunit from WT and Mal-deficient macrophages, indicates that Mal is *not* required for caspase-1 activation.[239] Nevertheless, the fact that caspase-1 does cleave Mal confirmes the assumption that there is some kind of 'interaction' between TLR signalling and inflammasomes by placing these two complexes in the same spatial context.

1.8.4.5 RAGE

All of the above mentioned Mal-mediated pathways are downstream of TLR2 and TLR4. MyD88 has been demonstrated to signal downstream of IL-1R, IL-18R, IFN-γ, and all TLRs except TLR3.[360] Mal signalling, therefore, appears to be more specific than MyD88 signalling, principally responding to LPS. Mal's association with the LPS receptor might explain why there is scant evidence of such promiscuity, because LPS contamination of ligand preparations is of serious concern.

The only evidence of a Mal–non-TLR receptor association is with the receptor for advanced glycation end products (RAGE).[243] Despite its name, RAGE is thought to respond not only to advanced glycation end products,[244] but also to an array of ligands including DNA, RNA, HMGB1,[245] amyloid fibrils[246] and S100 proteins.[247] RAGE signalling is known to induce a wide variety of responses including the typical TLR4 signalling targets ERK1/2, p38 MAP kinase, and NF-κB. How these pathways are activated by the very short cytoplasmic tail of RAGE, which lacks obvious signalling motifs, was a source of intrigue until Sakaguchi *et al.* provided evidence that ligand activation leads to the cytoplasmic tail of RAGE being phosphorylated at Ser391 by protein kinase C (PKC)-ζ. Phosphorylated RAGE can then bind Mal or MyD88.[243]

1.8.4.6 TLR3 inhibition

All TLRs are known to signal via MyD88 with the notable exception of TLR3. TLR3 instead uses the adaptor TRIF to activate antimicrobial responses via IRF3 and IRF7. In 2007, MyD88 was shown to negatively regulate TRIF-dependent phosphorylation of JNK and thereby the activation of human corneal epithelial cells.[248] In 2009 the same inhibition of poly(I:C)-induced TLR3 signalling was observed in Mal-deficient macrophages.[215] One possible mechanism of this negative regulation of TLR3 signalling by Mal might include IRAK-2. Mal could sequester IRAK-2 such that it is less accessible for TLR3 signalling. The purpose of such sequestration is unknown.

1.8.5 Negative regulators of Mal signalling

There are many negative regulators of TLR signalling. Pathogens are a major source of inhibitory molecules designed to dampen antimicrobial activation of TLRs, but it is believed that the host also produces an array of TLR inhibitors that suppress proinflammatory pathways to prevent immunopathology.

SOCS1 and caspase-1 are not the only negative regulators of Mal. Unlike the active forms, kinase inactive forms of IRAK-1 and IRAK-4 have no effect on Mal degradation, indicating that Mal degradation by phosphorylation and ubiquitination is also promoted by these IRAKs.[249] Also, the transmembrane TIR domain-containing IL-1-like receptor protein STL2 is known to inhibit TLR4 signalling by sequestering Mal, and TRIAD3A is an E3 ubiquitin ligase involved in the proteolytic degradation of TLR4 that is reported to downregulate Mal signalling but not MyD88 signalling.[250]

1.8.6 The TLR signalling adaptors and *B. pertussis*

MyD88 has been associated with host protection from various parasites, viruses and bacteria, such as *Staphylococcus aureus*[251] and *Listeria monocytogenes*,[252] but there is no published data examining the course of *B. pertussis* infection in any TLR adaptor-defective mice. The only TLR-defective animals to be infected with *B. pertussis* are C3HeJ mice, in which there is a more severe phenotype relative to C3HeN mice.

1.9 Mal and BACTERIAL INFECTION

Mal signalling pathways have been mostly studied *in vitro* using LPS or lipopeptides for specific activation of TLR2 and TLR4 pathways. Only a select few mouse pathogens have been used to gain an understanding of the function of Mal *in vivo*. The general importance of Mal in protection against bacterial infection is evident because certain bacterial species can subvert host immune responses by specifically targeting Mal or other Mal pathway molecules for negative regulation and enhancement of bacterial virulence (Figure 1.3).

Brucella species are known to make a TIR domain containing protein (TcpB) with significant homology to mammalian TIR domains including Mal[253] indicating that TcpB may act as a mimic. TcpB subverts TLR signalling[254] by degrading Mal[255] through enhancement of poly-ubiquitination.[256]

$Mal^{-/-}$ mice are defective in their immune response to *E. coli* infection of the lungs, with a higher bacterial burden in the lungs, more bacterial dissemination to the spleen, and earlier mortality.[257] Similar increases in virulence were also found when $Mal^{-/-}$ mice were infected with *Klebsiella pneumoniae,* but not with the flagellated bacteria *Pseudomonas aeruginosa*.[258] Studies detailed in section 1.9.3 outline conflicting data on

the role of Mal in Salmonella infection, and there is currently no published information regarding a role for Mal in response to *B. pertussis* infection.

1.9.1 The Human Mal D96N mutation

In 2009, Nagpal *et al.* published data from a screen of known Mal mutations.[259] The researchers discovered that Mal D96N results in a loss of the bridging function of Mal. In this study, LPS induced TNF production by Mal D96N macrophages was commensurate with Mal knockouts, and there was largely ablated NF-κB luciferase activity in mutant HEK293T cells. A subsequent study by George *et al.* supported these findings and

A. Phenotype of Mal-deficient mice

Pathogen	Disease	Effect
E.coli	Lung pathology, Bacteraemia	Protective
Pseudomonas aeruginosa	None	Redundant
Klebsiella pneumoniae	Klebsiella pneumonia	Protective
Salmonella enterica serovar Typhimurium	Salmonellosis	Redundant Protective
Bordetella pertussis	Whooping cough	UNKNOWN

B. Human Mal S180L disease association

Pathogen	Disease	Effect
Mycobacterium Tuberculosis	Tuberculosis	Protective Redundant
Streptococcus pneumoniae	Invasive pneumococcal disease	Protective
Trypanosoma cruzi	Chronic Chagas Cardiomyopathy	Protective
Plasmodium falciparum	Malaria	Protective
Haemophilus influenzae b	Vaccine failure	Protective
HIV-1	AIDS	Protective
N/A	System Lupus Erythematosus	Protective
N/A	Rheumatoid arthritis	Redundant
N/A	Behçet's disease	Protective

Figure 1.3 Mal and disease association

The relationship between various pathogens, diseases and the protective role of Mal. **A** is based on published evidence of infection models of $Mal^{-/-}$ mice. **B** is based on studies to assess the disease association with human heterozygote S180L MAL SNP. (*) is our own unpublished observation.

also found that the corresponding heterozgygous SNP (rs8177400) was present in 0.97% of a white German population.[260] In this study there was found to be no association between the SNP and lymphoma risk, but a more recent Chinese study found an association between Mal D96N and susceptibility to tuberculosis.[261]

In combination with structural studies of Mal–TIR,[210] these studies indicate that D96N is present in the MyD88-binding region of Mal and not on the TLR-interactive surfaces. The D96N mutation specifically interferes with the bridging function of Mal. Although Nagpal *et al.* compared Mal D96N to $Mal^{-/-}$ cells, in that study the only reported analysis was of MyD88 bridging function, i.e., NF-κB activation. Future research might compare Mal D96N mutant cells with $Mal^{-/-}$ cells, with special attention to the recently identified non-bridging components of Mal signalling.

1.9.2 The Human MAL S180L polymorphism

In 2007 Khor *et al.* catalogued functional variants of MAL present in UK, Vietnamese and African populations.[262] One of these SNPs encodes a serine instead of a leucine at position 180 (S180L). Data from Ferwerda *et al.* indicates that primary cells from LPS-primed individuals with the S180L polymorphism produce more proinflammatory cytokines.[263] Khor *et al.* found that, relative to both S180 and L180 homozygotes, heterozygote S180/L180 Mal conveys protection from bacteraemia, tuberculosis, malaria and pneumococcal disease, indicating a 'Goldilocks' model of TLR signal transduction, in which a 'just right' strength of signalling is beneficial for the host. Too little cytokine signalling, as in S180 homozygotes, might allow unfettered bacterial colonisation, and too much cytokine signalling, as in L180 homozygotes, might inhibit tolerance or negative feedback mechanisms designed to prevent systemic inflammatory response syndrome (SIRS) or related immunopathology.

There is some controversy as to the actual effect that MAL S180L has on disease incidence or severity. Nejentsev *et al.* found no evidence of association between Mal S180L and tuberculosis.[264] The two studies use different methods of statistical analysis and are based on different populations. A separate study of Colombian individuals found that the MAL S180L polymorphism is protective against tuberculosis.[265] But a meta-analysis by Miao *et al.* supports Nejentsev *et al.*, who found no association with tuberculosis.[266]

Despite the ongoing controversy in relation to tuberculosis, heterozygous S180L MAL has also been associated with Behçet disease,[267] systemic lupus erythematosus (SLE),[265] protection from serious infection in HIV-1 patients,[268] and a lower risk of *T. cruzi*-infected individuals developing chronic Chagas cardiomyopathy.[269] Rheumatoid arthritis is not believed to be associated with the MAL S180L SNP,[270] showing expected disease specificity. Ladhani *et al.* report a fascinating association between the failure of the *Haemophilus influenzae* serotype b (Hib) vaccine in British patients with a MAL SNP (rs1893352) with strong linkage disequilibrium with MAL S180L.[271] This is some of the best evidence yet for a genetic explanation for general vaccine failure, with Mal playing a central role.

The diverse array of infections associated with MAL SNPs is not likely to be coincidental. Because of the heterogeneity of populations in Africa and the very small frequency of polymorphism in some regions, it is clear that meta-analyses and back analysis of data sets that were acquired for different purposes are not sufficient to determine true disease associations with the S180L MAL polymorphism.

1.9.3 Mal and the barrier

Mal is entirely dispensable, and even detrimental, for host protection from intravenous administered *Salmonella enterica* serovar Typhimurium.[272,273] However, when *Salmonella* is administered via its natural route of infection, orally, Mal is protective.[274] The function of the intestinal barrier might determine this distinction between oral and intravenous infection.

Mal signalling pathways have been studied predominantly in cells of the macrophage–monocyte lineage, which function principally in inflammation and activation of the immune system. The role of Mal in other cell types and in induction of other responses is less-well documented.

The 'barrier' is a generic term to describe the protective separating layer of packed cells bound by tight junctions that line key mucosal sites of infection, such as the lungs and intestinal epithelial layers. Bacterial breach of the barrier results in dissemination of bacteria into otherwise sterile compartments, including the blood (bacteraemia or septicaemia), which in turn can cause deadly sepsis.

In 1993 it was discovered that LPS perturbs pulmonary vascular endothelial barrier function by inducing the depolymerisation of F-actin and consequently interfering with intercellular gap formation.[275] In 1997 the same group added that this effect on the barrier is mediated by tyrosine phosphorylation,[276] and in 2008 it was shown that the SRC family kinases (SFK) c-SRC, FYN and YES are activated by LPS, and that they tyrosine-phosphorylate zonula adherens proteins, thus permeabilising the barrier via the paracellular pathway.[277] Another study found that the barrier of pulmonary microvessel endothelial monolayers can be perturbed with lipoteichoic acid (LTA), a Gram-positive bacterial cell wall component known to be sensed by TLR2,[278] providing further evidence that both TLR2 and TLR4, the two TLRs that interact with Mal, are involved in lung barrier regulation.

A more decisive role for Mal in endothelial barrier permeability was shown by demonstrating that LPS activation of the SFKs is mediated by TRAF6,[279] a known component of Mal signalling cascades. In this study siRNA-induced silencing of Mal, or over-expression of dominant negative Mal, in human lung microvascular endothelial cells (HMVEC) blocked this 'LPS effect'.

The 'LPS effect' has also been demonstrated on epithelial cells indicating that Mal might also play a role in perturbing epithelial tight junctions. However, counter to the 'LPS effect', protein kinase C (PKC) is known to induce tight junction formation, strengthening the barrier[280,281] *in vitro* between cultured human nasal epithelial cells.[282] Corr *et al.* showed that this phenomenon can be activated by Mal.[274] By using Mal bone marrow chimeras to rule out the role of Mal in macrophage–monocyte lineage cells, the susceptibility of Mal-defective mice to oral *Salmonella* infection was demonstrated to be due to a faulty Mal–PKC–barrier axis, thus demonstrating that Mal *strengthens* the intestinal epithelial barrier against bacterial infection. Counter to this are studies indicating that PKC dephosphorylates occludin and increases tight junction permeability of epithelial cells.[283,284] The apparent conflict over the role of TLRs, MyD88 and Mal in relation to barrier permeability in some of these studies will probably be proven to be a factor of differential regulation by the different cell types and pathogens tested.

It is currently unclear from the literature how the lung barrier is regulated in response to *B. pertussis* infection, or if there is a role for Mal in the process.

1.10 PROJECT AIM

There are currently no data relating to the role that TLR adaptors play in modulating the immune response to *B. pertussis*. Circumstantial evidence of a role for Mal in *B. pertussis* infection comes from TLR4-defective mice. There is evidence that alveolar macrophages are vital for control of bacteria during the initial stages of *B. pertussis* infection.[117] Furthermore, *B. pertussis*-infected TLR4-defective C3HeJ mice have higher bacterial growth, higher infiltration of immune cells and immune mediated damage, relative to TLR4-sufficient C3HeN mice.[115,117]

However, advancements in our understanding of TLR signalling suggest that TLR4-defective mice and cells would be deficient in both Mal signalling and TRIF-dependent signalling. Furthermore, Mal potentially signals via TLR2 and participates in signalling networks that are independent of MyD88. Therefore, an investigation of $Mal^{-/-}$ mice and cells might provide valuable information on the role of TLR signalling adaptors in the immune response to *B. pertussis* infection.

Differences in the pathogenesis of *B. pertussis* between $Mal^{-/-}$ and published observations of TLR4-defective mice might even contribute to our understanding of the importance of anti-bacterial TLR2 signalling, and TRIF-dependent responses.

The aim of this project was to comprehensively characterise the immune response to *B. pertussis* infection in $Mal^{-/-}$ mice and cells, and to expand upon any aspect of novel immune or microbiological activity. A major objective was to focus on the inflammatory cells and cytokines of the lungs, the bacterial killing ability of those populations, and their role in clearance of *B. pertussis*.

Chapter Two

Materials and Methods

2.1 MATERIALS

Ammonium chloride lysis solution

0.77 g ammonium chloride (Sigma) dissolved in 100 ml dH_20 and filter sterilised.

Cell culture medium

Roswell Park Memeorial Institute-1640 medium (RPMI; Biosera) was supplemented with 10% heat inactivated (56°C, 30 min) foetal calf serum (FCS, Biosera), 100 µg/ml penicillin/streptomycin (Sigma).

EDTA (pH 8.0, 25mM)

0.93 g EDTA (Sigma) dissolved in 50 ml ddH_20

ELISA blocking solution

1 g bovine serum albumin (BSA) added to 100 ml 1× PBS

ELISA stopping solution (1M H_2SO_4)

26.47 ml 18 M H_2SO_4 with 473.26 ml dH_2O.

ELISA washing buffer

500 ml 20× PBS, 5 ml Tween (Sigma) made up to 10 L with dH_2O.

FACS buffer

500 ml of sterile 1× PBS supplemented with 2% FCS.

MACS buffer (pH 7.2)

10 ml FCS, 5 ml 25 mM EDTA added to 500 ml sterile 1× PBS.

Phosphate buffered saline (PBS)

20× stock was used for ELISA solutions: 800 g sodium chloride (NaCl, Sigma), 92 g sodium hydrogen phosphate (Na_2HPO_4, Sigma), 20 g potassium dihydrogen phosphate (KH_2PO_4, Sigma), 20 g potassium chloride (KCl, Sigma) all dissolved in 5L dH_2O and adjusted to pH 7. Sterile PBS (Sigma) was used for mouse injections, FACS buffer, MACS buffer, lavages and perfusions.

2.2 MICE

Female specific pathogen-free C57BL/6 and C57BL/6 $Mal^{-/-}$ mice[209] were bred in house or obtained from Harlan UK Ltd (Bicester, UK). Mice were maintained according to the regulations and guidelines of the Irish Department of Health, and treated according to ethical procedures controlled by the Irish Medicines Board (IMB). Mice were 7–12 weeks old and age-matched at the beginning of experiments. Mice were sacrificed by cervical dislocation or asphyxiation with CO_2. With the exception of *B. pertussis* infection, no experimental procedures were carried out on live mice.

2.3 METHODS

2.3.1 Cell isolation and culture

Unless otherwise stated, mammalian cells were separated by centrifugation at 242×*g* at 4°C for 5 mins in PBS, RPMI or DMEM. Supernatants were tipped off and cells were resuspended in the same media. Cells were counted by haemocytometer with at least 100 events recorded per sample, and without counting dead cells staining positive for 1/10 trypan blue. During protocols, cells were kept on ice and not left

sitting for long periods of time. Long term culture of cells was at 37°C with 5% CO_2 in humidified incubators.

2.3.2 Red blood cell lysis

Red blood cells from single cell suspensions were lysed using 1–5 ml warm ammonium chloride lysis solution for 3 mins. Cells were then washed with an excess of RPMI or PBS and centrifugation (242×*g*, 5 mins). Cell pellets were then resuspended in fresh medium.

2.3.3 *B. pertussis* culture

Frozen stocks of *B. pertussis* (strain Tohama 1; 338) were thawed and 100 µL was plated onto horse blood (Cruinn, Dublin, Ireland) BG agar plates. Plates were incubated at 37°C for 4 days. Loops of the bacterial growth were then added to liquid bacterial culture medium in an Erlenmeyer flask. Flasks were shaken at 37°C for 24 hours. 10 ml of that culture was then added to 90 ml of fresh bacterial culture medium and shaken at 37°C for another 24 hours.

Horse blood BG agar plates:

250 ml ddH_2O, 2.5 ml glycerol (BDH, VWR International, IL, USA) and 7.5 g Bordet Gengou agar (Becton, Dickinson and Company, Oxford, England) were mixed and then autoclaved at 121°C for 15 mins and then allowed to cool to around 50°C. Meanwhile defibrinated horse blood was warmed to 37°C, and 0.1 g Cephalexin (Sigma, Wicklow, Ireland) was added to 10 ml of ddH_2O and warmed to mix. 1 ml of 10 mg/ml cephalexin was added to 100 ml of horse blood. This mixture was then added to the agar mix, swirled, and 20 ml was plated into Petri dishes. Cooled and set plates were sealed and kept at 4°C until use.

Liquid bacterial culture medium:

The bacterial culture medium was 99% Stainer and Scholte (S&S) medium with 1% Supplement. S&S was made by mixing 10.72 g L-glutamic acid (Sigma, Wicklow, Ireland), 0.24 g L-proline (Sigma, Wicklow, Ireland, 2.5 g NaCl, 0.5 g KH_2PO_4, 0.2 g KCl (Merck, Nottingham, UK), 0.1 g $MgCl_6.H_20$ (Sigma, Wicklow, Ireland), 0.02 g $CaCl_2.2H_20$ (Sigma, Wicklow, Ireland), 1.525 g Tris base (Sigma, Wicklow, Ireland) and ddH_2O up to 1L. pH was then adjusted to 7.3 with 6N HCl and the medium was either filtered or autoclave sterilised then stored at 4°C for up to two weeks. Supplement was made by dissolving 0.4 g L-cystine (Sigma, Wicklow, Ireland) in 1 ml 6N HCl on a rotator for 2 hours then adding that to 0.1 g $FeSO_4.7H_20$ (Sigma, Wicklow, Ireland), 0.2 g ascorbic acid (Sigma, Wicklow, Ireland), 0.04 g nicotinic acid, 1 g reduced glutathione, and 100 ml ddH_2O. Supplement was filter sterilised and stored at 4°C for up to 2 weeks.

2.3.4 *B. pertussis* infection

4–6 erlenmeyer flasks of bacterial liquid culture were combined into 2 large centrifuge flasks. The tubes were then centrifuged (Sorval R45C) at 11,000 rpm for 15 mins at 4–20°C. Bacterial pellets were resuspended in 100 ml of 1% Casein salts. Dilutions of 1:20 and 1:50 were made of the culture in 1% casein salts for analysis of optical density by spectrophotometer at 600nm. Density was compared to historical standard curve for calculation of total bacterial number. Bacterial cultures were then volume adjusted to make a concentration of $1x10^9$ *B. pertussis* per ml.

As previously described,[79] liquid *B. pertussis* culture was placed in a tube and attached to a home-made nebuliser for infection of mice. Mice were placed in a specially constructed perspex box and aerosolised bacteria were pumped into the box for 15 mins. The mice were then left in the box

for a further 10 mins after the pump was switched off. 2 hours after infection, test mice were euthanised and their lungs were removed for calculation of CFU (as described below) to ensure consistent infection of mice, and consistency between experiments.

Casein salts: 6 g NaCl (Merck chemicals, Nottingham, UK) was added to 10 g casamino acids (Becton, Dickinson and Company, Oxford, England) and 1L ddH_2O. pH was adjusted to 7.1 with 1N NaOH and autoclaved at 115°C for 15 mins.

2.3.5 Calculation of *B. pertussis* CFUs

At least three organs were removed from infected mice and homogenised with a rotor stator homogeniser. Homogenate was diluted 1:10, 1:100, 1:1000 and 1:10,000 in casein salts and plated onto BG blood agar plates for enumeration of total CFUs per organ.

2.3.6 ELISA

Lung homogenates were diluted and used for detection of cytokines and chemokines by ELISA duosets (R&D systems, Ireland) or as otherwise stated in Table 2.1. 0.1% Tween-20 PBS was used as wash buffer. 0.16M H_2SO_4 was used as a stop solution. Concentration was calculated by colorimetric change with horse radish peroxidase and TMB, relative to 1:10 dilution standards of purified cytokine according to manufacturer's instructions on a Versamax spectrophotometer (Molecular devices, UK).

Table 2.1 ELISA antibodies, standards and blocking solutions.

Cytokine	Capture Antibody	Detection Antibody	Top standard	Supplier
IL-1β	4 μg/ml	2.5 μg/ml	1000 pg/ml	R&D
IL-23	4 μg/ml	100 ng/ml	2500 pg/ml	R&D
IL-27	2 μg/ml	1 μg/ml	1000 pg/ml	R&D
IL-10	4 μg/ml	300 ng/ml	2000 pg/ml	R&D
IL-12p40	1 μg/ml	1 μg/ml	2500 pg/ml	BD Pharmingen
IL-12p70	4 μg/ml	400 ng/ml	1500 pg/ml	R&D
IL-17A	2 μg/ml	400 ng/ml	1000 pg/ml	R&D
IFN-γ	1 μg/ml	1 μg/ml	10 ng/ml	BD Pharmingen
TNF-α	800 ng/ml	200 ng/ml	2000 pg/ml	R&D
GM-CSF	2 μg/ml	50 ng/ml	500 pg/ml	R&D
IL-6	1 μg/ml	1 μg/ml	5000 pg/ml	BD Pharmingen

2.3.7 Lung cell isolation

Broncho-alveolar lavage fluid (BAL-F) was isolated by inserting a cannula into the trachea of euthanized mice and injecting three 1 ml volumes of ice cold PBS without rupturing the lungs. BAL-F was then centrifuged at 300×*g* for 5 mins. Cells were then resuspended in ice-cold PBS. Alternatively lungs were homogenised by being cut into small pieces then rotated for 30 mins at 37°C in RPMI containing Collagenase IV (life Technologies, UK), DNase I (Sigma) and Brefeldin A (BioLegend, UK). Digested lung material was then passed through a 100 μm filter. Red blood cells (recipe) were lysed with red blood cell lysis buffer (0.829 g NH_4Cl, 0.109 g $KHCO_3$, 0.037 g disodium ethylenediaminetetracetic acid (EDTA; Sigma; 100ml H_2O, pH: 7.3–7.4) and leukocytes were collected for analysis.

.3.8 FACS

$1x10^6$ cells in 50 μL PBS were stained with 0.5 μL F_c Block (anti-CD16, anti-CD32), then surface stained with fluorescent conjugated anti-mouse antibodies for various surface markers (CD49b, NK1.1, γδ-TCR, CD3, CD4, CD8, CD11b, F4/80, Ly6C, Ly6G, MHC II, Siglec-F and CD11c) (eBioscience, Hatford, UK) for 20 mins on ice. Cells were incubated with 0.5 μL LIVE/DEAD aqua (Invitrogen, Ireland) as per manufacturer's instructions, then centrifuged at 300×*g* for 3 mins in FACS tubes. For intracellular FACS analysis, cells were resuspended in Fix/Perm (eBioscience Ltd, UK) for 20 mins as per manufacturer's instructions. Antibodies for intracellular cytokines were added in permeabilisation buffer (IFN-γ, IL-17A, IL-17F, IL-4, IL-10. FMOs and non-specific isotype antibodies as controls were also used). FACS samples were analysed in 100 μL volumes of FACS buffer or permeabilisation buffer (eBioscience Ltd, UK). Samples were calibrated with antibody labelled single stained

Table 2.2 FACS antibodies

Specificity	Clone	Fluorochrome	µl/1x10^6 cells	Supplier
IL-17A	eBio17B7	PE	0.25	eBioscience
IFN-γ	XMG1.2	PE	0.25	eBioscience
IL-4	11B11	PE	0.25	BD Pharmingen
CD4	GK1.5	PE, FITC	0.25	BD Pharmingen
CD3	145-2C11	FITC	0.25	eBioscience
CD19	ID3	APC	0.25	eBioscience
CD80	16-10A1	PE	0.25	BD Pharmingen
CD86	GL1	PE	0.25	BD Pharmingen
γδ-TCR	GL3	PE, APC	0.25	BD Pharmingen
CD11b	M1/70	APC-Cy7	0.25	eBioscience
CD11c	N418	APC	0.25	eBioscience
F480	BM8	PeCy7	0.25	eBioscience
Siglec-F	E50-2440	PE	0.25	BD-Pharmingen
GR1	RB6-8C5	PE	0.25	eBioscience
MHC-II/IA-IE	MS/114.15.2	Pacific Blue	0.05	eBioscience
Ly6C	HK1.4	PerCP-Cy5.5	0.25	eBioscience
Ly6G	1A8	Pacific Blue	0.25	Biolegend

beads and run on a BD FACSCalibur or FACSCanto 2. Data were analysed using FloJo software, and plotted with Prism.

2.3.9 *In vitro* infection of macrophages with *B. pertussis*

Bone marrow-derived macrophages (BMDM) were made from tibias and femurs of both back legs of mice. Bone marrow was flushed with a 3 ml syringe and 21-gauge needle into Dulbecco's Modified Media (DMEM, Sigma, Wicklow, Ireland) supplemented with 10% FCS. Red blood cells were lysed with RBC lysis buffer (0.829 g NH_4Cl, 0.109 g $KHCO_3$, 0.037 g disodium ethylenediaminetetracetic acid (EDTA) (Sigma), 100 ml H_2O, pH: 7.3–7.4) and remaining cells were washed and resuspended at a concentration of $1x10^6$/ml in DMEM supplemented with 10% FCS and 20% M-CSF-containing L929 supernatant (L929, fibrosarcoma cell line was cultured at a density of 0.5×10^6/ml in RPMI medium DMEM, (Sigma, Wicklow, Ireland) for 7 days. The supernatant was harvested and filter sterilised). 10 ml of cells were incubated in 10 cm non-tissue culture-treated dishes for 6 days. On day 6, supernatant was removed and 5 ml of ice cold PBS was added to each dish. BMDMs were gently dislodged from dish, by scraping. Cells were counted and reseeded at a density of 0.5×10^6 cells/ml and 2 ml/well on a 6-well dish and incubated overnight in DMEM, 10% FCS, L929. 24 hours later heat-killed *B. pertussis* or live *B. pertussis* was added and cultures were incubated for two or 24 hours at 37ºC with 5% CO_2. Cell culture supernatants were removed for cytokine ELISA. BMDM were then washed four times and then lysed in ddH_2O, and lysate was plated onto horse blood agar plates for 4 days (as described above) in order to enumerate intracellular bacteria.

2.3.10 Histology

At least three lungs and livers were removed at various times after aerosol infection with *B. pertussis* (as described above) and placed into 10% neutral-buffered formalin for tissue sectioning, and haemotoxylin and eosin staining. Sections were performed by Joseph Cassidy, UCD.

2.3.11 ROS assay

Cells were stained with CellRox deep red (Life Technologies) reagent *in situ* in culture dishes according to manufacturer instructions prior to being scraped from the culture plates and analysed by FACS in the FL-2 channel of a FACS Canto (BD). Cells were co-stained with Live/Dead Aqua to separate cells based on viability.

2.4 Statistical analysis

GraphPad Prism software was used for comparison of WT and $Mal^{-/-}$ by student's T test, two-way Anova with Bonferroni post-tests, or Logrank tests for statistical significance (as marked in figure legends).

Chapter Three

Mal prevents fatal *B. pertussis* infection

3.1 INTRODUCTION

The initial immune response to pulmonary infection is likely to involve innate immune cells that reside in the alveolar spaces, such as DCs, AMs and epithelial cells. In the case of *B. pertussis* infection, early innate immune responses probably limit bacterial numbers so that subsequent adaptive immune responses can more easily eliminate the pathogen. A better understanding of the innate immune responses that prevent serious *B. pertussis* infection might help in the design of a better P_a vaccine, a worthy pursuit given the apparent failures of the current widely used P_a vaccine, and the recent global resurgence of severe *B. pertussis* infection.[5]

Little is known of the interaction between *B. pertussis,* its products and mammalian PRRs. Generally, PRR-activated signalling pathways induce secretion of proinflammatory cytokines and chemokines that recruit inflammatory cells. Pulmonary inflammation is characteristic of *B. pertussis* infection in mice and also in humans.[8] The only well-characterised *B. pertussis*-TLR interaction is *B. pertussis* LPS–TLR4. TLR-deficient HEK 293 cells were transfected with different TLRs and stimulated with *B. pertussis* LPS.[285] Only TLR4-transfected cells responded, but the response was muted relative to that induced by *E. coli* LPS, suggesting that *B. pertussis* LPS has low bioactivity. Glucosamine modification of *B. pertussis* LPS phosphate groups has been shown to be important for TLR4 activation, possibly indicating that *E. coli* LPS binds TLR4 via a different mechanism to *B. pertussis* LPS, thereby explaining differences in bioactivity of *E. coli* and *B. pertussis* LPS.[286]

B. pertussis grows more rapidly and reaches higher CFUs in the lungs of TLR4-defective C3HeJ mice than in C3HeN mice,[115,117] and TLR4 is known to mediate recruitment of neutrophils to the lungs of mice after *B. pertussis* infection.[287] To date, C3HeJ mice are the only known PRR pathway-defective genotype to be infected with *B. pertussis*.

The TLR adaptor Mal (TIRAP), therefore, might be expected to contribute to protective immunity to *B. pertussis* as it is required for signalling via the MyD88-dependent TLR4 pathway. Mal signalling is known to be important for human protective immunity to lung infection as the MAL S180L short nucleotide polymorphism (SNP) is associated with susceptibility to *M. tuberculosis* infection, and other diseases.[262]

Further evidence of a role for Mal in protective immunity to bacterial infection is provided by reports that *E. coli*[257] and *K. pneumoniae*[258] cause severe disease in $Mal^{-/-}$ mice. Neither of these studies included detailed analysis of the lung cell populations that initially respond to infection. Although it was clear from these studies that Mal has a protective role against lung bacterial infection, the mechanism of this protection was unclear.

Mal is required to maintain the intestinal epithelial barrier, thereby preventing severe *S. typhimurium* infection in mice.[274] Data indicate a possible mechanism of Mal-mediated protection from bacterial infection, but the function, cellularity, structure and context of the intestinal epithelium is distinct from the mucosae of the lungs.

3.1.1 Aims of chapter 3

The aim of this chapter was to determine if Mal plays a role in protection against *B. pertussis* infection, and to characterise any differences in pathology or immune responses in *B. pertussis* infected $Mal^{-/-}$ mice compared with WT mice.

3.2 RESULTS

Experiments were designed to systematically assess the kinetics of *B. pertussis* lung infection, the host pathology, and the immune response associated with infection of $Mal^{-/-}$ mice in comparison with WT mice.

B. pertussis virulence factor expression is required for lung colonisation and also for recognition by the host innate immune response. For this reason, maintenance of suitable bacterial culture conditions was vital. To enumerate bacteria and to assess virulence of colonies, *B. pertussis* was plated onto BG blood agar prior to aerosol-infection, and homogenised lungs were also plated onto BG blood agar 2 hours after all *in vivo* infections. **Figure 3.1** depicts an example of virulent *B. pertussis* colonies, which appear small, bright and raised, with a 'halo' of haemolysis evident in the surrounding agar. Avirulent *B. pertussis* colonies are known to be dull, large and flat, with no halo effect.

3.2.1 Mal prevents *B. pertussis* lung and liver pathology

To determine the role of Mal in the pathogenesis of virulent *B. pertussis* infection, WT and $Mal^{-/-}$ mice were aerosol-infected. 7 days later mice were CO_2 euthanized, dissected and examined for thoracic and abdominal gross organ pathology (**Figure 3.2**). This preliminary assessment of the effect of Mal on *B. pertussis* infection revealed that, like uninfected WT and $Mal^{-/-}$ mice, after 7 days *B. pertussis* infection WT mice had relatively healthy, bright pink lungs (arrow A), and deep-red, shiny livers (arrow C), whereas $Mal^{-/-}$ mice had darker pink and patterned lungs (arrow B), and washed out, dull red livers (arrow D). These results indicated lung-specific and systemic pathology in *B. pertussis*-infected $Mal^{-/-}$ mice, thereby warranting closer examination of the protective role of Mal.

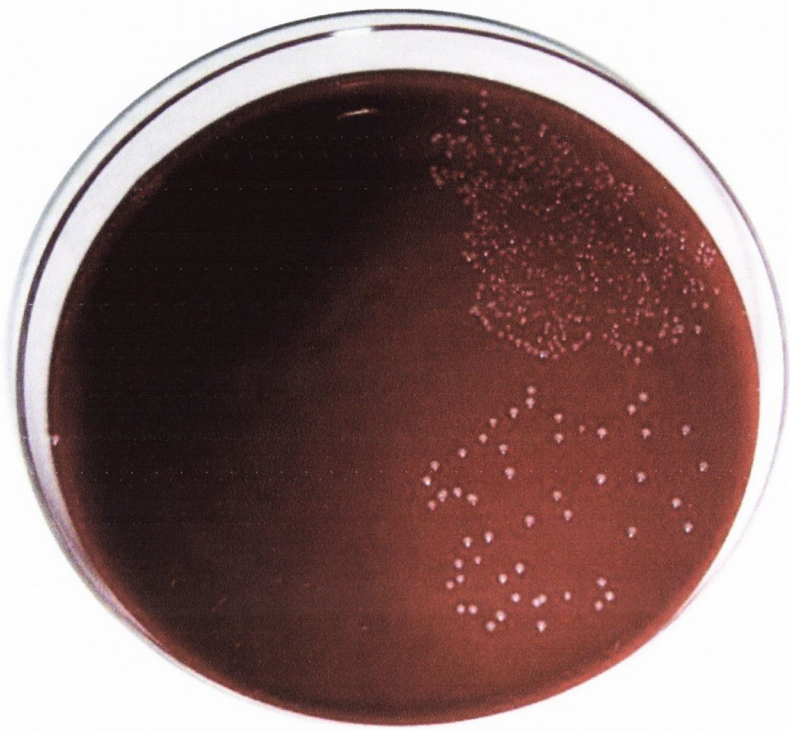


Figure 3.1 Cultured *B. pertussis* and lung isolates were virulent

Homogenised lung was diluted and plated onto BG horse blood agar plates. *B. pertussis* colonies always appeared as small, dome-shaped, shiny, and pearly white, and were surrounded by areas of haemolysis in the agar, indicating that bacteria were virulent. The plate depicted is a representative plate.

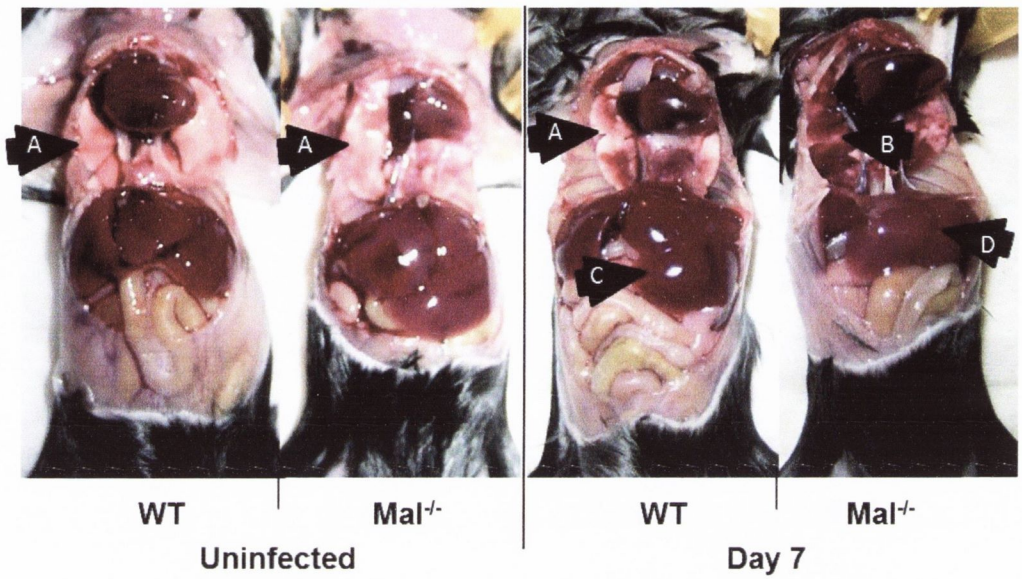


Figure 3.2 Mal prevents *B. pertussis* mediated gross organ pathology

At 7 days post-infection mice were dissected. Gross visual examination revealed that WT mice and uninfected $Mal^{-/-}$ mice had healthy organs, whereas there was significant organ pathology obvious in the $Mal^{-/-}$ mice 7 days after infection. Specifically, the lungs of uninfected WT and $Mal^{-/-}$ mice and day 7 infected WT mice **(A)** maintained a paler pink colour and uniform texture, whereas the lungs from infected $Mal^{-/-}$ mice **(B)** were darker, more delicate, and appeared to have patchy areas of an unidentified nature. The livers of the infected WT mice **(C)** appeared to have a healthy, deep red, shiny surface texture, whereas the livers of $Mal^{-/-}$ mice **(D)** were faded red and were not shiny.

3.2.2 Mal prevents *B. pertussis*-related fatality

Because there was organ pathology evident in *B. pertussis* infected $Mal^{-/-}$ mice at day 7 of infection, it was decided to longitudinally determine the severity of the infection in $Mal^{-/-}$ mice compared with WT mice.

All WT mice survived *B. pertussis* infection, whereas only 55% of $Mal^{-/-}$ mice survived (**Figure 3.3**). In all experiments, fatalities occurred in the third week of infection. Prior to death or euthanasia $Mal^{-/-}$ mice appeared weak, docile and hunched, whereas WT mice appeared normal. Mice that survived beyond the third week of infection were physically and behaviourally normal, and there were no subsequent fatalities. For this reason experiments were terminated 6 weeks after infection.

.2.3 Mal controls *B. pertussis* infection in the lungs

The kinetics of *B. pertussis* growth in WT and $Mal^{-/-}$ mice was assessed by estimation of viable *B. pertussis* colony forming units (CFUs) at various times post-infection (**Figure 3.4**). 2 hours after *B. pertussis* infection there were consistently equivalent numbers of viable bacteria in the lungs ($10^{4.2}$) of both mouse strains. By 24 hours and 4 days post-infection, *B. pertussis* had proliferated in both mouse genotypes but the bacterial load in the lungs of $Mal^{-/-}$ mice was significantly higher (more than 10-fold) than in the lungs of WT mice at day 4 of infection, indicating that proliferation in the lungs of WT mice was inhibited by Mal-dependent host immune responses. Peak lung load in both genotypes occurred at 7 days post-infection, with almost 10^7 bacteria in the lungs of $Mal^{-/-}$ mice compared with approximately 40-fold fewer bacteria ($10^{5.4}$) in the lungs of WT mice.

14 days after infection the bacterial load in the lungs of WT mice had dropped below 10^5 suggesting that WT host immune responses were active in clearing bacteria, whereas the $Mal^{-/-}$ mice failed to control the infection—the bacterial lung load was still around 10^7. Reduction of

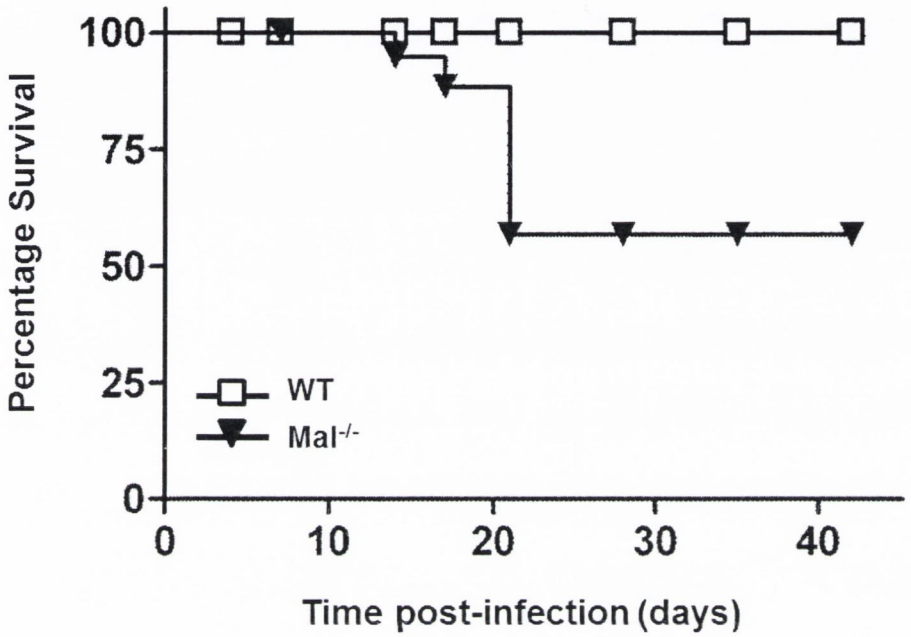


Figure 3.3 Mal prevents *B. pertussis*-related fatality

20 WT and 20 $Mal^{-/-}$ mice were infected with *B. pertussis* by aerosol challenge with 1×10^9 *B. pertussis* per ml. Mice were checked daily for severe morbidity or mortality and survival percentage was plotted over the course of 6 weeks. Data presented are pooled from three individual experiments. Logrank test confirmed that survival curves for WT and $Mal^{-/-}$ mice were significantly different $^{**}P=0.0051$.

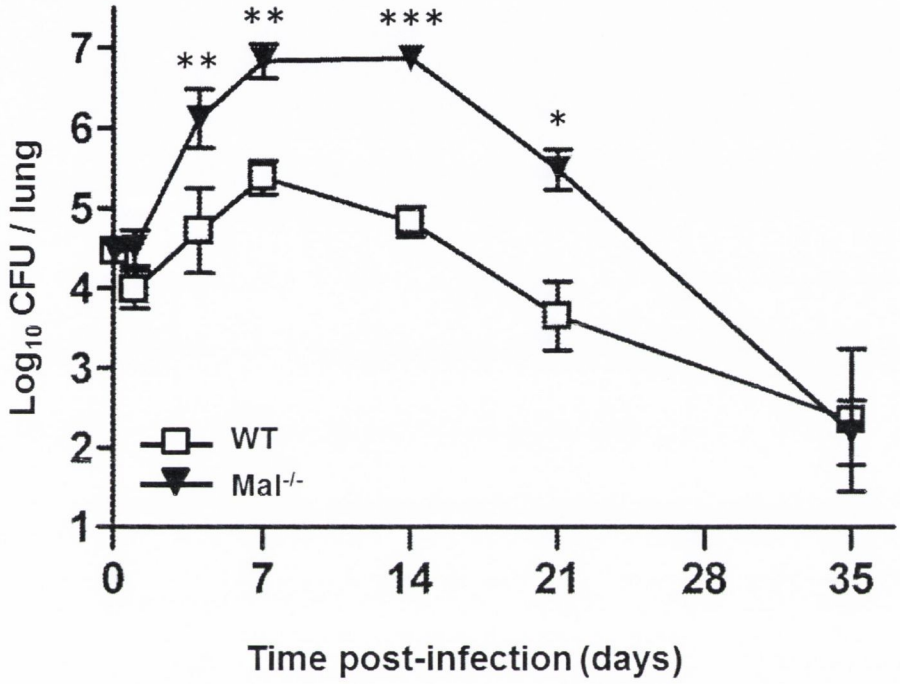


Figure 3.4 Mal is required to control *B. pertussis* infection

WT and Mal$^{-/-}$ mice were exposed to a 20 minute aerosol challenge with 1×10^9 *B. pertussis* per ml. At various times post-infection lungs were removed, homogenised, diluted and cultured on BG blood agar plates with ciproflaxin. *B. pertussis* colonies were counted to enumerate total lung bacterial load. Data are the mean ± SEM from n = 3 mice, from one experiment representative of 8 separate experiments. *P<0.05, **P<0.01, ***P<0.001 statistical analysis by 2-way ANOVA with Bonferroni test.

bacterial numbers in Mal$^{-/-}$ mice was not observed until 21 days post-infection. However, 45% of Mal$^{-/-}$ mice had died by day 21 (Figure 3.3), therefore CFU enumeration from the lungs of Mal$^{-/-}$ mice at day 21 and day 35 are skewed, as they estimate only the number of bacteria in surviving Mal$^{-/-}$ mice, i.e., those mice that display a resistant phenotype. Although the number of CFUs in Mal$^{-/-}$ mice was equivalent to the WT mice on day 35, surviving mice of either genotype had fewer than 1,000 viable *B. pertussis* per lung.

3.2.4 Mal prevents severe *B. pertussis*-related pulmonary inflammation

The results in figure 3.2 and figure 3.3 showed *B. pertussis*-related fatality of Mal$^{-/-}$ mice and gross organ pathology at day 7 of the infection. To get a better idea of inflammatory responses to the enhanced colonisation of the lungs of Mal$^{-/-}$ mice, histological sections of *B. pertussis*-infected mice were made. Lungs from infected WT and Mal$^{-/-}$ mice were fixed, sectioned, and stained with haematoxylin and eosin (H&E). Uninfected lungs from WT and Mal$^{-/-}$ mice appeared morphologically indistinguishable **(Figure 3.5, A–B)**. Following 24 hours of *B. pertussis* infection there was evidence of alveolar wall thickening, indicating limited inflammation **(Figure 3.5, C–F)**. However, there were no significant histological differences between lungs from WT and Mal$^{-/-}$ mice at this stage of infection.

4 days after *B. pertussis* infection there was severe localised inflammation in the lungs of Mal$^{-/-}$ mice, whereas lungs of WT mice appeared normal **(Figure 3.5, G–H)**. In the lungs of Mal$^{-/-}$ mice there was evidence of perivascular cuffing and infiltration of alveoli, with cells that morphologically resemble neutrophils and macrophages. Inflammation was evident in the lungs of *B. pertussis*-infected WT mice by day 7 of the infection, but this inflammation began to resolve by day 14 **(Figure 3.5, I–L)**. By contrast, the inflammation in the lungs of Mal$^{-/-}$

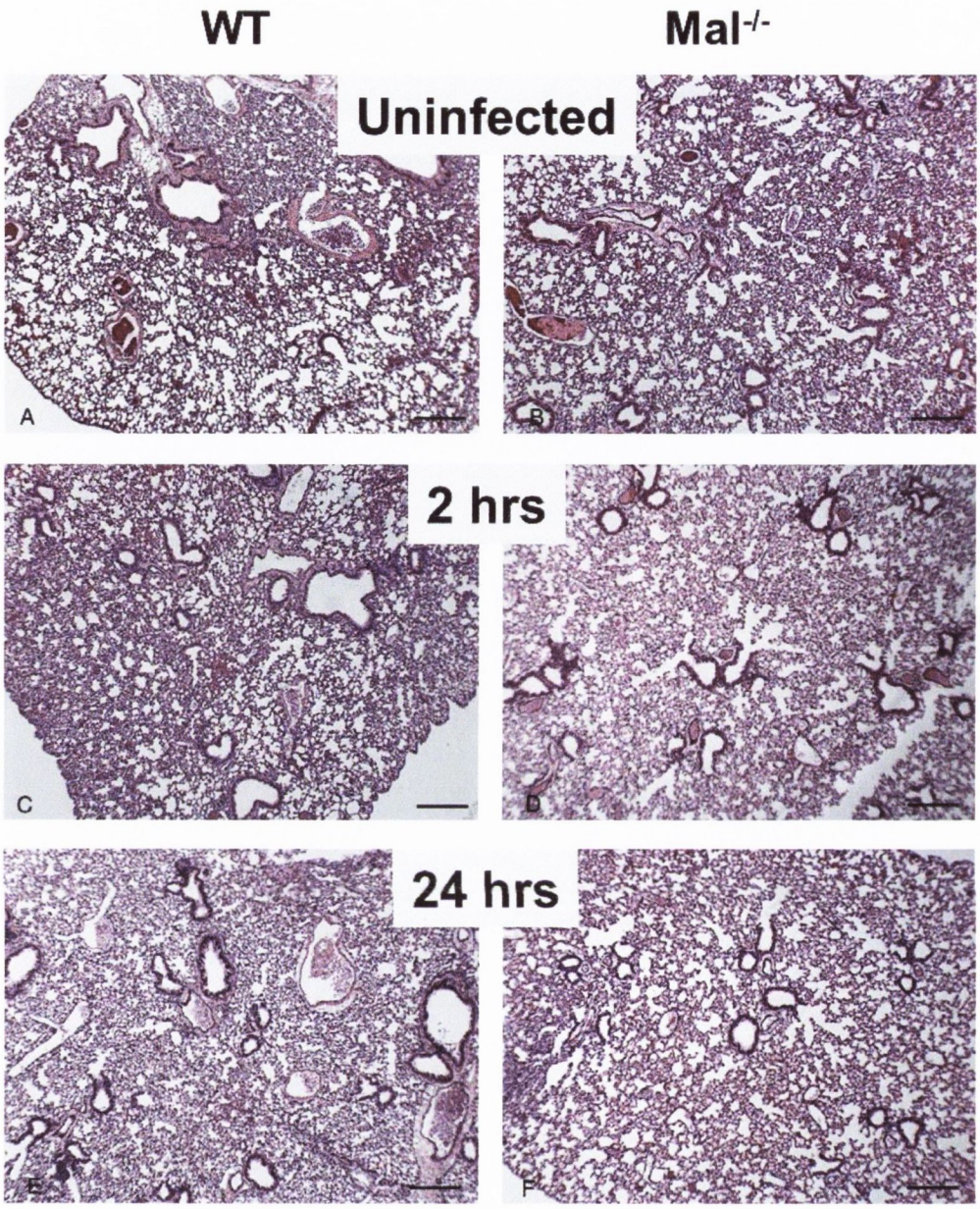


Figure 3.5 A–F Uninfected lungs from Mal$^{-/-}$ mice are normal

Low magnification photomicrographs illustrating the indistinguishable histopathological features of lungs from uninfected WT (A) and Mal$^{-/-}$ (B) mice, 24 hours after *B. pertussis* infection of WT (C,E) and Mal$^{-/-}$ (D,F) mice. Haematoxylin and eosin stain. Scale bars = 250 μm.

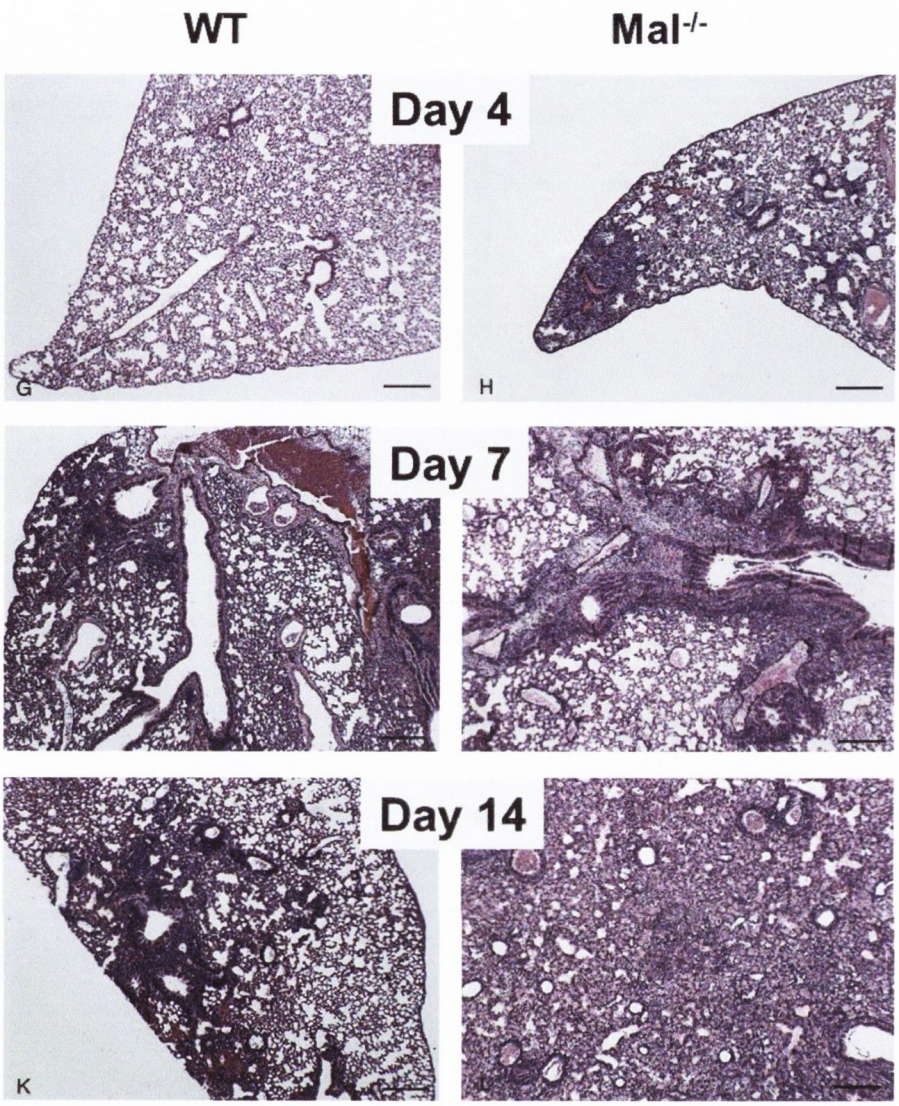


Figure 3.5 G–L Mal prevents severe *B. pertussis*-induced lung inflammation

Low magnification photomicrographs. Earlier development and more severe pulmonary inflammation in $Mal^{-/-}$ (H,J,L) than WT (G,I,K) mice. Alveoli infiltrated by neutrophils and macrophages and membrane breakdown at day 7 of *B. pertussis* infection in $Mal^{-/-}$ mice (H). Inflammation resolving in WT mice at day 14 (K), but in $Mal^{-/-}$ mice flooding of alveoli by cellular (neutrophils and macrophages) and proteinaceous exudate (L). Haematoxylin and eosin stain. Scale bars = 250 μm.

mice did not resolve by day 14. This inflammation included a proteinaceous exudate and possible alveolar damage. Histological examination of lungs from day 21 of infection indicated that inflammation remained unresolved in the lungs of $Mal^{-/-}$ mice, even at late stages of infection (data not shown).

3.2.5 Mal is required for an early innate 'burst' of anti-*B. pertussis* cytokines

Because the lung histology indicated a severe inflammatory infiltration in response to *B. pertussis* infection of the lungs, particularly in $Mal^{-/-}$ mice, and especially at late stages of infection (Figure 3.5), it was decided to determine the nature of the inflammatory response. The focus was the late cytokines typically produced by T_H cells, and the early innate cytokines that can promote differentiation of naïve T cells. WT and $Mal^{-/-}$ mice were infected with *B. pertussis* and lungs were removed at various times over the course of a 2 week infection. The experiment was ended at 2 weeks as that was the time at which approximately half of all $Mal^{-/-}$ mice died from infection (Figure 3.3).

Lungs were homogenised and homogenates were diluted (where necessary) for determination of lung cytokines by ELISA. Cytokines that are indicative of infiltrating T_H response-type, such as IL-17A, IL-17F, IL-22 (T_H17), and IFN-γ (T_H1), and IL-6, had similar kinetics of induction (**Figure 3.6**) to the bacterial growth kinetics (Figure 3.4). These cytokines were not largely detectable at early stages of infection but were upregulated at late stages of infection (day 7–14), with very high concentrations detected in the lungs of $Mal^{-/-}$ mice. This indicated a significant inflammatory immune response at late stages of *B. pertussis* infection. Other cytokines that are typically derived from innate resident lung cells, (DCs, macrophages and epithelial cells), including IL-12p70,

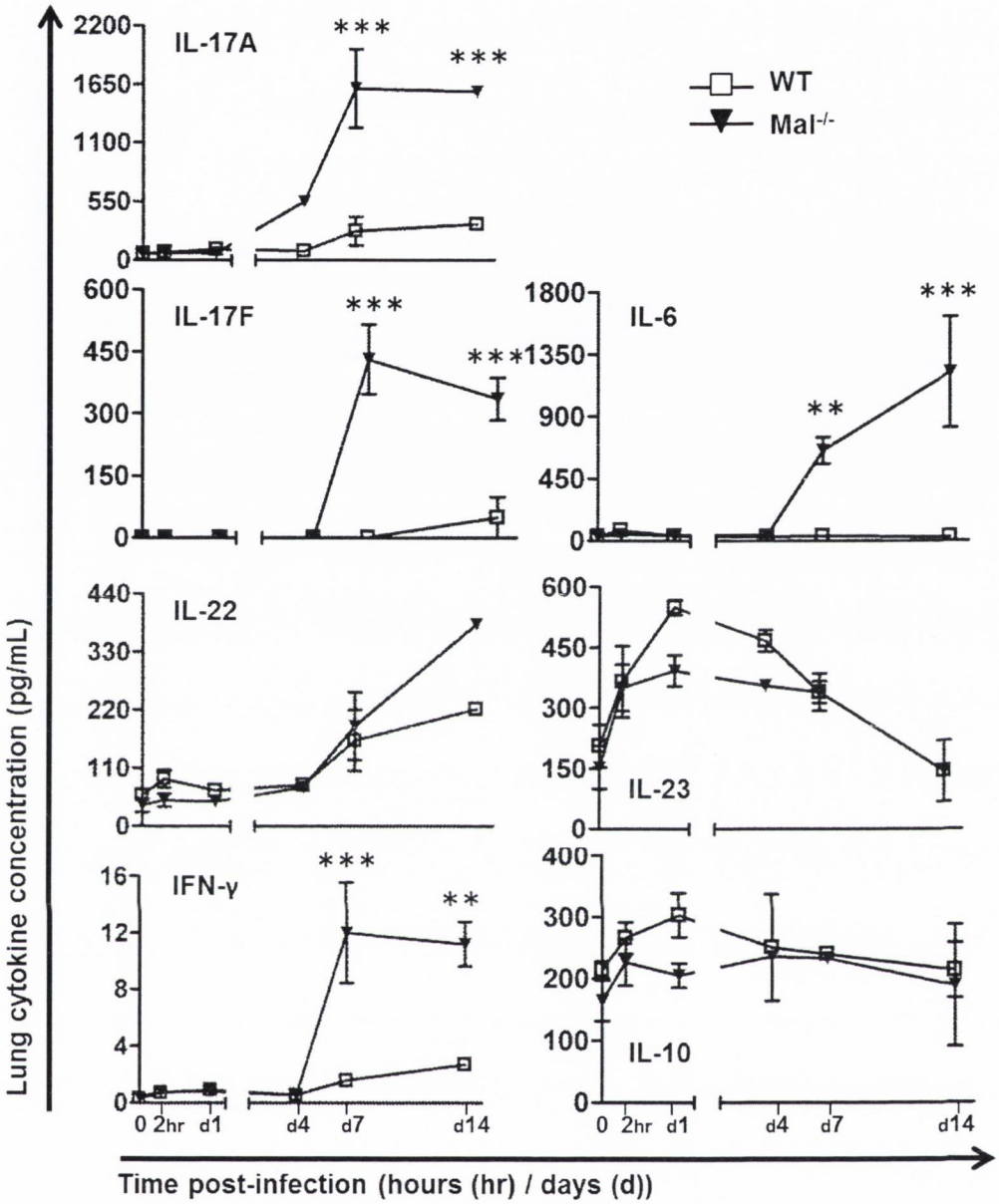


Figure 3.6 Enhanced *B. pertussis* induction of proinflammatory cytokine responses in Mal$^{-/-}$ mice

WT and Mal$^{-/-}$ mice were exposed to a 20 minute aerosol challenge with 1×10^9 *B. pertussis* per ml. At various times post-infection a single lung lobe was homogenised into 1 ml PBS, diluted, and used for cytokine ELISAs. Data are the mean ± SEM from n = 3 mice, from one experiment that is representative of three separate experiments. *P<0.05, **P<0.01, ***P<0.001 statistical analysis by 2-way ANOVA with Bonferroni test.

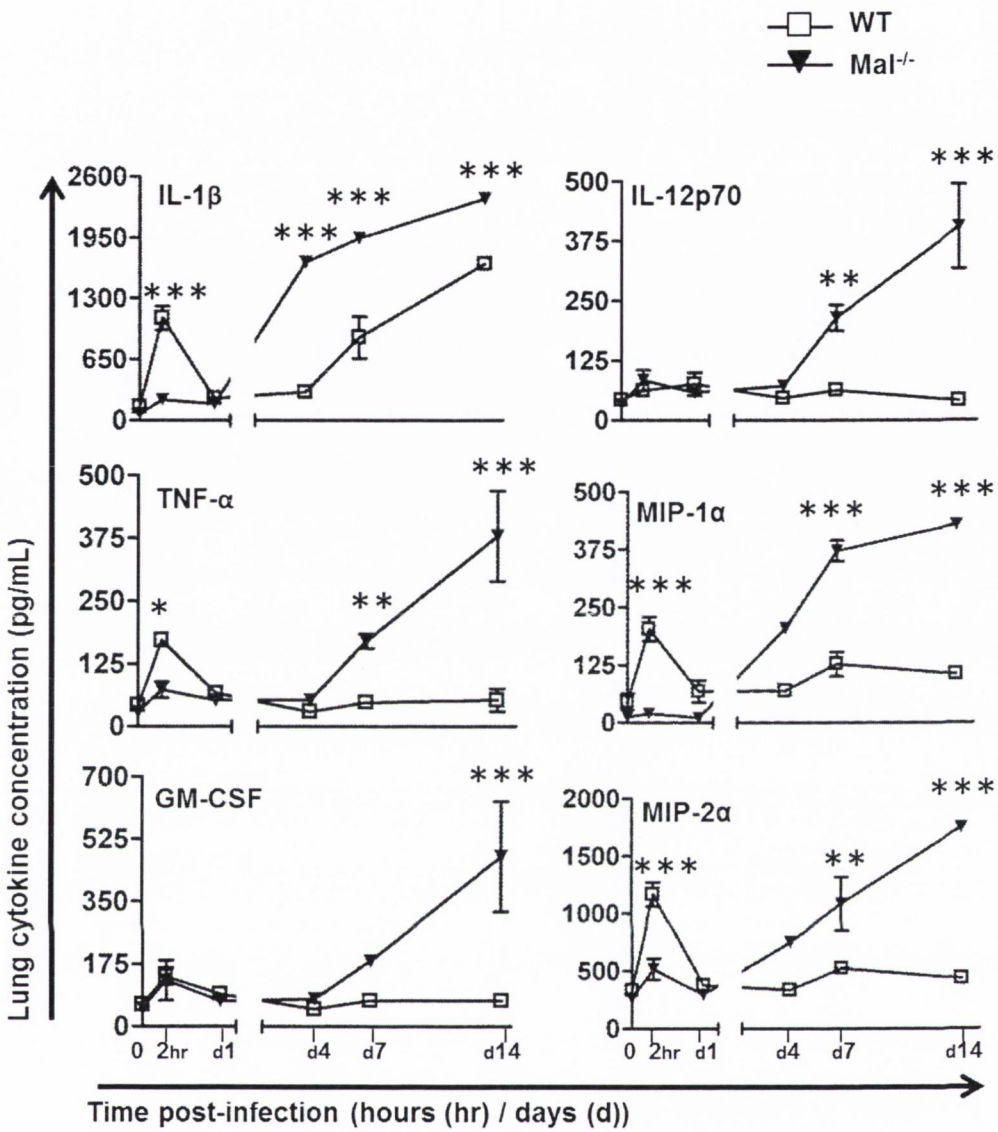


Figure 3.7 Mal is required for early innate cytokines responses post-*B. pertussis* infection

WT and Mal$^{-/-}$ mice were exposed to a 20 minute aerosol challenge with 1×10^9 *B. pertussis* per ml. At various times post-infection a single lung lobe was homogenised into 1 ml PBS, diluted, and used for cytokine ELISAs. Data are the mean ± SEM from $n = 3$ mice, from one experiment that is representative of three separate experiments. *P<0.05, **P<0.01, ***P<0.001 statistical analysis by 2-way ANOVA with Bonferroni test.

M-CSF, IL-1β, TNF, MIP-1α and MIP-2α, displayed a different pattern of expression in the lungs of *B. pertussis*-infected mice (**Figure 3.7**).

These cytokines were produced in a short 'burst' (henceforth referred to as the 'cytokine burst') in WT mice, that was evident 2 hours after infection, and then no longer evident by 24 hours post-infection (except IL-12p70, which was still evident at 24 hours). Despite the fact that bacterial numbers at 2 hours post-infection were equivalent in both mouse genotypes (Figure 3.4), the burst of innate IL-1β, TNF, MIP-1α and MIP-2α was significantly muted in the lungs of $Mal^{-/-}$ mice compared with WT mice. More than 6-fold more IL-1β, 2-fold more TNF, 2.5-fold more MIP-1α, and 10-fold more MIP-2α was detected in the lungs of WT mice compared with the lungs of $Mal^{-/-}$ mice.

Other innate cytokines that were evident in the initial cytokine burst, including IL-10 and IL-23 (**Figure 3.6**), were detected at very similar concentrations in the lungs of WT and $Mal^{-/-}$ mice 2 hours after infection, but by 24 hours there was more IL-10 and IL-23 production detected in the lungs of WT mice compared with $Mal^{-/-}$ mice. GM-CSF and IL-12p70, which were evident in the cytokine burst at 2 hours post-infection, were detected at equivalent concentrations in the lungs of WT and $Mal^{-/-}$ mice **(Figure 3.7)**.

3.2.6 Rationale for lung cell isolation technique and FACS gating

Because there were clear differences in cytokine concentrations detected in *B. pertussis*-infected lungs from WT and $Mal^{-/-}$ mice, it was decided to characterise the cell subsets present in the lungs after infection. The aim was to identify the cells responsible for the cytokine burst (Figure 3.7), and also the cells responsible for the high concentrations of IL-17A and IFN-γ, and other cytokines produced in the late stages of infection (Figure 3.6).

Two methods of cell isolation were employed, based on enzymatic and mechanical homogenisation of lungs or on broncho-alveolar lavage (BAL).

Homogenisation of lungs allows isolation of cells from alveolar spaces as well as the interstitial cells and cells attached to endothelial surfaces. A significant limitation of this crude technique is the creation of debris and cell death. This technique is best for identification of infiltrating cells as most inflammatory cells do not generally enter the alveoli. For this reason this method was employed to analyse NK cells and T cell subsets. The gating strategy for the FACS analysis of lung homogenate γδ T cells, NKT cells, $CD4^+$ T helper cells, $CD8^+$ cytotoxic T cells, and NK cells is presented in **figure 3.8**. CD49b was used as a marker of NKT cells, despite the fact that not all NKT cells are $CD49b^+$. There was no access to the ideal reagent, CD1d tetramer, which definitively identifies iNKT cells. Here the term 'NKT cell' is interchangeable with '$CD49b^+$ T cell', but not with the general consensus of an NKT cell.

BAL is a much cleaner method of isolating cells with better viability than lung digestion. However, a limitation of the method is that it only isolates cells present in the alveolar spaces. BAL is ideal for analysis of resident alveolar cells and inflammatory infiltrating cells that enter the alveoli. For this reason the technique was used to analyse alveolar macrophages, pulmonary DC, neutrophils, eosinophils, inflammatory monocytes and exudate macrophages. The gating strategy for the FACS analysis of these populations is presented in **figure 3.16**. Both methods of lung cell analysis used FACS with debris gated out by FCS-A vs. SSC-A, doublets gated out by FSC-A vs. FSC-H, and dead cells gated out as determined by staining with propidium iodide (PI) or Live/Dead Aqua (LDA). FMOs and isotype control antibodies were used to set baselines prior to experimentation, and elsewhere as necessary. The data were validated with fluorescence minus one (FMO) controls **(Supp. figure. 1)** and IgG_1 or IgG_{2a} isotype antibody controls **(Supp. figure. 2** and **supp fig. 3)**.

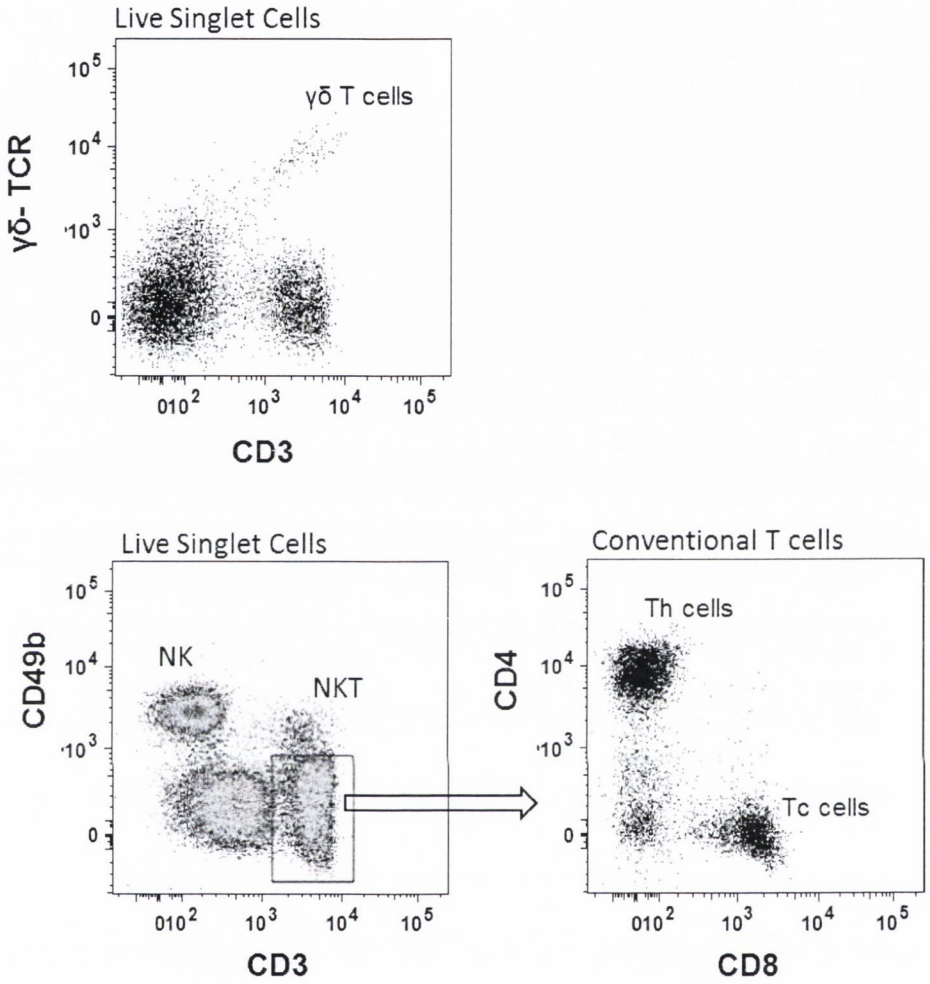


Figure 3.8 FACS Gating Strategy for NK cells and T cell subsets

Lungs were mechanically and enzymatically digested. Red blood cells were lysed. Leukocytes were analysed by FACS for conventional $CD4^+$ or $CD8^+CD3^+CD49b^-$ $\gamma\delta$–TCR^- T cells, $CD3^-CD49b^+$ NK cells $CD3^+CD49b^+$ NKT cells and $CD3^+\gamma\delta$–TCR^+ $\gamma\delta$ T cells. Prior to end-gating, cells were selected for FCS-A vs SSC-A to gate out junk material, FCS-A vs FCS-W to gate out doublet cells, and FCS-A vs Live/Dead Aqua or PI to gate out dead cells. Antibodies used were CD3–FITC, $\gamma\delta$-TCR–APC, CD49b–PE, CD4–APC-Cy7, CD8–PerCpCy5.5.

3.2.7 Enhanced infiltration of T cells into the lungs of *B. pertussis* infected Mal$^{-/-}$ mice

FACS analysis of cells prepared by homogenisation of lungs revealed that the total number of γδ T cells, CD4$^+$ T cells and CD8$^+$ T cells was equivalent in uninfected WT and Mal$^{-/-}$ mice (**Figure 3.9**). There was also no detectable increase in these cell types over the course of the first 4 days of *B. pertussis* infection. In WT mice there was no change in this number at day 7 of infection. However, there was an increase in the total number of leukocytes in the lungs of Mal$^{-/-}$ mice by day 7. By the peak of cellular infiltration at day 14 there were significantly more leukocytes (10-fold more) in the lungs of Mal$^{-/-}$ mice compared with WT mice **(Figure 3.9)**. Between day 7 and day 21 of infection there were significantly more CD4$^+$ T cells (up to 10-fold more) and γδ T cells (up to 10-fold more) in the lungs of Mal$^{-/-}$ mice compared with WT mice. There were also more CD8$^+$ T cells detected in the lungs of infected Mal$^{-/-}$ mice compared with WT, but the difference was not significant.

3.2.8 IL-17A-producing and IFN-γ-producing T cells in *B. pertussis*-infected Mal$^{-/-}$ mice

ELISA of lung homogenates indicated that there were much higher concentrations of IL-17 and IFN-γ in *B. pertussis*-infected Mal$^{-/-}$ mice compared with infected WT mice (Figure 3.6). This could be explained by the observation that there were more T cells in the lungs of Mal$^{-/-}$ mice at late stages of infection (Figure 3.9). To confirm this, and to determine the source of the cytokines, it was decided to analyse the different T cell subsets for production of IL-17A and IFN-γ, by intracellular FACS analysis. The method employed was *ex vivo* incubation with Brefeldin A, but without the commonly used *in vitro* stimulants, PMA and ionomycin. There were

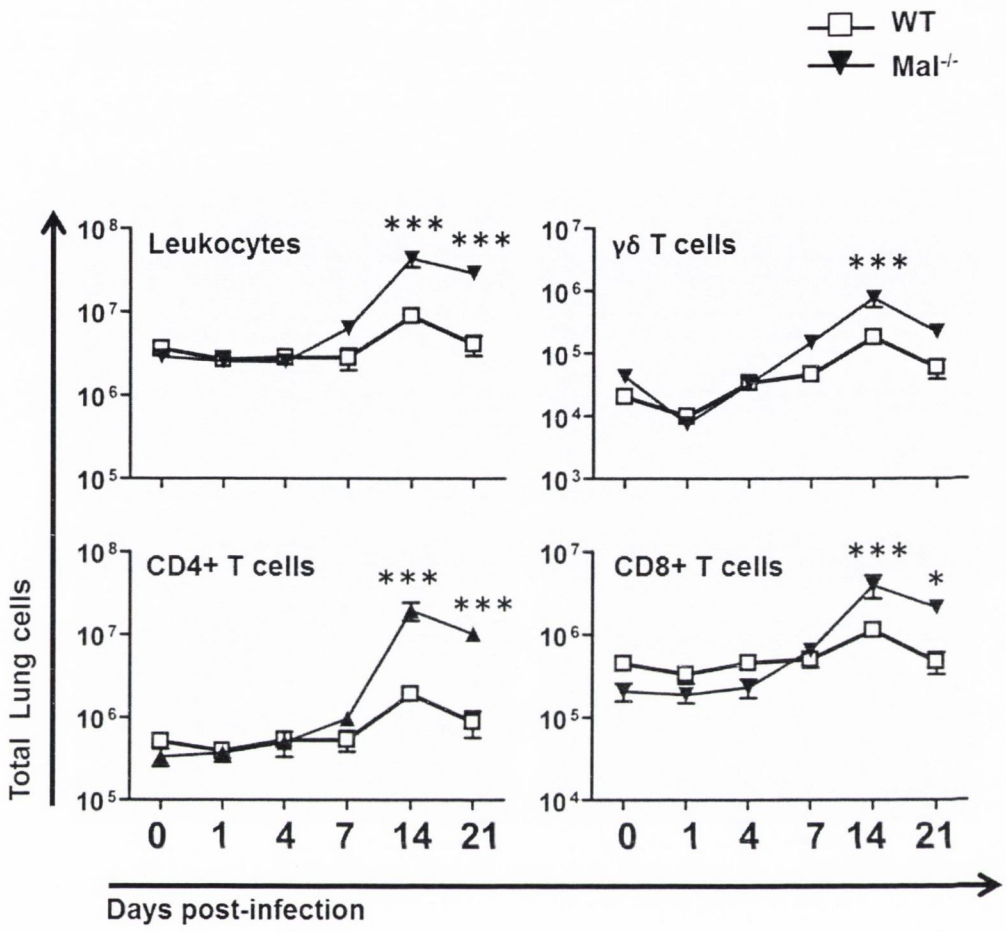


Figure 3.9 Enhanced lung T-cell infiltration in late stages of *B. pertussis* infection of Mal$^{-/-}$ mice

WT and Mal$^{-/-}$ mice were exposed to a 20 minute aerosol challenge with 1×10^9 *B. pertussis* per ml. At various times after infection lungs were mechanically and enzymatically digested. Red blood cells were lysed and isolated leukocytes were analysed by FACS. Data are the mean ± SEM from n = 3 mice, from one experiment that is representative of three separate experiments. *P<0.05, **P<0.01, ***P<0.001 statistical analysis by 2-way ANOVA with Bonferroni test.

significantly more IFN-γ^+CD4$^+$ T cells and IFN-γ^+CD8$^+$ T cells detected in the lungs of Mal$^{-/-}$ mice compared with WT mice at day 14 and day 21 of *B. pertussis* infection (**Figure 3.10**).

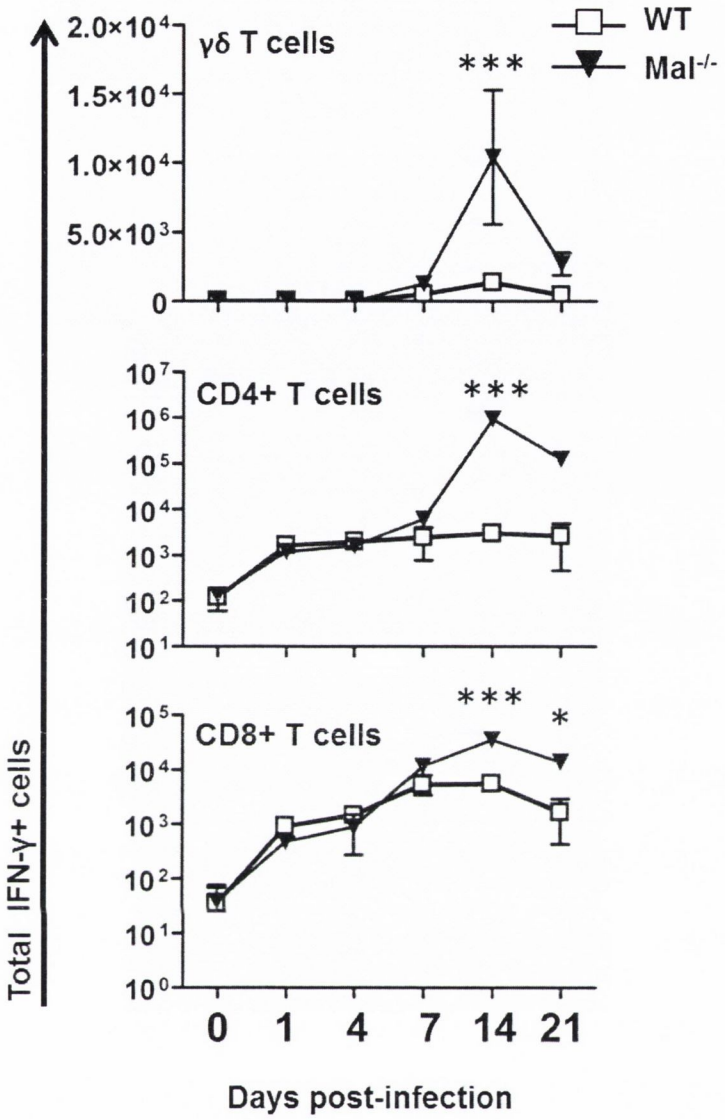
here were also more IFN-γ^+ $\gamma\delta$ T cells and more IL-17A-producing CD4$^+$ T cells, CD8$^+$ T cells and $\gamma\delta$ T cells at late stages of infection in the lungs of Mal$^{-/-}$ mice compared with WT mice, but these differences were not significant (**Figure 3.10** and **3.11**). Infiltration into the lungs by these cytokine-producing cells correlated temporally with the increased cytokine production detected by ELISA of lung homogenates (Figure 3.6).

3.2.9 Increased frequency of cytokine producing cells in the lungs of *B. pertussis*-infected Mal$^{-/-}$ mice

As NK cells and NKT cells are resident and recruited cells in the lungs, and are also known to produce IL-17A and IFN-γ, it was decided to assess the frequency of cytokine producing cells.

7 days after infection only 2.5% of $\gamma\delta$ T cells from WT mice, but almost 50% of $\gamma\delta$ T cells from Mal$^{-/-}$ mice produced IL-17A (**Figure 3.12**). NK cells were not significant IL-17A producers at this stage of infection, and only a small percentage of conventional $\alpha\beta$ T cells produced IL-17A. Less than 1.5% of NKT cells from WT mice, but as many as 12% of NKT cells from Mal$^{-/-}$ mice produced IL-17A.

The percentage of IFN-γ^+ cells was also assessed. Although there was a greater percentage of IFN-γ^+ cells in the Mal$^{-/-}$ mice compared with WT mice, IFN-γ was mainly produced by conventional $\alpha\beta$ T cells, NK cells and NKT cells (**Figure 3.13**), and not by $\gamma\delta$ T cells. This is in contrast to the data showing that almost 50% of $\gamma\delta$ T cells from Mal$^{-/-}$ mice produced IL-17A. Also the percentage of IFN-γ-producing NK, NKT, and $\gamma\delta$ T cells was only around 3% of the total of those cell populations, indicating a possible *B. pertussis*-related IL-17A production bias in Mal$^{-/-}$ lungs.



igure 3.10 Enhanced infiltration of IFN-γ-producing cells in lungs from *B. pertussis*-infected Mal$^{-/-}$ mice

WT and Mal$^{-/-}$ mice were exposed to a 20 minute aerosol challenge with 1×10^9 *B. pertussis* per ml. At various times post-infection lungs were mechanically and enzymatically digested. Red blood cells were lysed. Leukocytes were permeabilised and fixed for intracellular FACS analysis. Cells were stained with anti-IFN-γ FACS antibody. Data is the mean ± SEM from n = 3 mice, from one experiment that is representative of three separate experiments. *P<0.05, **P<0.01, ***P<0.001 statistical analysis by 2-way ANOVA with Bonferroni test.

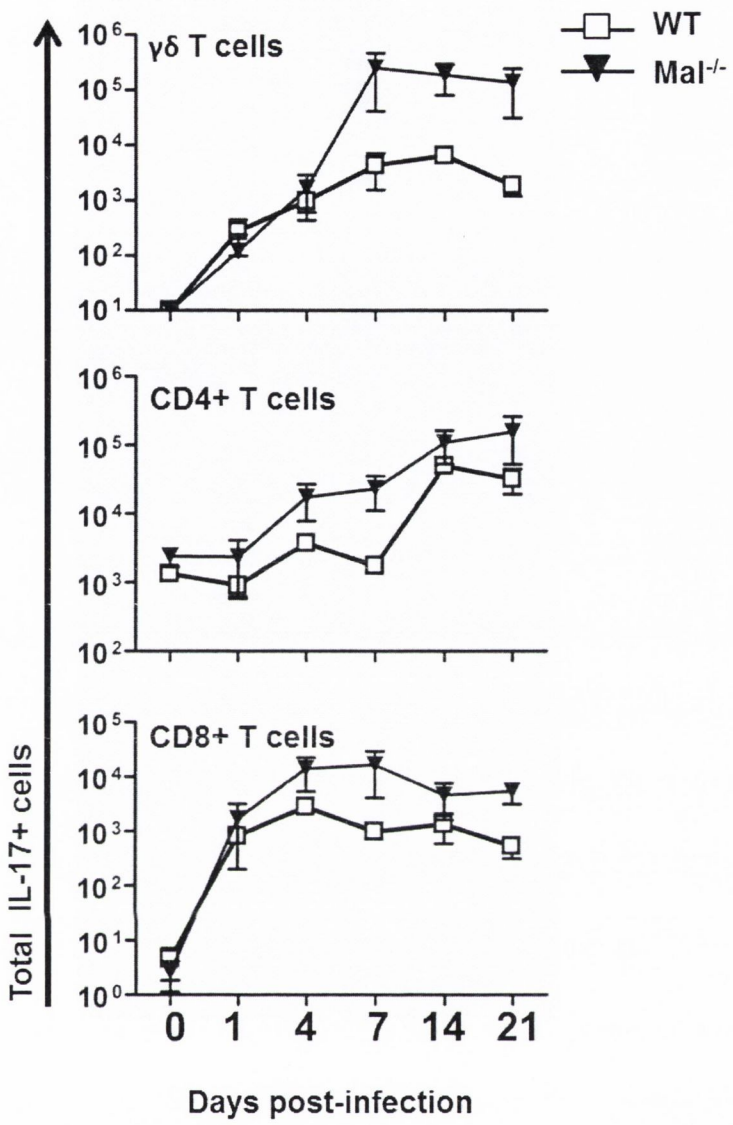


Figure 3.11 Enhanced infiltration of IL-17A-producing cells in lungs from *B. pertussis*-infected Mal$^{-/-}$ mice

WT and Mal$^{-/-}$ mice were exposed to a 20 minute aerosol challenge with 1×10^9 *B. pertussis* per ml. At various times post-infection lungs were mechanically and enzymatically digested. Red blood cells were lysed. Leukocytes were permeabilised and fixed for intracellular FACS analysis. Cells were stained with anti-IL-17A FACS antibody. Data is the mean ± SEM from n = 3 mice, from one experiment that is representative of three separate experiments. *P<0.05, **P<0.01, ***P<0.001 statistical analysis by 2-way ANOVA with Bonferroni test.

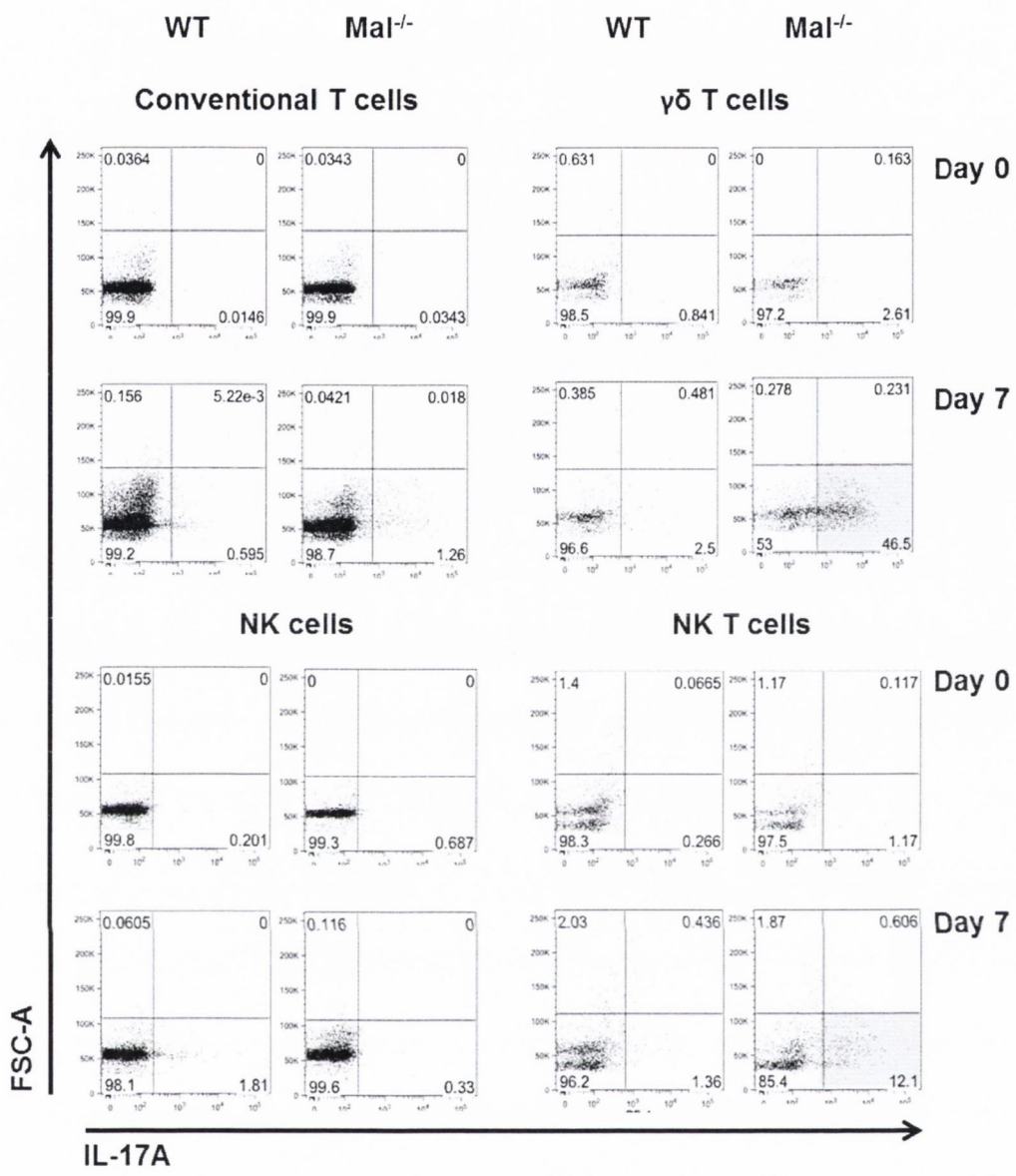


Figure 3.12 Increased frequency of IL-17A-producing γδ T cells and NKT cells in the lungs of *B. pertussis*-infected Mal$^{-/-}$ mice

WT and Mal$^{-/-}$ mice were exposed to a 20 minute aerosol challenge with 1×10^9 *B. pertussis* per ml. At day 7 post-infection lungs were mechanically and enzymatically digested. Red blood cells were lysed and leukocytes were permeabilised and fixed for intracellular FACS analysis. Cells were stained with anti-IL-17A FACS antibody. Data are from a representative experiment in which at least three mice of each genotype were used at each time point. Shaded panels are to draw attention to important data.

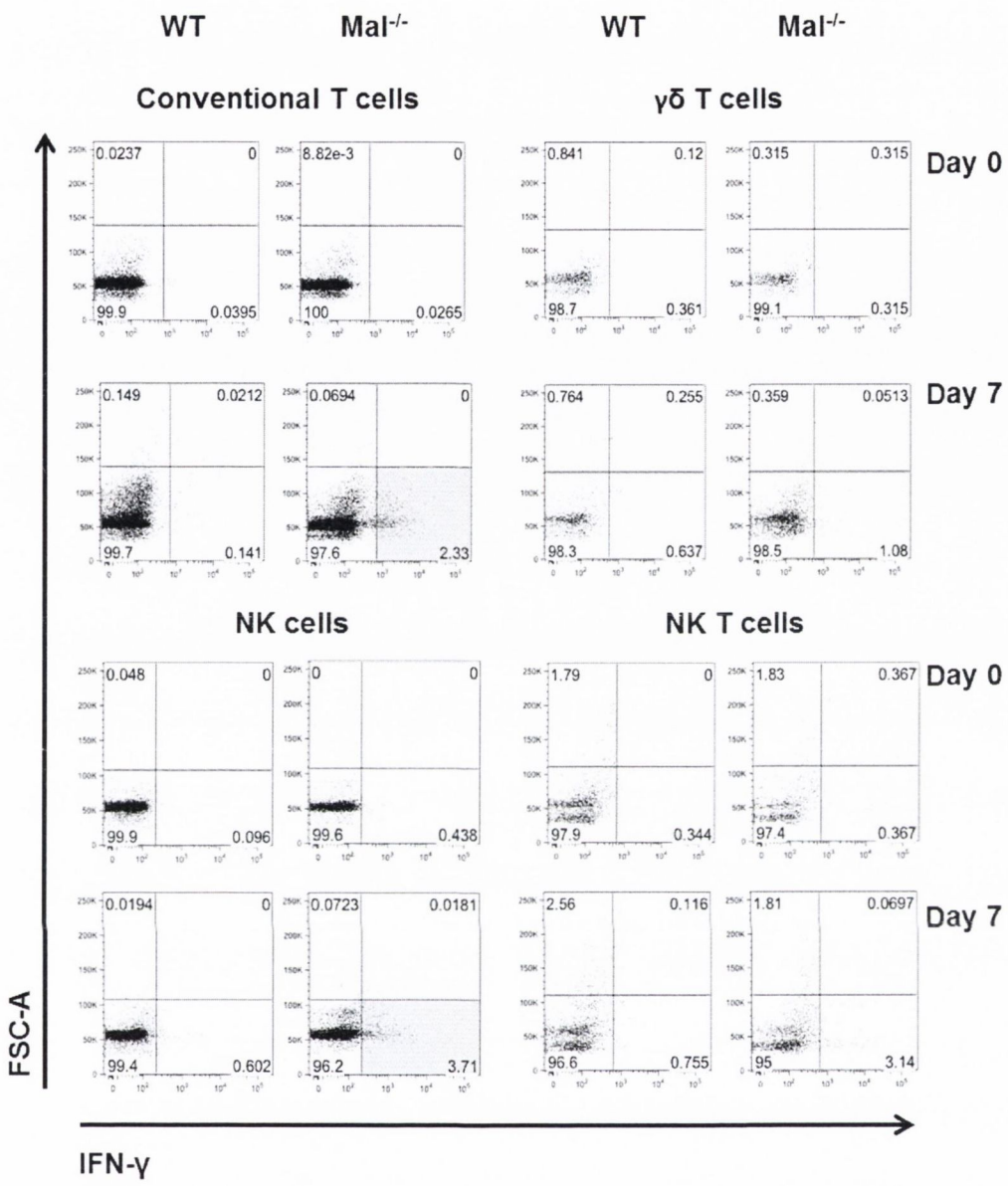


Figure 3.13 Increased frequency of IFN-γ-producing T cells and NK cells in the lungs of Mal$^{-/-}$ mice

WT and Mal$^{-/-}$ mice were exposed to a 20 minute aerosol challenge with 1×10^9 *B. pertussis* per ml. At day 7 post-infection lungs were mechanically and enzymatically digested. Red blood cells were lysed and leukocytes were permeabilised and fixed for intracellular FACS analysis. Cells were stained with anti-IFN-γ FACS antibody. Data are from one representative experiment in which at least three mice of each genotype were used at each time point. Shaded panels are to draw attention to important data.

3.2.10 Increased absolute number of cytokine-producing cells in the lungs of *B. pertussis* infected Mal$^{-/-}$ mice

Absolute cell numbers correlated with the ELISA data that showed a peak of cytokine production 7 days after *B. pertussis* infection. There were significantly more IFN-γ^+ conventional T cells and NK cells detected in lungs of Mal$^{-/-}$ mice compared with WT mice (**Figure 3.14**). WT and Mal$^{-/-}$ mice also had significantly more IFN-γ^+ conventional T cells and NK cells in their lungs compared with uninfected control mice. While there were more IFN-γ^+ $\gamma\delta$ T cells and NKT cells detected in the lungs of infected Mal$^{-/-}$ mice compared with WT, the differences were not significant.

Analysis of the total number of IL-17A-producing cells in the lungs of *B. pertussis* infected WT and Mal$^{-/-}$ mice revealed a significant correlation with the ELISA data (Figure 3.6) and the FACS percentage data (Figure 3.12), which indicated that a primary source of IL-17A production in the lungs was $\gamma\delta$ T cells. There were as many as 23,000 IL-17A-producing $\gamma\delta$ T cells detected in infected Mal$^{-/-}$ lungs compared to less than 1,000 in the WT (**Figure 3.15**). There were also significantly more (4-fold more) IL-17A-producing conventional T cells in the lungs of Mal$^{-/-}$ mice compared with the lungs of WT mice, and significantly more IL-17A$^+$ conventional T cells and $\gamma\delta$ T cells of infected mice compared with uninfected mice. There were more IFN-γ^+ NKT cells in the lungs of infected Mal$^{-/-}$ mice compared with WT, but this difference was not significant.

Together these FACS data (Figure 3.9–3.15) indicate that there was a significant increase in the percentage and absolute number of IFN-γ-producing conventional CD4$^+$ and CD8$^+$ T cells and NK cells, and IL-17A-producing conventional T cells and $\gamma\delta$ T cells in the lungs of *B. pertussis* infected Mal$^{-/-}$ mice, compared to the lungs of WT mice.

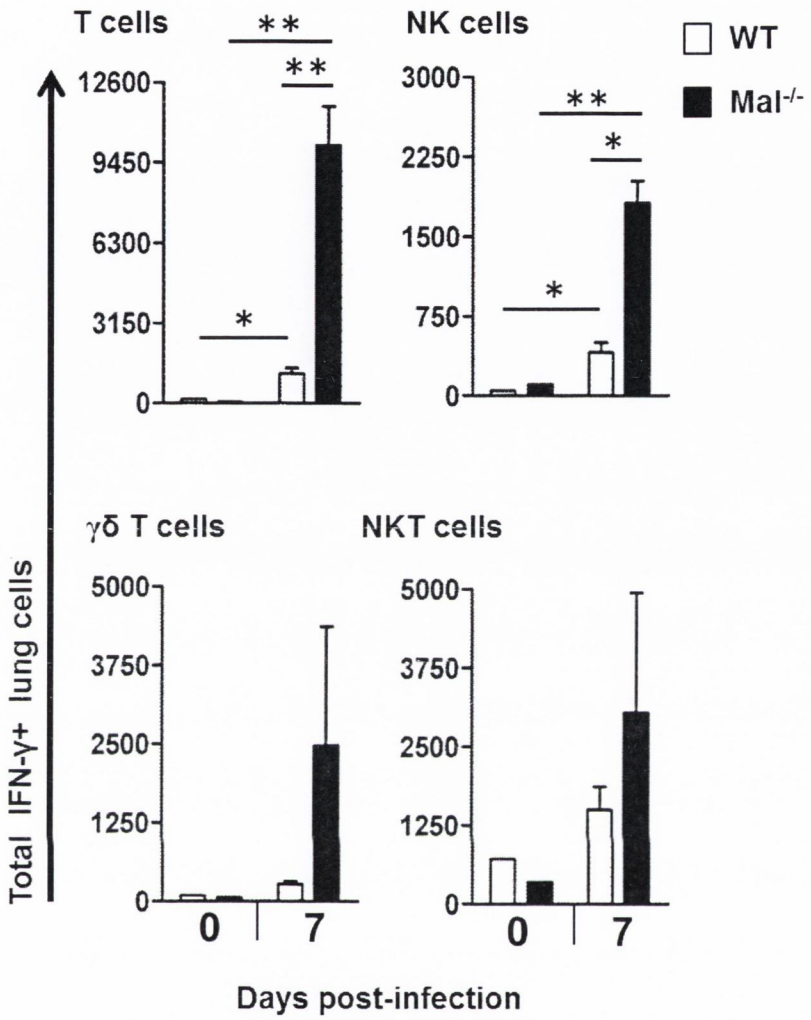


Figure 3.14 Enhanced number of IFN-γ-producing cells in the lungs of *B. pertussis*-infected Mal$^{-/-}$ mice

WT and Mal$^{-/-}$ mice were exposed to a 20 minute aerosol challenge with 1×10^9 *B. pertussis* per ml. At day 7 post-infection lungs were mechanically and enzymatically digested. Red blood cells were lysed and leukocytes were permeabilised and fixed for intracellular FACS analysis. Cells were stained with anti-IFN-γ FACS antibody. Data are the mean ± SEM from n = 3 mice, from one experiment that is representative of two separate experiments. *P<0.05, **P<0.01, ***P<0.001 statistical analysis by 2-way ANOVA with Bonferroni test.

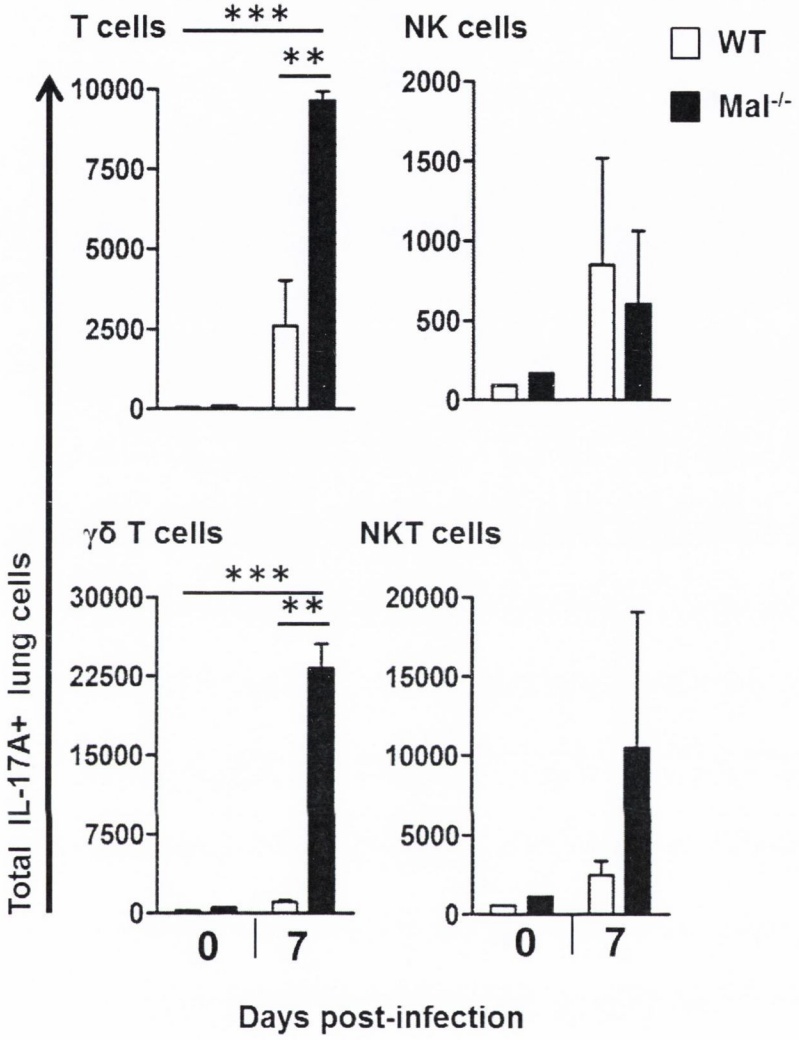


Figure 3.15 Enhanced numbers of IL-17A-producing T cells in the lungs of *B. pertussis*-infected Mal$^{-/-}$ mice

WT and Mal$^{-/-}$ mice were exposed to a 20 minute aerosol challenge with 1×10^9 *B. pertussis* per ml. At day 7 post-infection lungs were mechanically and enzymatically digested. Red blood cells were lysed and leukocytes were permeabilised and fixed for intracellular FACS analysis. Cells were stained with anti-IL-17A FACS antibody. Data are the mean ± SEM from n = 3 mice, from one experiment representative of two separate experiments. *P<0.05, **P<0.01, ***P<0.001 statistical analysis by 2-way ANOVA with Bonferroni test.

3.2.11 *B. pertussis* infection depletes the resident alveolar macrophage population in Mal$^{-/-}$ mice

Having shown that the cellular source of IL-17A in the lungs of *B. pertussis*-infected mice was mainly T cells (Figure 3.15), and the source of IFN-γ was T cells and NK cells (Figure 3.14), the next aim was to identify the source of the early burst of IL-1β, TNF, MIP-1α and MIP-2α that had been observed by ELISA of lung homogenates from WT mice 2 hours after infection (Figure 3.7). Because the innate cytokine burst occurred at 2 hours post-infection, and not later, it was hypothesised that resident lung cells could be the source. Lung resident AMs, which are known to produce those proinflammatory cytokines, are present principally in the alveolar spaces, therefore, it was decided that the best method of cell isolation was BAL. The FACS gating strategy for BAL-F was described previously and is presented in **figure 3.16**. BAL-F was taken at various times post-*B. pertussis* infection. Cells were analysed by FACS and the percentage of each population was determined. In uninfected mice more than 90% of recovered cells were AMs **(Figure 3.17)**. The other cells consisted mainly of neutrophils, monocytes and DCs. In Mal$^{-/-}$ mice there was a slightly larger percentage of these cells.

Following *B. pertussis* infection there was significant infiltration of Ly6G^{+}Ly6C^{+} neutrophils detected in the lungs of WT and Mal$^{-/-}$ mice, however this was earlier in the Mal$^{-/-}$ mice (Day 1) compared with the WT mice (Day 3) **(Figure 3.18)**. Furthermore, after 14 days of *B. pertussis* infection, the proportion of neutrophils reached approximately 75% of all cells recovered from the BAL-F of Mal$^{-/-}$ mice, compared with just 50% in the BAL-F from WT mice **(Figure 3.17** and **3.18)**. Concurrent with the enhanced infiltration of neutrophils in the lungs of *B. pertussis*-infected Mal$^{-/-}$ mice, there was a significant decrease in the percentage of resident AMs **(Figure 3.17** and **3.19)**. While the percentage of AMs did decrease in

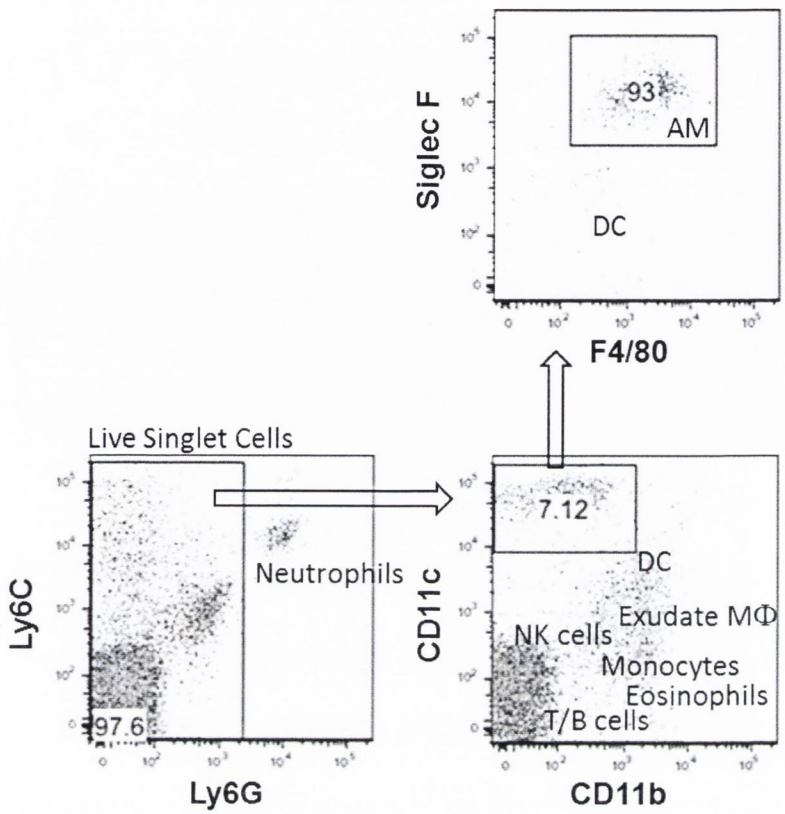


Figure 3.16 BAL-F FACS gating strategy

Example from a *B. pertussis* infected WT lung. Prior to end-gating, cells were selected for FCS-A vs SSC-A to gate out junk material, FCS-A vs FCS-W to gate out doublet cells, and FCS-A vs Live/Dead Aqua to gate out dead cells. Gating of individual populations was dependent on expression of the following surface markers.

AM: $FSC^{hi}SSC^{hi}CD11c^{+}SiglecF^{+}CD11b^{lo}F4/80^{+}MHC\text{-}II^{lo\text{-}med}$ autofluorescent,
DC: $CD11c^{+}F4/80^{-}SiglecF^{-}CD11b^{+}MHC\text{-}II^{hi}$,non-autofluorescent
Monocytes: $CD11c^{-}CD11b^{+}Ly6C^{+}Ly6G^{-}F4/80^{+}$,
Neutrophils: $CD11b^{+}Ly6G^{+}Ly6C^{+}F4/80^{-}$
Exudate macrophage: CD11b+CD11c-$F4/80^{hi}$Ly6C-
Eosinophils: $SSC^{hi}MHC\text{-}II^{lo}$CD11b+$SiglecF^{+}$

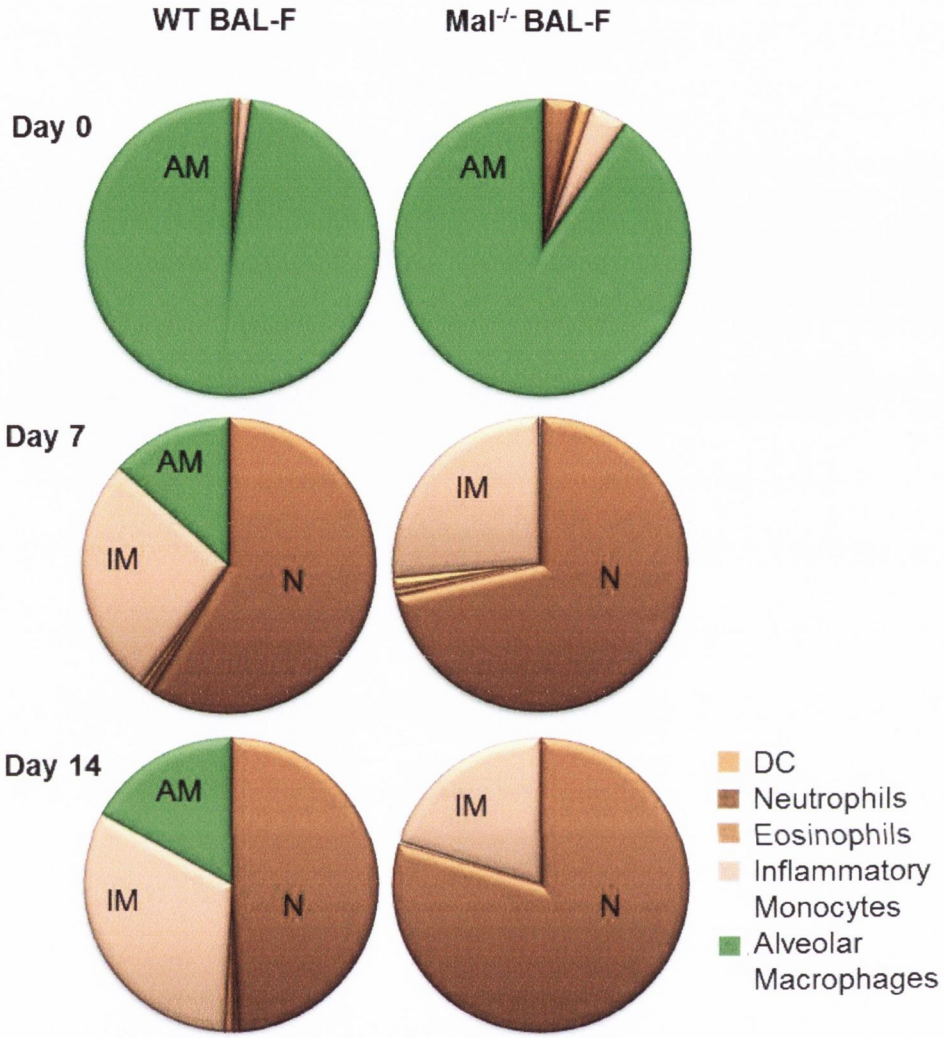


Figure 3.17 AM are absent from *B. pertussis*-infected Mal$^{-/-}$ mice

WT and Mal$^{-/-}$ mice were exposed to a 20 minute aerosol challenge with 1×10^9 *B. pertussis* per ml. BAL-F was taken from mice at various times post-infection and FACS analysed for various cell subsets. Data are the mean of at least 3 mice per genotype per time point.

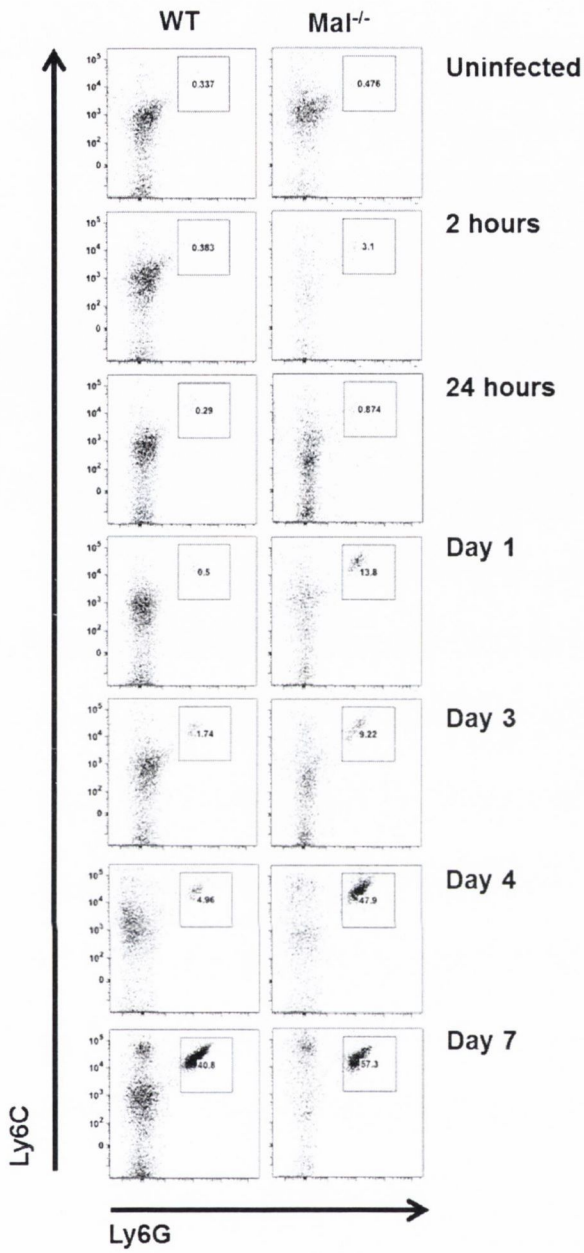


Figure 3.18 *B. pertussis*-induced neutrophilia is more pronounced in Mal$^{-/-}$ lungs

WT and Mal$^{-/-}$ mice were exposed to a 20 minute aerosol challenge with 1×10^9 *B. pertussis* per ml. BAL-F was taken from mice at various times post-infection and FACS analysed for various cell subsets. The result is representative of two experiments.

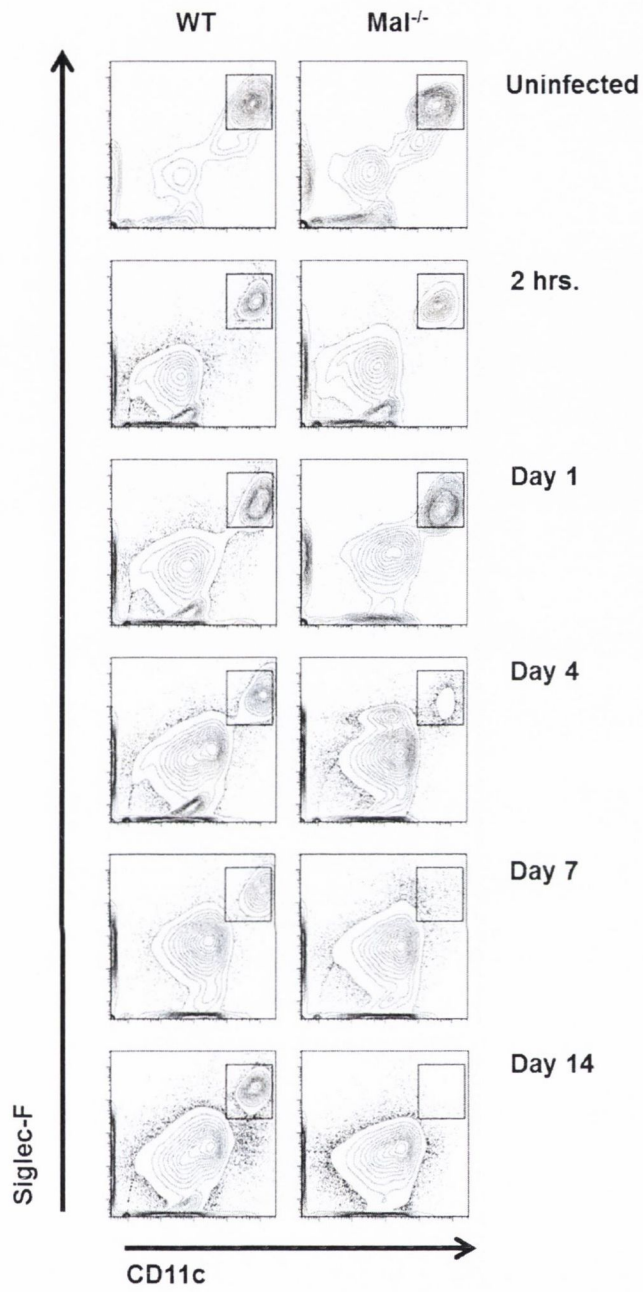


Figure 3.19 *B. pertussis* depletes Mal$^{-/-}$ alveolar macrophages from BAL-F

WT and Mal$^{-/-}$ mice were exposed to a 20 minute aerosol challenge with 1×10^9 *B. pertussis* per ml. BAL-F was taken from mice and FACS analysed for various cell subsets. FACS plots are representative of at least three mice per genotype per time point. The experiment was repeated and is consistent.

the lungs of WT mice, the reduction in percentage $Mal^{-/-}$ AMs was evident at day 4 of infection, and by day 7 AMs were not detectable in the lungs of $Mal^{-/-}$ mice. In contrast, AMs constituted 10–15% of the cells in BAL-F from WT mice as late as day 14 of *B. pertussis* infection. Assessment of absolute numbers of cells in the lungs confirmed that there was an influx of neutrophils (day 14, approx. WT = 150,000, $Mal^{-/-}$ = 1,000,000) and inflammatory monocytes (day 14, approx. WT = 150,000, $Mal^{-/-}$ = 800,000) following *B. pertussis* infection, and that there were many more at late stages of infection (**Figure 3.20**). There was also a decline in the absolute number of AMs in the lungs of $Mal^{-/-}$ mice. 7 days after *B. pertussis* infection AMs were almost undetectable in BAL-F from $Mal^{-/-}$ mice.

The absolute number of AMs was lower in the BAL-F from $Mal^{-/-}$ uninfected mice compared to WT mice (approx. 65%), possibly explaining the defect in 'burst cytokines' detected in those mice 2 hours after infection. There was also a small increase in the number of eosinophils and DCs in both genotypes but these numbers remained low throughout the course of the infection. These findings indicate that Mal plays an important role in maintenance of AM numbers before and after *B. pertussis* infection, and that these cells might be vital for anti-*B. pertussis* immunity.

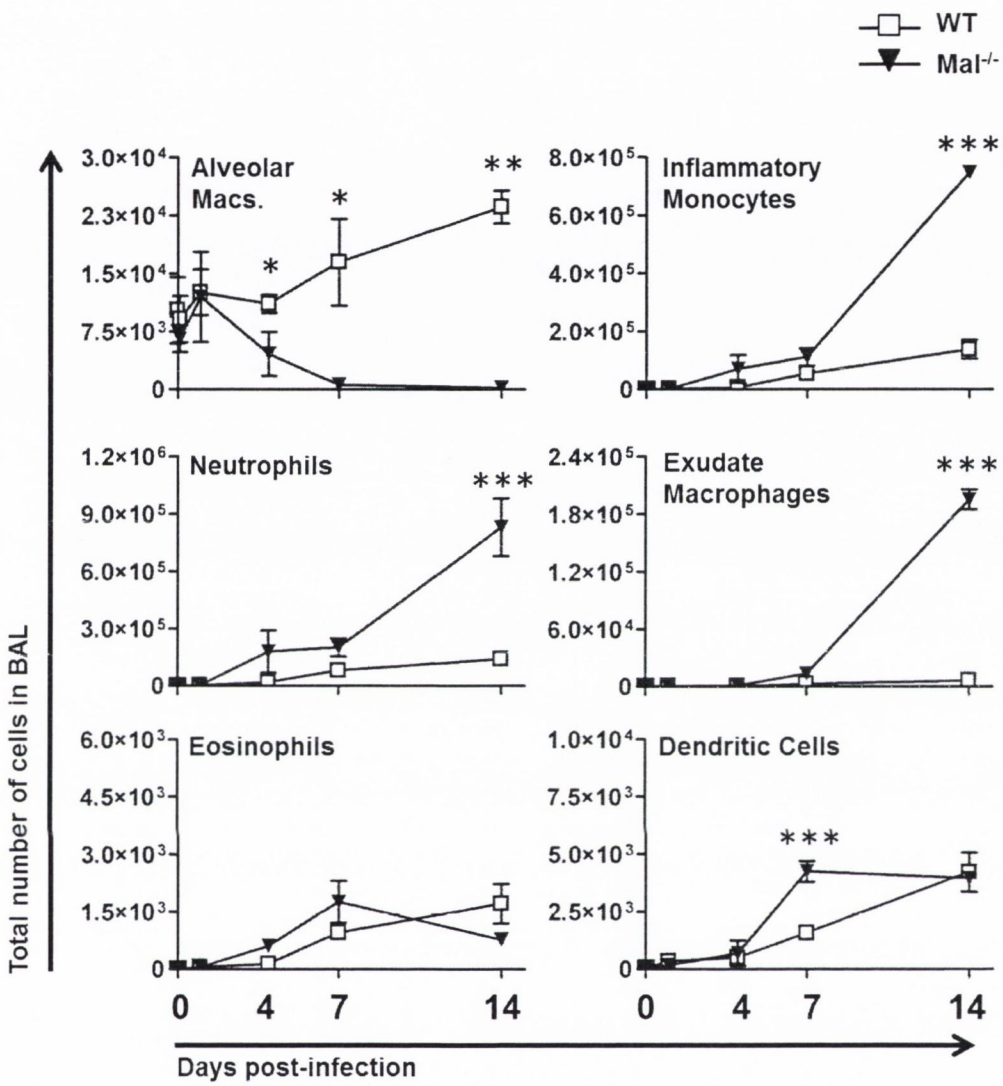


Figure 3.20 *B. pertussis* infection depletes AMs from Mal$^{-/-}$ mice

WT and Mal$^{-/-}$ mice were exposed to a 20 minute aerosol challenge with 1×10^9 *B. pertussis* per ml. BAL-F was taken from mice at various times post-infection and FACS analysed for various cell subsets. Data are the mean ± SEM from n = 3 mice, from one experiment that is representative of three separate experiments. *P<0.05, **P<0.01, ***P<0.001 statistical analysis by 2-way ANOVA with Bonferroni test.

3.3 DISCUSSION

Although Mal is known to protect mice from lung infection with *E. coli*[257] and *K. pneumoniae*,[258] and the S180L MAL SNP is known to be involved in susceptibility to *M. tuberculosis* infection in humans,[262] the mechanism of Mal-mediated protection from lung bacterial infection remains incompletely characterised.

The present study showed that Mal plays a vital role in protection from *B. pertussis* infection of the airways. An initial burst of Mal-dependent cytokines two hours after *B. pertussis* infection of the lungs preceded lower bacterial numbers and a lower incidence of pathology and fatality in WT mice compared with $Mal^{-/-}$ mice. It was also discovered that *B. pertussis* infection of the lungs caused significantly more immigration of inflammatory neutrophils and monocytes, and IL-17A$^+$ T cells and IFN-γ^+ T cells and IFN-γ^+ NK cells in $Mal^{-/-}$ mice compared with WT mice. Conversely it was discovered that resident AMs increase in numbers in the lungs of infected WT mice but completely disappear from the lungs of infected $Mal^{-/-}$ mice.

The resurgence of *B. pertussis* in vaccinated populations[2] is evidence that our understanding of this host–pathogen interaction is incomplete. Further characterisation of the immune response to *B. pertussis* and the factors that determine susceptibility are required. Although TLRs are thought to be vital for host protection from lung bacterial infection, only TLR4-defective C3HeJ mice have been used as a model for *B. pertussis* susceptibility.[117] There are no other reports of TLR or TLR signalling gene-defective mice infected with *B. pertussis*. Although the signalling adaptor Mal signals in response to TLR4, it can also signal downstream of TLR2. TLR4 can induce the TRIF–TRAM pathway, which Mal cannot.

The results presented in this chapter systematically characterised the cellular immune response to *B. pertussis* infection, and the associated

bacterial growth kinetics and subsequent gross pathology, with special attention to significant variance between WT and $Mal^{-/-}$ mice. The fact that almost half $Mal^{-/-}$ mice were found to die as a result of *B. pertussis* infection, whereas WT mice were completely resistant, indicates that Mal is a vital protective TLR adaptor required for immunity to initial *B. pertussis* exposure.

In the late stages of *B. pertussis* infection very high bacterial numbers in lungs from $Mal^{-/-}$ mice correlated with an equally robust immune response that included infiltration of inflammatory cells. This is consistent with the published data on *B. pertussis* infection of TLR4-defective C3HeJ mice.[115,117] A significant point of variance between the immune response to *B. pertussis* infection in $Mal^{-/-}$ and C3HeJ mice was that significant production of IFN-γ was not detected in C3HeJ mice, as it was in $Mal^{-/-}$ mice. IL-17A was not measured in the C3HeJ study. This could indicate that *B. pertussis* activation of the TRIF–TRAM pathway is needed for optimal T_H1 responses. However, the $Mal^{-/-}$ mice used in this study were on a C57Bl/6 background and so are not directly comparable with C3HeJ mice. The boosted IFN-γ in the $Mal^{-/-}$ relative to C3HeJ mice might be specific to the C57Bl/6 background of $Mal^{-/-}$ mice.

Although *B. pertussis*-infected $Mal^{-/-}$ mice had stronger T_H cell responses compared with WT mice late in infection, these differences are probably related to the superior bacterial growth in $Mal^{-/-}$ mice, or the associated lung damage. Thereby, the T_H cell responses might be unrelated to Mal signalling. Instead, the major protective function of Mal during the course of *B. pertussis* infection could be in the very early stages of infection. As early as two hours after *B. pertussis* infection, it was evident that there was a defect in the ability of resident cells from the lungs of $Mal^{-/-}$ mice to produce a 'burst' of IL-1β, TNF, MIP-1α, and MIP-2α. This burst is characteristic of the WT response to *B. pertussis* infection and might be vital for host protection. The proinflammatory cytokines and chemokines

produced in the burst are known to be crucial determinants of subsequent immune responses, as they are involved in initiating immune responses to infection, including the recruitment of inflammatory phagocytes and the activation of bacterial killing mechanisms. A similar early cytokine burst was also seen in TLR4-sufficient C3HeN mice and not in C3HeJ mice,[117] indicating that unlike the late cytokine responses, the early cytokine burst is likely an immediate host-protective TLR4-activated Mal-dependant response to *B. pertussis* LPS. The early cytokine burst is probably produced by epithelial cells, AMs and pulmonary DCs, all of which are known to express surface TLR2 and TLR4, and Mal. AMs, in particular, have been shown to be potentially important modulators of *B. pertussis* infectivity, as *B. pertussis* can escape phagolysosomal degradation in AM endosomal compartments.

An important observation made in this chapter was that the number of AM in WT mice increased 2-fold in the first 2 weeks of *B. pertussis* infection. Conversely, in $Mal^{-/-}$ mice these cells were completely absent by the 4^{th} day of *B. pertussis* infection, and the number of AMs did not subsequently recover, despite recruitment of other inflammatory cells. It is possible AMs are a vital source of the early burst of cytokines in WT mice, and that they are required for clearance of *B. pertussis*, and thereby for host survival.

It was discovered that there were approximately 65% as many AMs in the BAL-F from uninfected $Mal^{-/-}$ mice compared with uninfected WT mice. There are a number of explanations for this finding. Because activation of AMs is thought to lead to a loosening of integrin $\alpha v\beta 6$-mediated binding to the alveolar epithelium,[288] it is possible that in a $Mal^{-/-}$ mouse there are fewer activation signals relative to the WT mice, potentially causing a stronger association of AMs with the alveolar membranes. However, this is an unlikely explanation. Infection should inhibit the membrane interaction such that AMs are recoverable over the course of *B. pertussis* infection. In fact, fewer AMs were recovered from infected $Mal^{-/-}$ mice. An alternative

and more plausible explanation is that there are constitutively fewer AMs in $Mal^{-/-}$ mice due to homeostatic differences that relate to the proliferation of AM progenitors or general viability.

If the deficiency in absolute numbers of AMs in the lungs of $Mal^{-/-}$ mice is real, and not related to BAL-F recovery, it might be partly responsible for the lack of burst cytokines observed in *B. pertussis*-infected $Mal^{-/-}$ lungs. However, there were sufficient AMs recovered from the BAL-F of $Mal^{-/-}$ mice 2 hours after infection that, if Mal does not also play a role in activation of those cells, some burst would be expected.

The most obvious explanations for the diverging pattern of AM numbers in WT and $Mal^{-/-}$ mice are *B. pertussis*-induced death of AMs in $Mal^{-/-}$ mice, or a decrease in the rate of replenishment. Alternatively there might be an increase in the rate of emigration of AMs out of the lungs of $Mal^{-/-}$ mice, possibly as a result of the increased inflammatory profile in the lungs of those mice. Although the dogma suggests that AMs are not efficient APCs, and that pulmonary DC fulfil that role, evidence indicates that AMs can migrate to the mediastinal lymph nodes to present bacterial antigens, and that they might be the first cells from the lungs to do so.[144]

Because *B. pertussis* is known to modulate programmed cell death mechanisms of AMs and other cells via ACT and other virulence factor functions,[33,34,35,285] it is of importance to determine if the 'disappearing AMs' in *B. pertussis* infected $Mal^{-/-}$ mice are emigrating, if they are being killed by the bacteria, or if they are activating host-protective programmed cell death to prevent bacterial spread. A better understanding of AM population maintenance in response to *B. pertussis* infection might explain phenomena such as the lack of burst cytokines, the uncontrolled growth of *B. pertussis*, and the incidence of fatality, all observed in $Mal^{-/-}$ mice and not in WT mice. AMs, which constitute 95% of all alveolar resident cells, could be a vital cell type for determining susceptibility to *B. pertussis* infection. More importantly, the observed loss of AMs in *B. pertussis*-

infected $Mal^{-/-}$ mice might also occur as a consequence of other bacterial infections. Further investigations could reveal novel mechanisms of general bacterial pulmonary pathogenesis.

In summary, this chapter has shown that Mal is required **1)** to limit *B. pertussis* growth in the lungs, **2)** to prevent *B. pertussis*-mediated organ pathology and fatality, **3)** to prevent *B. pertussis*-related AM depletion, and **4)**, for an important innate burst of *B. pertussis*-induced cytokines. These findings have created further questions, such as, what is the cellular source of the innate cytokine burst? What is the role of Mal in the innate cytokine burst? What role does Mal play in AM homeostasis and population maintenance? What Mal-dependent cellular mechanisms prevent *B. pertussis* growth in the lungs?

Chapter Four

Mal is required for anti-*B. pertussis* alveolar macrophage effector functions

4.1 INTRODUCTION

Macrophages are morphologically heterogeneous and can be found in almost every part of the body. Highly differentiated and specialised tissue-resident macrophages, such as resident AMs, are distinct from exudate macrophages, which are infiltrating monocyte-derived cells that migrate to sites of infection or damage.

Macrophages are one of the most important PRR-expressing cells. They commonly express TLR2 and TLR4, the TLRs upstream of the signalling adaptor Mal. Much of our knowledge of TLR and Mal function has been determined by *in vitro* experimentation with macrophages;[360] however, little is known of macrophage function *in vivo* because tissue macrophages are difficult to isolate in sufficient numbers and purity without adversely affecting their viability or function. Furthermore, macrophage populations are often heterogeneous *in vivo,* making determination of function difficult. Consequently, most studies of macrophage function use cell lines or *in vitro* differentiated cells, such as BMDMs.

4.1.1 Macrophage classification

Functional characterisation of macrophages was first based on phagocytic and antigen-presenting capability, as well as inflammatory cytokine production.[289] Classification of the different functional macrophage types was linked to the mechanism of their activation. Classically-activated macrophages were the first type of macrophage to be described and were defined as those macrophages that are primed by IFN-γ,[289] and subsequently activated by TNF or a TNF-inducer, such as a TLR agonist. It is now understood that this kind of activation, which is dependent on STAT1,[290] supports T_H1 immune responses via IL-12 production. These cells are often referred to as classically activated M1-type macrophages, or simply M1 macrophages, in reference to the T cell terminology.

In 1992 Gordon *et al.* showed that macrophages could be activated by an 'alternative' mechanism, by adding IL-4 to cultures *in vitro*.[291] IL-13 can similarly affect macrophage activation. Such alternative activation pathways are known to be dependent on STAT6.[290] Consistent with the T-cell nomenclature, alternatively-activated macrophages are often referred to as M2 macrophages. It should be noted that tissue macrophages *in vivo* do not fit into this neat classification system.

4.1.2 Macrophage function

Although identification of M1 and M2 macrophages can be made based on expression of typical alternatively activated M2 genes, such as *Fizz1* and *Ym1*,[292] macrophages are usually identified based on their effector function and cytokine secretion profiles.

M1 macrophages are typically involved in the production of the proinflammatory cytokines IL-1β, TNF, IL-6, IL-12 and IL-23, reactive oxygen species (ROS), and the induction of antibacterial T_H1 responses. M2 macrophages are known to express the scavenger receptors and mannose receptors, and produce anti-inflammatory cytokines such as IL-10 and the IL-1 receptor antagonist (IL-1Ra). M2 cells are known to be important for tissue remodelling and repair mechanisms, such as VEGF-mediated angiogenesis, as well as the characteristic induction of T_H2 responses.

M1 and M2 macrophages also have distinct metabolic activity. There are differences in glucose metabolism,[293] and the use and storage of iron intermediates.[294] The best understood metabolic distinction between M1 and M2 cells relates to their usage of arginine. Unlike M1 macrophages, M2 macrophages are unable to produce nitric oxide (NO), because arginase prevents the inducible nitric oxide synthase 2 (iNOS2)-mediated

conversion of arginine to NO,[295] preventing efficient NO-dependent intracellular killing of bacteria. Arginase-activity in M2 macrophages contributes to the tissue and cell repair mechanisms that define the cell type via the T_H2 cytokine-induced synthesis of ornithine and polyamines.

Although still an area of contention, Alberto Mantovani championed the idea that M1 and M2 polarisation is highly plastic and reversible.[296,297] His theory is based on an understanding that macrophage phenotype is heavily influenced by the immediate tissue micro-environment. Tissue macrophages, such as AMs, can be anti-inflammatory tissue-remodelling cells that rapidly become proinflammatory, or even tissue-damaging cells, if activated by pathogens.

4.1.3 Macrophage killing function

Elie Metchnikoff is credited with having discovered the function of phagocytosis in 1882. The simple observation of cells 'eating' things is now understood as PRR-based recognition of pathogens by macrophages and other phagocytes, engulfment, and degradation within phagolysosomes containing antimicrobial peptides and various degradative enzymes. Proteasomal analysis reveals the depth of phagosomal complexity; one study identified more than 140 phagosomal proteins, most with unknown function.[298]

One mechanism of antibacterial activity is the production of NO and peroxynitrite, toxic free radicals that interfere with bacterial iron metabolism,[299] and induces damage to bacterial DNA.[300] However, the well-documented classical activation of macrophages by TNF and IFN-γ is fundamentally based on activation of the 'oxidative burst'.[301,302,303]

Coincident with phagocytosis, the oxidative burst is characterised by an increase in oxygen consumption and the NADPH-dependent formation of

reactive oxygen species (ROS). NADPH converts O_2 to the unstable superoxide anion (O_2^-),[304] which in turn leads to production of other ROS, including hypochlorous acid, hydrogen peroxide, hydroxyl radicals and peroxynitrite.[305]

The oxidative burst and phagolysosomal activation have been shown to be activated by a number of pathways downstream of TLRs, such as the MAPK pathway[306] and p38-dependent[307] phagosomal maturation of macrophages.[308] Mal-dependent TLRs play a role in intracellular bacterial killing. TLR4 is involved in anti-*B. pertussis* ROS production because intranasal administration of LPS can protect mice from concurrent exposure to *B. pertussis* by boosting H_2O_2 production and activating 'stimulated innate resistance',[287] and TLR2 is also known to be recruited to the phagosome of macrophages to enhance pathogen detection and drive bacteriolysis.[309]

Pathogens have evolved various molecular strategies to avoid sterilising phagocytosis. *Salmonella* is known to survive inside phagosomes,[310] by preventing the transport of NADPH oxidase;[311] *E. coli* can inhibit PI3K signalling;[312] *Yersinia pestis* inhibits actin rearrangements required for maturation of the phagosome;[313] and *M. tuberculosis* can inhibit phagolysosomal acidification by depleting the membrane-bound proton ATPase.[314,315] In response to these persistent intracellular bacterial infections, macrophages have evolved the ability to activate autophagosomal pathways. Autophagy plays a role in recycling cellular organelles and cytoplasmic components that are too large to be degraded by the proteasome. Part of this cleaning process includes the degradation and removal of intracellular pathogens, which could include *B. pertussis*. However, it should be noted that while autophagy by AMs has been shown to protect against *P. aeruginosa* in the lungs,[316,317] in a separate study Mal was found to be redundant for protection,[258] perhaps indicating that Mal is not critical for activation of autophagy.

4.1.4 Alveolar macrophages

The lungs are the most directly exposed mucosal site, with an estimated 10–60 litres of air inhaled every minute, depending on activity.[318] Since the resident lung immune cells are constantly exposed to environmental non-pathogenic micro-organisms, particulates, antigens and allergens, inflammatory mechanisms of activation must be tightly controlled. One of the most important cell types for prevention of lung inflammation in response to inhalation of innocuous material are the resident AMs.

AMs constitute approximately 95% of cells found in healthy BAL-F.[319] These frontline defenders reside in the alveolar spaces where they phagocytose particulates including bacteria, and initiate antimicrobial and inflammatory pathways to prevent serious infection, without causing immune-mediated damage to the delicate function of alveolar gas-exchange.

Early studies with rat AMs, pioneered by P. G. Holt and colleagues, identified that in a basal state AMs are a key anti-inflammatory cell. As early as 1978 Holt discovered that AMs actively suppress polyclonal T cell activation, even with as few as 1 AM per 1,000 T cells, although more significantly with ratios of less than 1:100.[320,321] Holt and Batty then demonstrated that AMs are deficient in their ability to present antigen to T cells,[322] and thereby deficient in inducing T cell proliferation.[323]

4.1.5 Alveolar macrophage killing function

Although AMs are often described as anti-inflammatory tissue remodelling, similar to M2 macrophages,[365] they are also, owing to their location, likely to be the first dedicated PRR-expressing immune cells to encounter deep bacterial lung infections. AMs are vital for the phagocytosis and clearance

of unwanted material, as well as the activation of localised inflammation in response to severe bacterial infection.

LPS-stimulated human and murine AMs produce inflammatory intermediates that are inhibited by TGF-β, including MIP-2α, IL-1β, IL-6 and NO.[324] Addition of hydrogen peroxide induces p38 MAPK activation, and prevention of TGF-β-mediated suppressive ERK and MAPK phosphatase-1 up-regulation, indicating that the oxidative burst mechanism of bacterial killing is a function of resident AMs.[324]

The bacteria that are known to be killed by AMs include *K. pneumoniae,*[325] *L. monocytogenes,*[326] and *M. tuberculosis.*[327] Although *B. pertussis* is found inside AMs *in vivo*, and clodronate depletion of AMs leads to more severe *B. pertussis* infection, *B. pertussis* has not yet been shown to be killed by AMs.

4.1.6 Aims of chapter 4

The aims of this chapter were to **1)** determine the effect of *B. pertussis* exposure on the activation of macrophages by assessing their killing potential and cytokine production, **2)** determine the role Mal plays in macrophage activation, and **3)** determine if AMs are the source of the Mal-dependent 'burst' of innate cytokines detected *in vivo* two hours after *B. pertussis* infection of WT mice.

4.2 RESULTS

AMs are a likely source of an early 'burst' of innate cytokines detected 2 hours after *B. pertussis*-infection of WT mice. Because this cytokine burst was defective in infected $Mal^{-/-}$ mice, it was decided to examine the effect of Mal on macrophage killing and cytokine production.

4.2.1 *B. pertussis*-induced cytokine production is defective in Mal$^{-/-}$ BMDMs

Viable AMs are difficult to isolate in sufficient numbers to conduct extensive dose-response experiments, therefore, BMDMs were first used to test the effect of *B. pertussis,* across a range of relative multiplicity of infection (MOI), on the production of cytokines. The cytokines tested included those that had previously been identified from the *in vivo* 'burst' in WT mice, or were deemed to be important macrophage-derived immunomodulating cytokines. The peak of cytokine production 2 hours after infection was found to be generated with an MOI of 2 or 20 **(Figure 4.1)**.

2 hours after *B. pertussis* infection BMDMs from WT mice produced significantly more MIP-1α, MIP-2α, TNF, IL-10 and GM-CSF compared with BMDMs from Mal$^{-/-}$ mice. At this early stage of infection there was no IL-1β, IL-6 or IL-23 detected in any of the culture supernatants from WT or Mal$^{-/-}$ BMDMs. At the optimal relative MOI for cytokine production, BMDMs from WT mice produced 3-fold more TNF, 2-fold more GM-CSF, 2.5-fold more IL-10, and 2.5-fold more MIP-2α than BMDMs from Mal$^{-/-}$ mice. BMDMs from WT mice produced up to 160pg/ml MIP-1α, whereas BMDMs from Mal$^{-/-}$ mice were completely deficient in MIP-1α production. By 24 hours post-infection, the peak of production for all cytokines tested was detected at relative MOIs of 20–2,000 **(Figure 4.2)**. The most significant defect in cytokine production by BMDMs from Mal$^{-/-}$ mice compared with WT was the production of IL-1β, IL-10 and TNF. MIP-1α production was variable at different MOIs, and there was significantly more

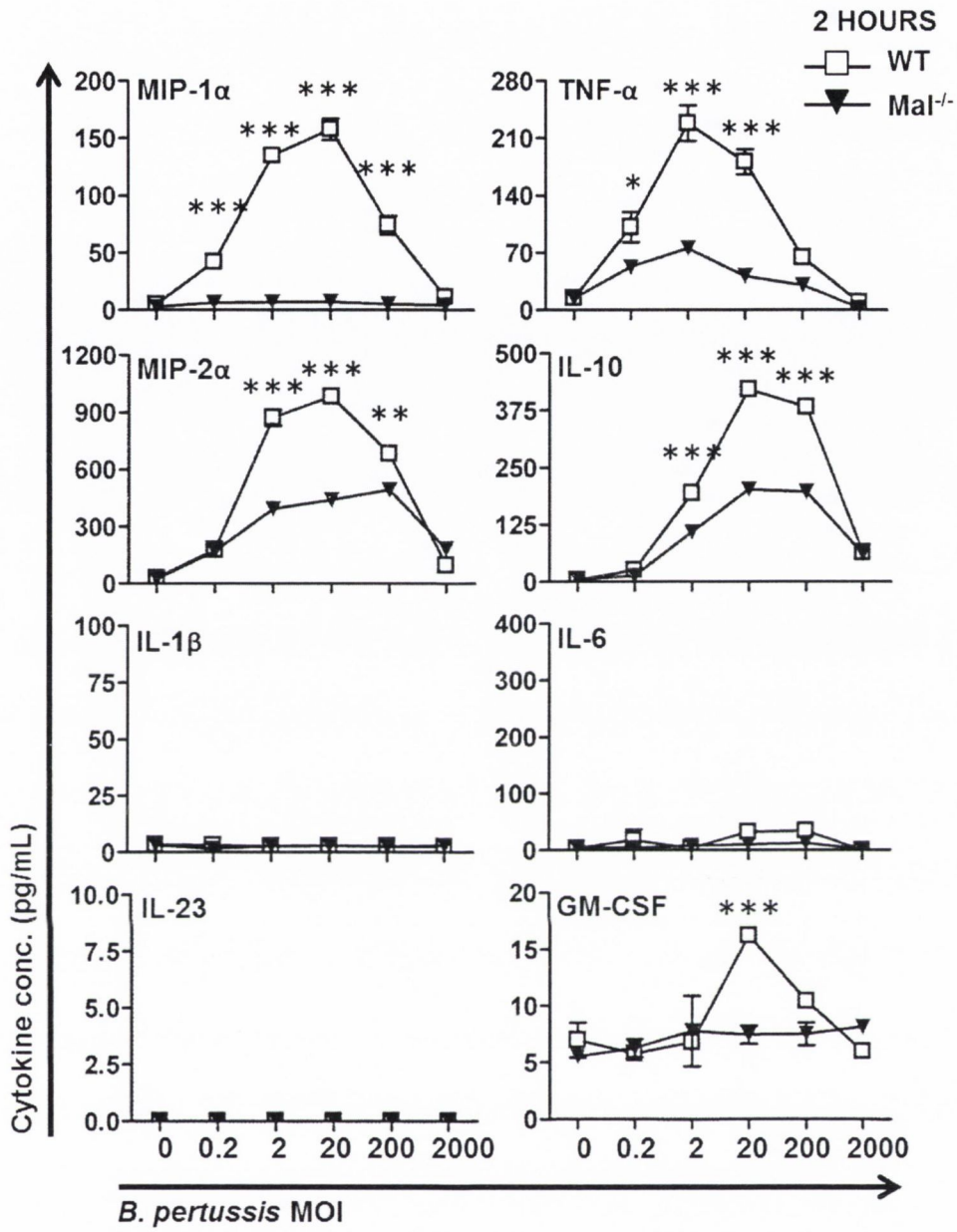


Figure 4.1 Mal is required for optimal early macrophage cytokine production

WT and $Mal^{-/-}$ BMDM were co-cultured with a range of relative MOI of live *B. pertussis* for 2 hours. Supernatants were removed for quantification of cytokines by ELISA. Data are the mean ± SEM from n = 3 mice, from one experiment that is representative of three separate experiments. *P<0.05, **P<0.01, ***P<0.001 statistical analysis by 2-way ANOVA with Bonferroni test.

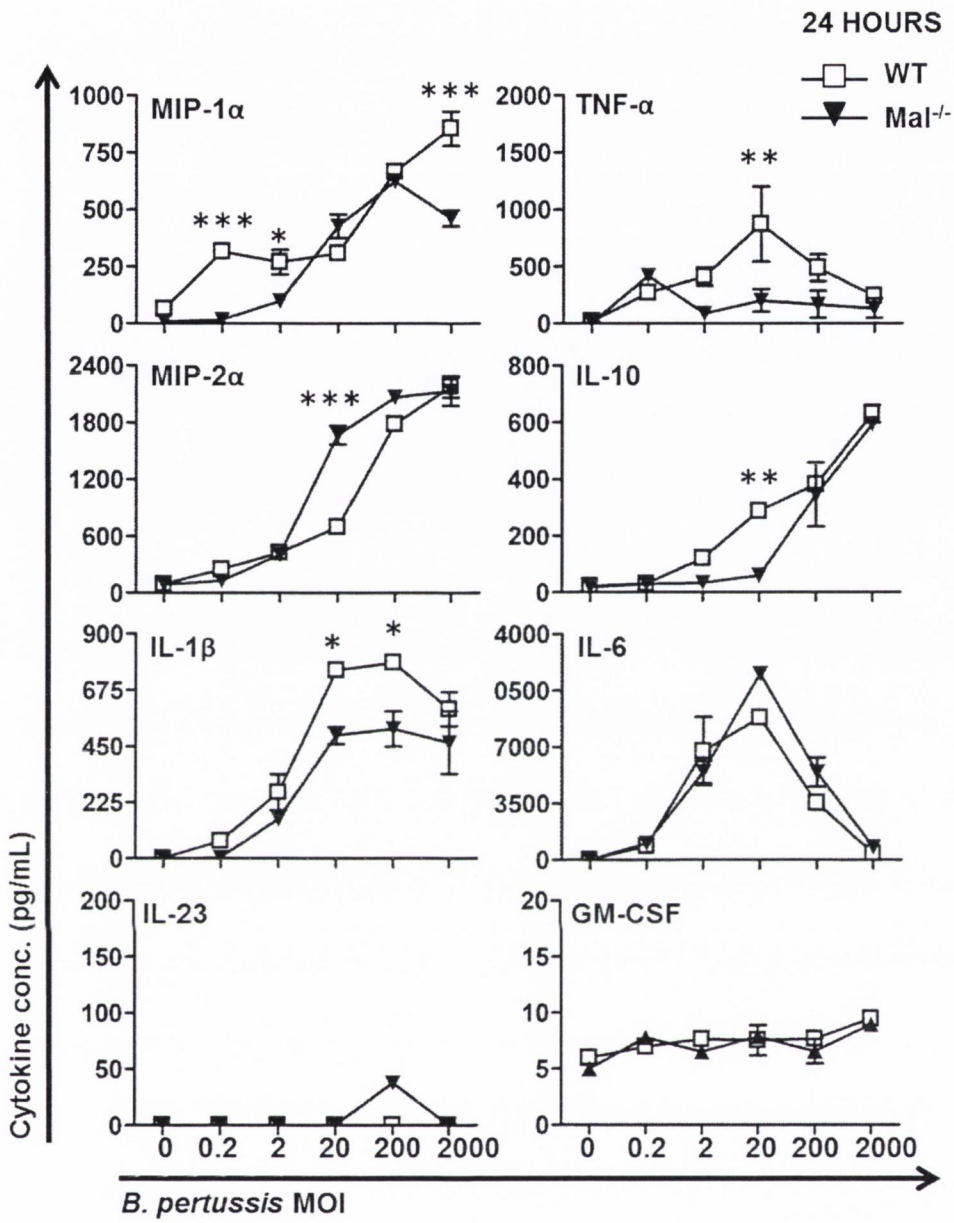


Figure 4.2 Mal is redundant for later macrophage cytokine production in response to *B. pertussis*

WT and $Mal^{-/-}$ BMDM were co-cultured with a range of relative MOI of live *B. pertussis* for 2 hours. Supernatants were removed for quantification of cytokines by ELISA. Data are the mean ± SEM from n = 3 mice, from one experiment that is representative of three separate experiments. *P<0.05, **P<0.01, ***P<0.001 statistical analysis by 2-way ANOVA with Bonferroni test.

MIP-2α and IL-6 produced by $Mal^{-/-}$ BMDMs compared with WT BMDMs.

These data were mostly consistent with the *in vivo* data, and indicated that a very early Mal-dependent burst of MIP-1α, MIP-2α, IL-10 and TNF occurs in response to *B. pertussis*, and that this is closely followed by Mal-dependent IL-1β production.

4.2.2 *B. pertussis*-induced cytokine production is defective in AMs from $Mal^{-/-}$ mice

Having determined the cytokines that are differentially regulated by Mal, and the optimal relative MOI for cytokine production by macrophages, AMs were purified from BAL-F from uninfected WT and $Mal^{-/-}$ mice for *in vitro* infection. The BAL-F from ten mice was pooled and incubated on plastic for two hours to allow AMs to adhere. Non-adherent cells were washed away and purity was determined by FACS analysis of scraped cells. Consistently, more than 95% of viable WT and $Mal^{-/-}$ cells isolated in this manner were large $CD11c^{+}CD11b^{-}Ly6G^{-}F4/80^{+}Siglec\text{-}F^{+}MHC\text{-}II^{int}$ autofluorescent cells, indicating that they were typical resident AMs **(Figure 4.3)**. An insignificant number of contaminants were either pulmonary DC or unidentified macrophage populations.

2 hours and 24 hours after *in vitro B. pertussis* infection of AMs the culture supernatants were taken to quantify cytokine production by ELISAs. After 2 hours with an optimal MOI of 10, AMs from WT mice produced large amounts of MIP-2α (2,000 pg/ml) and TNF (1,300 pg/ml) and a small amount of IL-6 (24 pg/ml) **(Figure 4.4)**. Consistent with the BMDMs, there was no early IL-1β production. AMs from $Mal^{-/-}$ mice produced only 100 pg/ml of MIP-2α and 50 pg/ml of TNF. IL-1β, IL-6 or IL-10 production was not detected.

By 24 hours, also with an optimal *B. pertussis* relative MOI of 10, there was detectable IL-1β in the AM supernatants **(Figure 4.5)**. AMs from WT

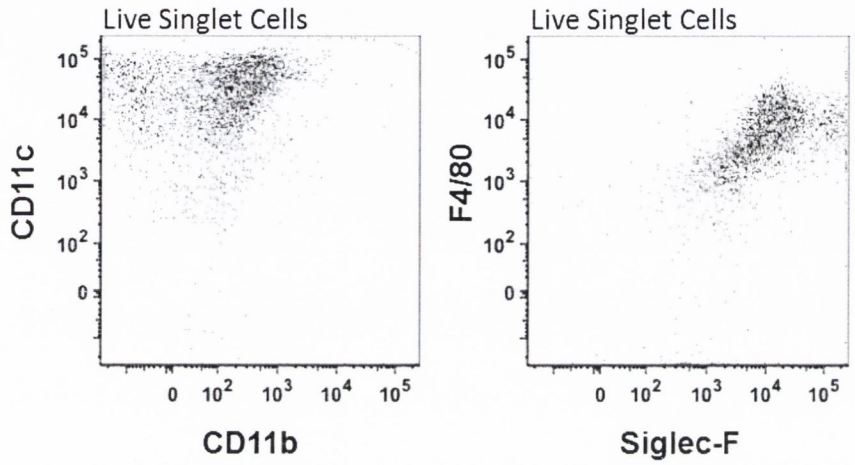


Figure 4.3 Plastic adherent BAL-F cells from uninfected mice are more than 95% alveolar macrophages

Alveolar macrophages were enriched from BAL-F by plating onto plastic for two hours at 37°C. Non-adherent cells were washed away and remaining cells were scraped from the plastic, then FACS analysis was performed. Cells were consistently $CD11c^{+}CD11b^{+}F4/80^{+}Siglec\text{-}F^{+}$, indicating >95% purity of alveolar macrophages, with minor contamination by pulmonary

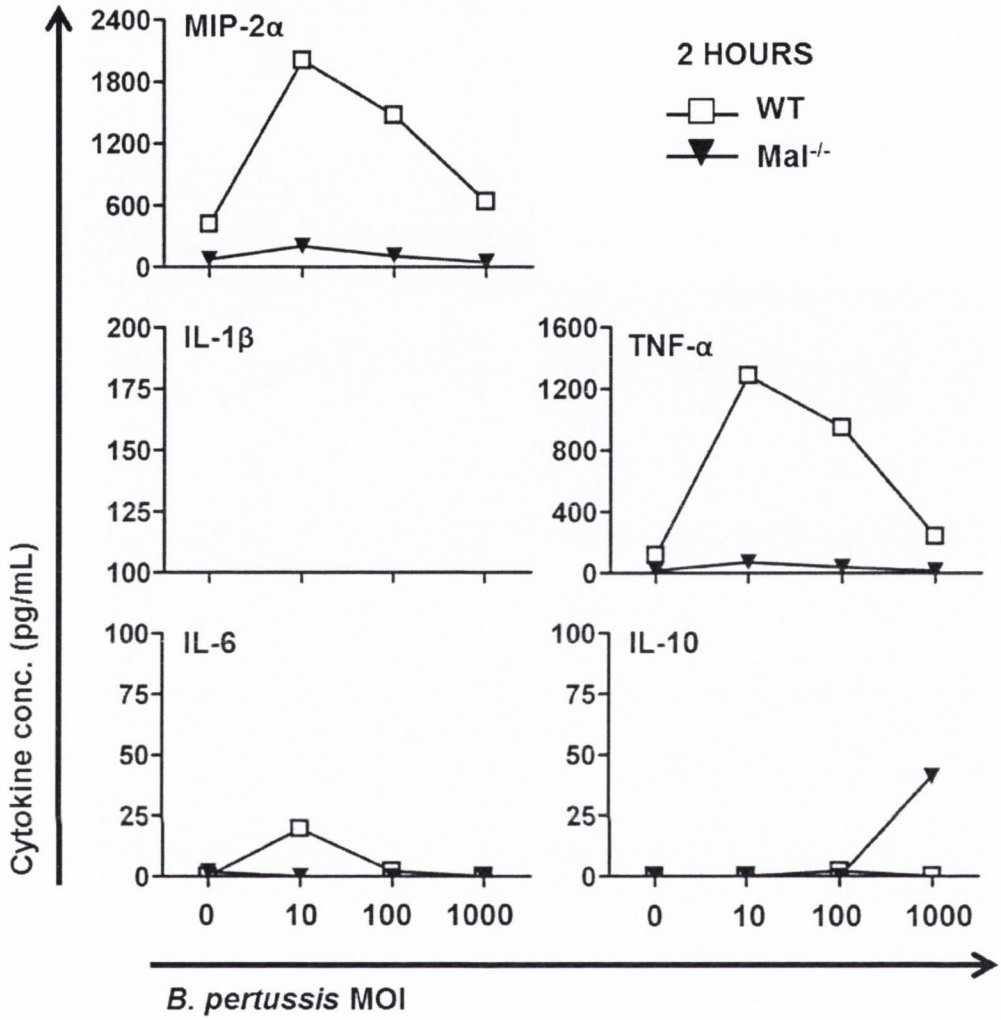


Figure 4.4 Defective early MIP-2α and TNF-α production by *B. pertussis*-infected alveolar macrophages from Mal$^{-/-}$ mice

AMs from ten WT or ten Mal$^{-/-}$ mice were pooled and co-cultured with live *B. pertussis* for 2 hours. Supernatants were removed for quantification of cytokines by ELISA. Data points are individual due to the difficulty of isolating sufficient AMs, and are representative of two separate experiments.

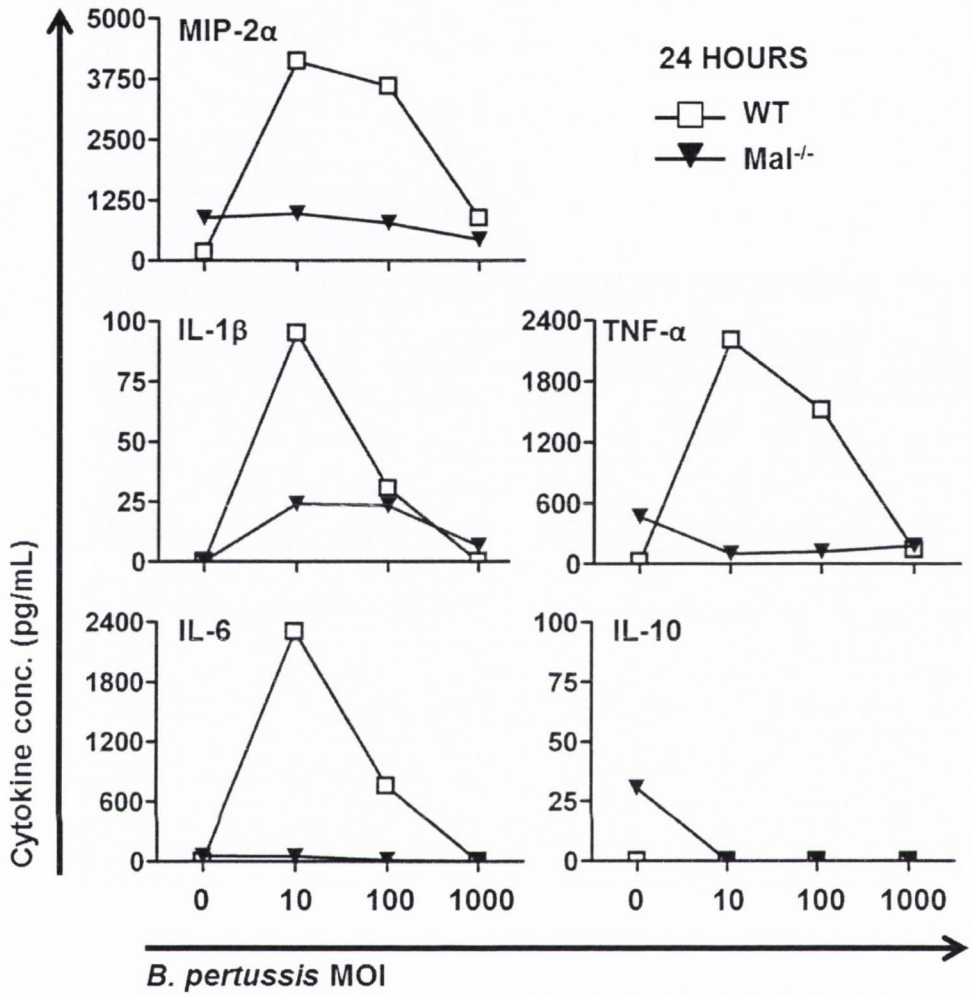


Figure 4.5 Defective cytokine production by *B. pertussis*-infected alveolar macrophages from Mal$^{-/-}$ mice

AMs from ten WT or ten Mal$^{-/-}$ mice were pooled and co-cultured with live *B. pertussis* for 24 hours before supernatants were removed for cytokine ELISA. Data are individual points due to the difficulty of isolating sufficient AMs, and are representative of two separate experiments.

mice produced 4-fold more IL-1β compared with AMs from $Mal^{-/-}$ mice. AMs from WT mice also produced large amounts of MIP-2α, TNF and IL-6, none of which were induced by *B. pertussis* infection of AMs from $Mal^{-/-}$ mice. Unlike BMDMs, there was no IL-10 production by AMs from WT or $Mal^{-/-}$ mice.

4.2.3 Mal is required to prevent intracellular proliferation of *B. pertussis* in macrophages

Although BMDMs and AMs have slightly different cytokine production profiles in response to *B. pertussis* infection, there were enough similarities, and the same cytokine production defects in cells from $Mal^{-/-}$ mice, that BMDMs were deemed a suitable model for analysis of antibacterial function.

We had hypothesised that a key anti-*B. pertussis* function of AMs was Mal-dependent phagocytic killing. Because *B. pertussis* is known to evade extracellular killing by residing in the intracellular compartments of AMs, intracellular killing ability was assessed *in vitro*.

To determine if Mal is involved in *B. pertussis* invasion of macrophages, extracellular bacteria were washed off cultures two hours after infection. Cells were lysed in water and plated on BG agar plates for enumeration of *B. pertussis* **(Figure 4.6)**. An MOI-dependent increase in the number of bacteria indicated that there was invasion of the macrophages. However, there was no significant numerical difference between the viable *B. pertussis* isolated from the intracellular compartments of BMDMs from WT compared with $Mal^{-/-}$ mice. After 24 hours there were more than 3-fold more bacteria inside BMDMs from $Mal^{-/-}$ mice compared with the BMDMs from WT mice **(Figure 4.6)**, indicating that while Mal is not involved in invasion, Mal does play a vital role in prevention of intracellular

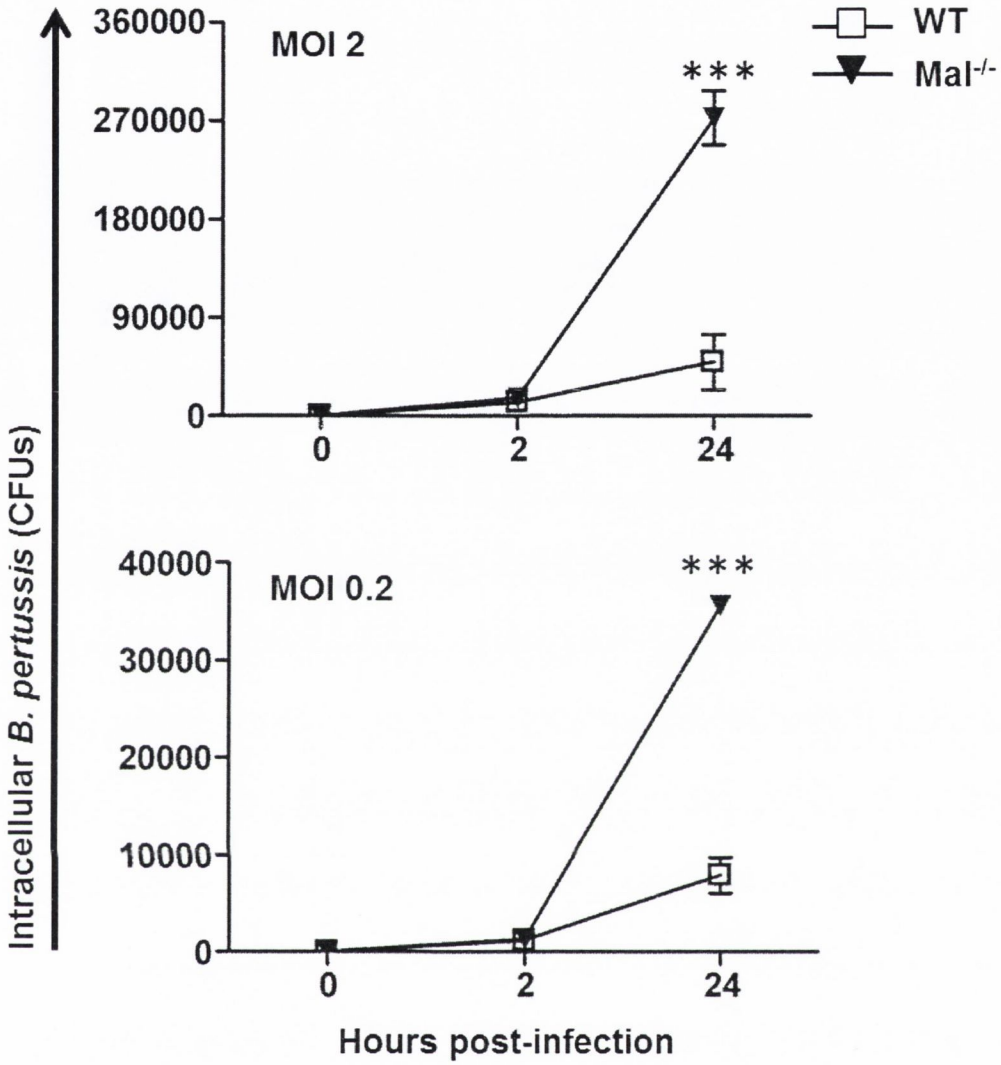


Figure 4.6 Mal prevents intracellular growth of *B. pertussis*

WT and $Mal^{-/-}$ BMDM were co-cultured with different *B. pertussis* MOI. At 2 hours post-infection supernatant was removed and cell monolayers were washed three times then lysed and plated onto BG blood agar for CFU enumeration. Data are the mean ± SD from at least three biological replicates from one experiment that is representative of 3 separate experiments. **P*<0.05, ***P*<0.01, ****P*<0.001 statistical analysis by 2-way ANOVA with Bonferroni test.

B. pertussis proliferation inside macrophages. A comparison of the bacterial CFUs inside the BMDMs two hours and 24 hours after infection revealed 10-fold proliferation in cells from $Mal^{-/-}$ mice, compared with only 4-fold proliferation inside BMDMs from WT mice.

4.2.4 The oxidative burst is defective in *B. pertussis*-infected macrophages from $Mal^{-/-}$ mice

Mal seemed to play a vital role in the intracellular suppression of *B. pertussis* growth in macrophages; therefore, it was decided to determine if the mechanism was based on inhibition of bacterial proliferation, or on direct killing of *B. pertussis*. To determine if Mal was involved in the activation of the oxidative burst, *B. pertussis*-induced ROS production by BMDMs was measured. BMDMs were infected with a relative MOI of 10. 3 hours and 6 hours after infection cells were stained with CellRox, a fluorescent dye that detects ROS in whole cells. Fluorescence intensity was measured by FACS analysis.

3 hours after *B. pertussis* infection there was a significant shift in the mean fluorescence intensity (MFI) of the CellRox staining of infected BMDMs ($10^{2.35}$) compared with uninfected BMDMs ($10^{2.1}$) from WT mice **(Figure 4.7)**. This shift indicated possible activation of the oxidative burst. There was no equivalent shift in the CellRox MFI ($10^{2.1}$) of infected BMDMs from $Mal^{-/-}$ mice, indicating that the oxidative burst response to *B. pertussis* is dependent on Mal.

After 6 hours in culture with *B. pertussis* the general shift in CellROX MFI that was evident at 3 hours was no longer obvious. However, at 6 hours there was a *B. pertussis*-induced Mal-dependent increase of CellRox staining of a small (FCS-A^{lo}) sub-population of BMDMs from WT mice. The shift in MFI of these cells was large and significant, from 10^2 to $10^{3.1}$, and was not detected in infected BMDMs from $Mal^{-/-}$ mice. The small size of

the CellROX^{+} BMDMs could indicate an advanced state of apoptosis. This suggests a correlation between oxidative burst activity and cell death.

Concurrent with the activation of the oxidative burst in BMDMs from WT mice there was significant cell death detected **(Figure 4.8)**. 35–40% of

B. pertussis-infected BMDMs from WT mice, and only 10% of infected BMDMs from Mal$^{-/-}$ mice, were dead after 6 hours of *B. pertussis* infection, suggesting that Mal signalling is involved in *B. pertussis*-induced cell death by an unknown mechanism. This supported a further correlation between cell death, activation of the oxidative burst, and intracellular killing of *B. pertussis*.

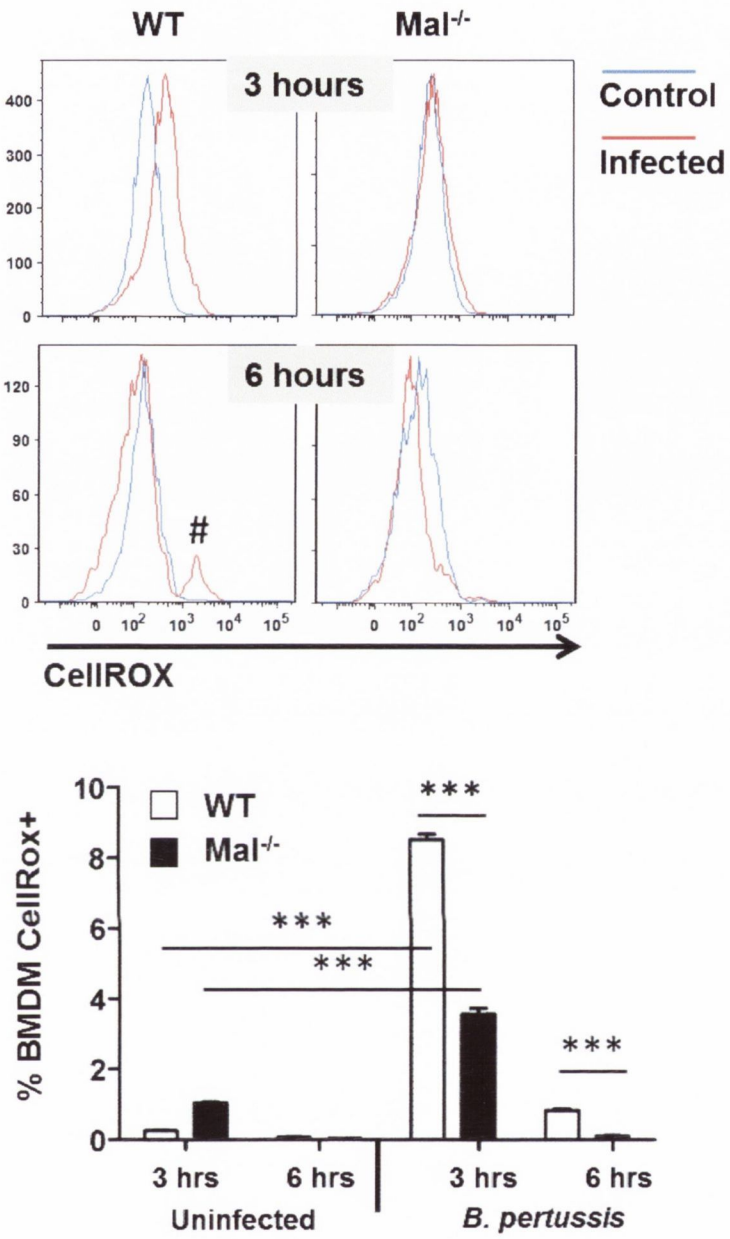


Figure 4.7 *B. pertussis*-induced ROS is dependent on Mal

WT and Mal$^{-/-}$ BMDM were co-cultured with *B. pertussis* MOI of 10 for 3 hours and 6 hours. BMDM were stained with Live/Dead Aqua and CellROX for FACS determination of viability and oxidative burst activity. Data are the mean ± SD from n = 3 mice, from one experiment. Unstainfed controls not shown. *P<0.05, **P<0.01, ***P<0.001 statistical analysis by 2-tailed unpaired Student's T test. # = FSC-A^{hi} population of CellROX^{+} cells.

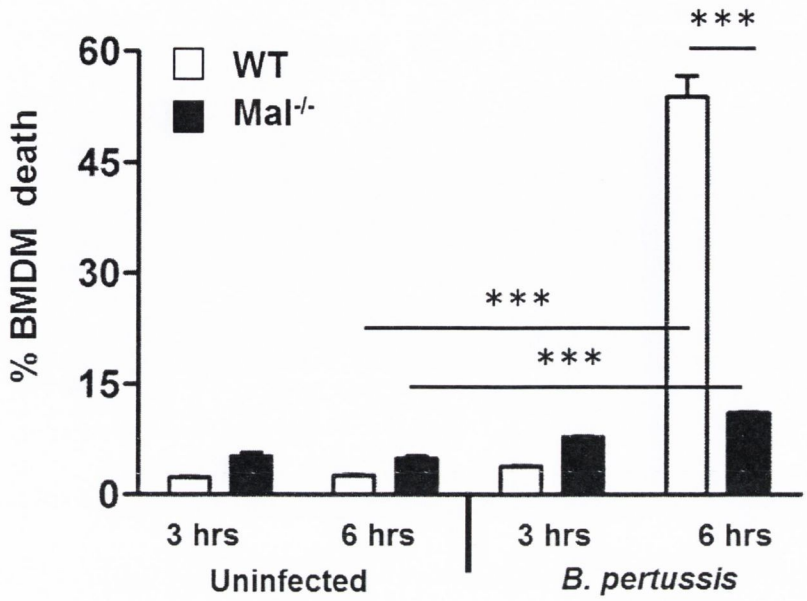


Figure 4.8 *B. pertussis* induces Mal-dependent macrophage death

WT and Mal$^{-/-}$ BMDM were co-cultured with *B. pertussis* MOI of 10 for 3 hours and 6 hours. BMDM were stained with Live/Dead Aqua and CellROX for determination of viability and oxidative burst activity. Data are the mean ± SD from n = 3 mice, from one experiment. *P<0.05, **P<0.01, ***P<0.001 statistical analysis by 2-tailed unpaired Student's T test.

4.3 DISCUSSION

In chapter three the role of Mal in an early burst of cytokine production in the lungs was characterised. After 2 hours *B. pertussis* was shown to induce significant production of IL-1β, TNF, MIP-1α and MIP-2α in the lungs of WT mice, and not in the lungs of $Mal^{-/-}$ mice. These cytokines were no longer detectable 24 hours after infection. The cellular source of the cytokines was unclear, although it was suspected to be innate resident alveolar cells such as AMs. However, only 65% as many AMs were recovered from the BAL-F of uninfected $Mal^{-/-}$ mice compared with uninfected WT mice, possibly indicating that the deficit in cytokine production was not Mal-dependent, but rather cell number-specific. Therefore, in this chapter the cytokine production potential of WT and $Mal^{-/-}$ macrophages was tested *in vitro*.

Consistent with the *in vivo* data, Mal was found to be vital for the initial early production of MIP-2α and TNF by *B. pertussis*-infected AMs *in vitro*. This indicated that the deficit in the innate cytokine burst in $Mal^{-/-}$ mice was an inherent defect in the ability of resident AMs to be activated, and to produce those cytokines in response to *B. pertussis* infection.

As might be expected in a comparison of purified cells relative to heterogeneous organs, there were some differences in the cytokine production profiles of *in vitro* infected AMs relative to the cytokine production in the lungs of infected mice. The most obvious difference was that AMs and BMDMs did not produce IL-1β 2 hours after infection, whereas IL-1β was detected in lung homogenates from *B. pertussis*-infected mice after 2 hours. IL-1β is known to require activation of the inflammasome by a second signal, other than a TLR agonist,[154] possibly indicating that epithelial cell-derived or DC-derived stimuli are required to drive optimal activation of AMs *in vivo*. However, IL-1β was produced *in vitro* 24 hours after infection, but only by AMs from WT mice.

B. pertussis infected AMs from $Mal^{-/-}$ mice were defective at producing MIP-2α and TNF. These two cytokines are both known to have autocrine function and could be responsible for subsequent defects in the immune response to *B. pertussis*. The autocrine function of TNF can prime the oxidative burst of phagocytic bacterial killing. Indeed, the results of the present study demonstrated that BMDMs from WT mice are superior to BMDMs from $Mal^{-/-}$ mice in controlling intracellular growth of *B. pertussis*. Furthermore, it was demonstrated that the potential mechanism for Mal-dependent killing could be activation of the oxidative burst. Infection of BMDMs from WT mice induced oxidative burst within 3 hours, as measured by CellROX staining, whereas invasion of BMDMs from $Mal^{-/-}$ mice with an equivalent number of bacteria did not lead to any alteration of CellROx staining 3 hours or 6 hours after infection.

After *B. pertussis* infection of BMDMs from WT mice there was activation of the oxidative burst, and by 6 hours in culture approximately 55% of the BMDMs were dead. Also at 6 hours, there was a population of $CellROX^{+}$ cells that were small in size, possibly indicating that they were apoptotic. Infected BMDMs from $Mal^{-/-}$ mice did not activate oxidative burst and there was only around 10% cell death. In conjunction with the evidence that Mal is required for BMDMs to kill intracellular *B. pertussis*, these results suggest that Mal is required for a sequence of events: cytokine production → phagocytosis → activation of the oxidative burst → bacterial killing → host cell death. Apoptosis[361] and autophagy[362] are recognised anti-bacterial cell death mechanisms and are known to involve TLR4 signalling. Either of these cell death mechanisms could therefore be dependent on Mal.

The results showed that BMDMs from WT mice are more susceptible to *B. pertussis*-induced cell death compared with $Mal^{-/-}$ BMDMs. These data might appear to conflict with the hypothesis that the disappearance of AMs from $Mal^{-/-}$ mice was a cell death-based phenomenon, however, while

there was no defect in the recruitment of neutrophils or monocytes in infected $Mal^{-/-}$ mice, it is possible that the lack of MIP-2α and GM-CSF, and other chemokines, is partly responsible for the disappearance of AMs from the lungs of *B. pertussis*-infected $Mal^{-/-}$ mice.

MIP-2α is an important chemokine for recruitment of inflammatory cells. Vitally, MIP-2α is also a known mobiliser of stem cells,[328] and stem cells are now thought to contribute to the homeostasis of AM numbers in the lungs.[146,147] Thereby, via homeostatic modulation of emigration or replenishment, the lack of MIP-2α and other cytokines *in vivo* might contribute to the disappearance of AMs from the lungs of *B. pertussis*-infected $Mal^{-/-}$ mice. *In vitro,* the function of MIP-2α and other cytokines would obviously have no such chemotactic effect. Furthermore, as cell death is related to cell activation, the short exposure time could be responsible for the lack of activation of macrophages *in vitro*. *In vivo*, over the course of two or more weeks, there might be Mal-independent mechanisms of *B. pertussis*-induced activation that could lead to cell death. Therefore, separate mechanisms of macrophage cell death might be functional *in vitro* and *in vivo*.

In summary, this chapter has **1)** identified a role for Mal in the production of cytokines and chemokines by AMs in response to *B. pertussis* infection, **2)** discovered that Mal is required for the intracellular inhibition of bacterial proliferation inside macrophages, **3)** discovered that the mechanism of this inhibition involves activation of the oxidative burst, and **4)** found that Mal-deficiency does not enhance *B. pertussis*-induced macrophage death *in vitro*.

As a means of understanding the observed *B. pertussis*-induced disappearance of AMs from the lungs of $Mal^{-/-}$ mice, the next chapter will examine possible Mal-dependent mechanisms for the maintenance of AM homeostasis in *B. pertussis*-infected mice.

Chapter Five

Mal in maintenance of AM homeostasis and prevention of *B. pertussis* dissemination

5.1 INTRODUCTION

Bacterial superinfections that cause pneumonia are common following influenza virus infection, and might be one of the principal causes of influenza-related fatality.[329] A study using a Balb/C mouse model indicates that the mechanism of susceptibility to influenza-related pneumonia involves the depletion of AMs.[330] The present study has demonstrated that AMs play a vital role in defence against bacterial infection of the lungs. Although bacteria can induce apoptosis of AMs, there is no evidence that bacterial infection can cause the complete ablation of AMs, and there is no mechanism to explain AM depletion.

B. pertussis-induced depletion of AMs from the lungs of $Mal^{-/-}$ mice, (Chapter three), is the first description of bacterial induced-depletion of AMs, and is the first study to implicate a particular gene in prevention of AM depletion. In this chapter the role of Mal in the maintenance of AMs in the lungs following *B. pertussis* infection has been examined, focusing on **1)** cell emigration, **2)** cell death, and **3)** homeostatic replenishment.

5.1.1 Emigration of alveolar macrophages

A possible explanation for the sudden disappearance of phagocytic cells from sites of active infection or inflammation is that they emigrate to draining lymph nodes to present antigens. Although AMs, which have intermediate MHC II expression, have been described as poor antigen presenters, they are known to be efficient at phagocytosis.[331] One benefit of killing bacteria post-engulfment, rather than simply destroying them in the extracellular humours with lytic granules, is that antigens can be processed and presented on surface-expressed MHC molecules. Because AMs have been recognised to be expert phagocytic cells, they should also be capable of presenting antigen to T cells.

Pulmonary DC are known to emigrate from the lungs and present antigens in the lung draining lymph nodes.[144] A similar function was described for AMs, which were detected in the mediastinal lymph nodes, even in uninfected mice. Upon lung infection with GFP-tagged *S. pneumoniae,* AMs were observed in increasing numbers in the lymph nodes, and were detected prior to other cell types, including pulmonary DC. Therefore, emigration could contribute to the disappearance of AMs from *B. pertussis*-infected $Mal^{-/-}$ lungs.

5.1.2 Cell death, apoptosis and autophagy

While cell death from necrosis and viral lysis are considered pathological, apoptosis and autophagy are thought to be mechanisms for host protection from severe infection. Such programmed cell death of macrophages can prevent intracellular proliferation of bacteria, and thereby function as a host protective mechanism to prevent the persistence of bacteria in compartments that evade extracellular immune responses.

B. pertussis toxins, including ACT,[33,34,35] are known to have apoptogenic effects on macrophages. Conversely *B. pertussis* LPS has been shown to prevent apoptosis of monocyte-derived DCs.[285] This apparent contrariety could be explained by the complex evolutionary nature of the interaction between host and pathogen. There is still confusion as to whether apoptosis of AMs is a pathogen-induced mechanism of extracellular colonisation, or a host-protective mechanism to prevent intracellular colonisation, or both.

Autophagy is now recognised to play a role in protection from persistent intracellular bacteria that evade oxidative burst and phagolysosomal degradation. Part of that protection, however, is not just anti-bacterial, but

cell death-mediated. Much of the cellular machinery that controls autophagy is shared and integrated with apoptosis pathways, indicating causal links between this otherwise pro-survival process, and cell death. It is now accepted that in certain contexts autophagy is a mechanism of host-protective cell death,[332] principally as a response to stress or nutrient deprivation, but also to persistent intracellular bacterial infections.

As various PRR pathways have been implicated in programmed cell death, apoptosis or autophagy could account for the disappearance of AMs from the lungs of *B. pertussis* infected $Mal^{-/-}$ mice.

5.1.3 Alveolar macrophage ontogeny and homeostasis

Regardless of whether AM numbers are depleted by cell death, apoptosis, autophagy, or by emigration out of the alveolar spaces, the most important determinant of immune cell number in any compartment is the maintenance of homeostasis, i.e., stable regulation of cell numbers. Cell death and emigration are continual processes in healthy individuals, as long as replenishment of those populations account for depletion. Therefore, it is vital to determine if Mal plays a role in regulation of cell death and/or emigration, or if it is more importantly involved as a master regulator of homeostasis. To understand the complex mechanism of resident AM homeostasis, it is necessary to understand lineage, differentiation state, and the source of cellular replenishment, areas that remain controversial due to the inherent complexity of analysing these processes *in vivo.*

Pioneering microscopy work showed that so-called 'alveolar macrophages' appear morphologically heterogeneous, particularly in size and granularity.[363] More recent FACS analysis has confirmed morphological heterogeneity. FACS has also been used to attribute some of that

heterogeneity to differential expression of key surface markers that are indicative of alternative macrophage lineages.

Definitive evidence for a monocyte origin of macrophages was first published in 1939 by Ebert and Florey. But those authors did not suggest that all macrophages are so-derived, rather they specifically focused on monocyte–macrophages found in inflammatory foci. The dogma that blood monocytes are the source of all macrophages, including tissue macrophages, would come 30 years later.[333]

The origin of AMs has been one of the greatest controversies in immuno-ontogeny and has confused our understanding of macrophage function for the past 40 years. Different studies have indicated that AMs are differentiated from blood monocytes,[334,335] that they are self-replicating and maintain their numbers within the lungs,[319,336] or that they are derived directly from bone-marrow stem-cell progenitors.[337]

Some of the most direct evidence against a monocyte origin of tissue macrophages came from studies of phylogeny and ontogeny.[338,339] These studies indicated that macrophages evolved prior to the evolution of monocytes, and during development macrophages derive directly from yolk-sac stem cells, also prior to the development of monocytes.

Further evidence for the ontogeny of AMs came from the study of monocytopenic mice. Mice made to be monocytopenic by exposure to bone marrow-focused irradiation maintained a resident AM population.[340] In that study the persistence of AM numbers was explained by self-replication of the population. In another study, it was demonstrated that *op/op* mice, a model of spontaneous osteoporosis, which are also monocytopenic, also have a sufficient quantity of tissue macrophages, if not quality.[341] This data indicated that under normal non-inflammatory conditions, blood-resident monocytes are not vital for homeostasis of AM numbers, and are not AM progenitors.

Using parabiosis and BrdU-labelling FACS experiments, two separate groups have now shown that AM ontogeny is dependent on GM-CSF and that AMs are almost entirely derived from $Ly6C^{hi}CD11b^{hi}$ foetal monocytes.[146,147] These foetal progenitors traffic to the lungs during early development. Subsequently, and without re-seeding by blood monocytes, AMs self-maintain in the lungs throughout life.

Despite such overwhelming evidence, those involved in the inflammatory stimulation of mice often confuse tissue resident AMs and monocyte-derived exudate macrophages that migrate into sites of inflammation. Most of this confusion stems from earlier work that characterised macrophage parameters, such as increased cytokine production or killing-ability in the context of infection. For example, as early as 1975 'alveolar macrophages' were shown to be activated by *L. monocytogenes.*[342] These cells had enhanced ability to ingest and kill bacteria. But as BAL-F was isolated 10-48 days after aerosol infection of the lungs they would have co-recovered inflammatory monocyte-derived exudate macrophages, primed to be proinflammatory, rather than a pure resident AM population. Therefore, it is necessary to reassess our comprehension of such literature in the context of the latest developments in our understanding of AM ontogeny and homeostasis.

Throughout the present text 'AM' or 'resident AM' refers specifically to autofluorescent, $CD11b^{-}CD11c^{+}Siglec\text{-}F^{+}Ly6G^{-}MHC\text{-}II^{lo–int}$ alveolar resident macrophages. The term 'exudate macrophage' is used specifically to refer to monocyte-derived macrophages, which are recruited to the lungs in response to infection. The term 'pulmonary macrophage' is used to refer to the heterogeneous population of AMs, exudate macrophages and interstitial macrophages.

5.1.4 Aims of chapter five

The aim of the experiments described in this chapter was to determine the role of Mal in macrophage emigration, cell death, autophagy or apoptosis, in response to *B. pertussis* infection.

5.2 RESULTS

Experiments in this chapter were designed to explain the disappearance of AMs from the lungs of *B. pertussis*-infected $Mal^{-/-}$ mice. The effect of Mal signalling on *B. pertussis*-induced apoptosis, cell death, or emigration of AMs was characterised. As presented in **figure 5.1**, AMs were identified by strict FACS gating of autofluorescent $F4/80^{+}CD11c^{+}MHC\text{-}II^{int}$ $CD11b^{lo}Ly6G^{-}Siglec\text{-}F^{+}$ cells.

5.2.1 AM death *in vitro*

As macrophages from $Mal^{-/-}$ mice are defective in their ability to kill intracellular *B. pertussis* (Figure 4.8), the simplest explanation for the disappearance of AMs from the lungs of $Mal^{-/-}$ mice (Figure 3.9.4) is cell death as a result of higher bacterial colonisation. However, in chapter four it was found that when infected with *B. pertussis in vitro,* WT BMDMs were surprisingly more susceptible to cell death than BMDMs from $Mal^{-/-}$ mice (Figure 4.11). To determine if this was a cell-specific phenomenon, AMs were isolated and co-cultured for 24 hours with a *B. pertussis* MOI of 10. Other time points were not examined due to the difficulty of isolating sufficient numbers of AMs. As with BMDMs (Figure. 4.11), *B. pertussis*-infected AMs from WT mice were more susceptible to cell death than infected AMs from $Mal^{-/-}$ mice **(Figure 5.2)**. Unsurprisingly, after 24 hours in culture the viability of uninfected AMs (50%) *in vitro* was low relative to BMDMs (80%). However, these experiments were considered valid as there was no significant difference in viability between uninfected AMs from WT mice and from $Mal^{-/-}$ mice.

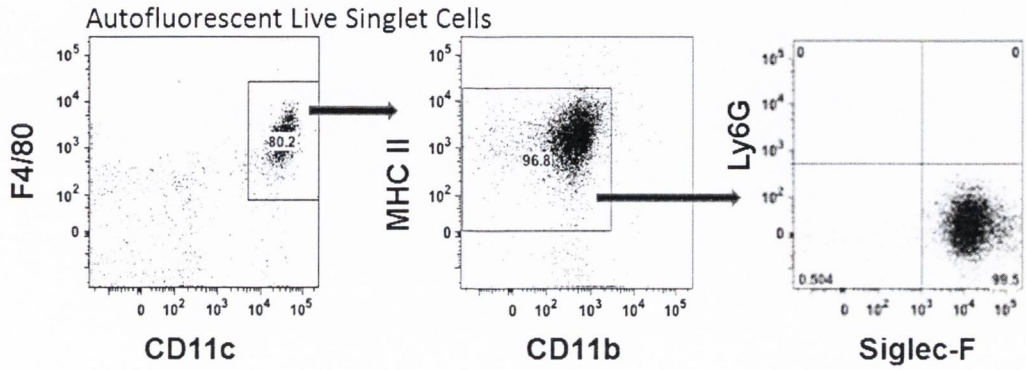


Figure 5.1 FACS gating strategy for AMs

Cells were F4/80^{+}CD11c^{+}MHC-II^{int}CD11b^{lo}Ly6G^{-}SiglecF^{+} autofluorescent cells. High MHC-II MFI is autofluorescence in the FITC channel, and is lower than the MFI of DC.

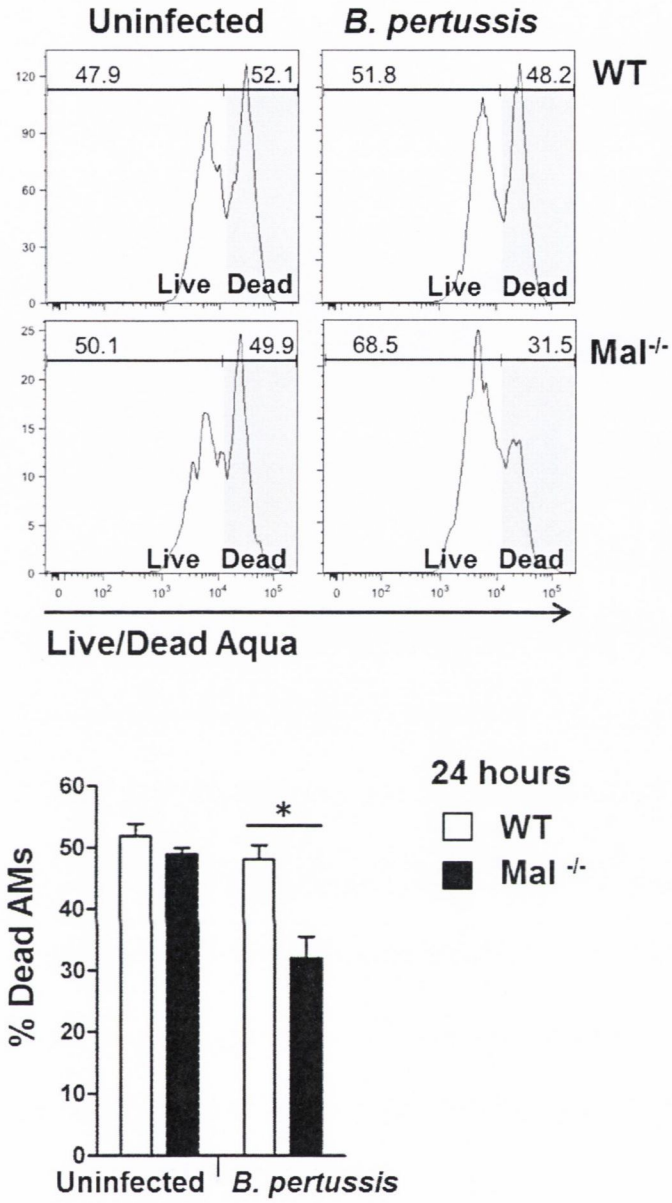


Figure 5.2 A role for Mal in survival of AMs *in vitro*

AMs were isolated by coating BAL-F on plastic for two hours. Non-adherent cells were washed away and adherent cells were infected with *B. pertussis* for 24 hours. Cells were stained with Live/Dead Aqua and analysed by FACS to determine the percentage of dead cells. Data are the mean ± SD from $n = 3$ replicates from one experiment. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ statistical analysis by 2-tailed unpaired Student's T test

5.2.2 AM death *in vivo*

Having determined a role for Mal in promotion of macrophage death in response to *B. pertussis* infection *in vitro*, the resident AM population was re-analysed to account for cell death and apoptosis following *B. pertussis* infection. The aim was to understand the apparent contradiction between the Mal-dependent increased incidence of cell death *in vitro* and the disappearance of AMs from $Mal^{-/-}$ mice *in vivo*.

WT and $Mal^{-/-}$ mice were infected with *B. pertussis*. At various times post-infection BAL-F was analysed by FACS using Annexin V as a measure of apoptosis, and Live/Dead Aqua as a measure of cell death. Using this method AMs *in vivo* could be separated into viable, dead, early apoptotic, or late apoptotic populations.

As had previously been discovered (Fig. 3.9.4), following *B. pertussis* infection there was a rapid decline in the percentage of AMs from the BAL-F of $Mal^{-/-}$ mice **(Figure 5.3 A–B)**. Also consistent with previous data there was no decline in the AM population in infected WT mice. AMs from $Mal^{-/-}$ mice constituted less than 10% of the total cells within the first 4 days of *B. pertussis* infection, whereas BAL-F from WT mice consisted mostly (65%) of AMs even after 7 days *B. pertussis* infection. The reduction in percentage of AMs in the BAL-F from WT mice was temporally consistent with the influx of monocytes and neutrophils between 4–7 days post-infection, indicating that unlike $Mal^{-/-}$ mice this reduction in percentage of AMs was not representative of total cell numbers.

Over the course of *B. pertussis* infection there was an increase in the percentage of apoptotic AMs **(Figure 5.4 A–B)** in WT and $Mal^{-/-}$ mice. However, the percentage increase was significantly greater in the $Mal^{-/-}$ mice compared with the WT mice. This difference was evident at all stages of infection, as early as 24 hours (approximately, Mal: 20%, WT: 6%), but

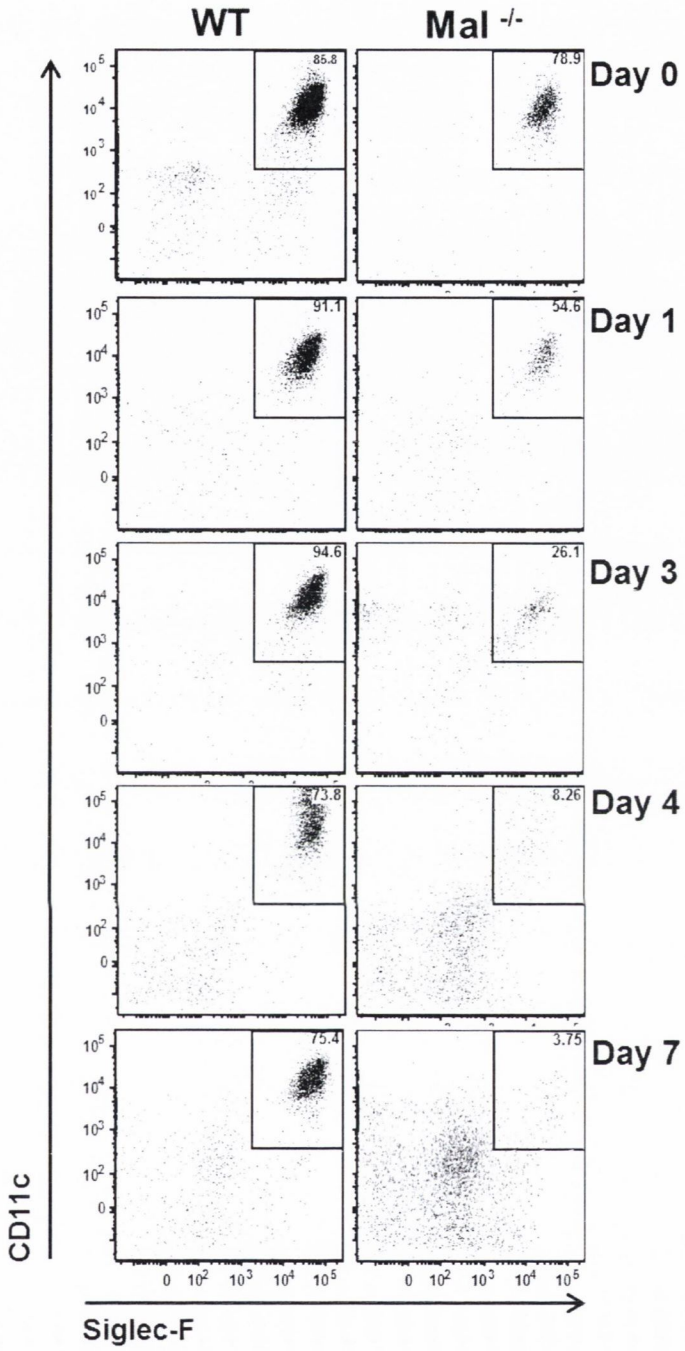


Figure 5.3A Loss of AMs in lungs of *B. pertussis*-infected Mal$^{-/-}$ mice

WT and Mal$^{-/-}$ mice were exposed to a 20 minute aerosol challenge with 1×10^9 *B. pertussis* per ml. At various times post-infection BAL-F was collected and FACS analysed for the frequency of AMs. Data are the mean from n = 3 mice, from one experiment that is representative of two separate experiments.

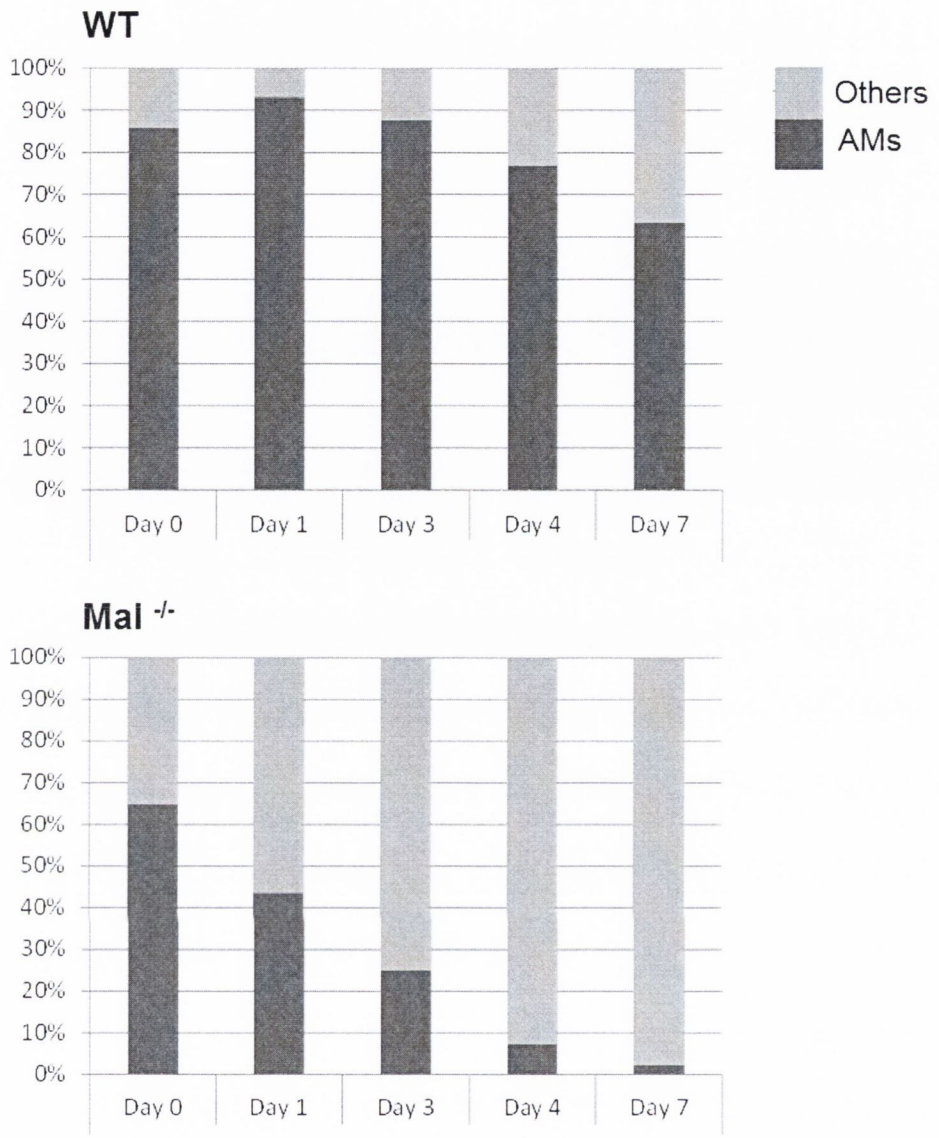


Figure 5.3B Mal prevents *B. pertussis*-induced disappearance of AMs

WT and $Mal^{-/-}$ mice were exposed to a 20 minute aerosol challenge with 1×10^9 *B. pertussis* per ml. At various times post-infection BAL-F was collected and FACS analysed for the frequency of various cell subsets. Data are the mean ± SEM from *n* = 3 mice, from one experiment that is representative of two separate experimen

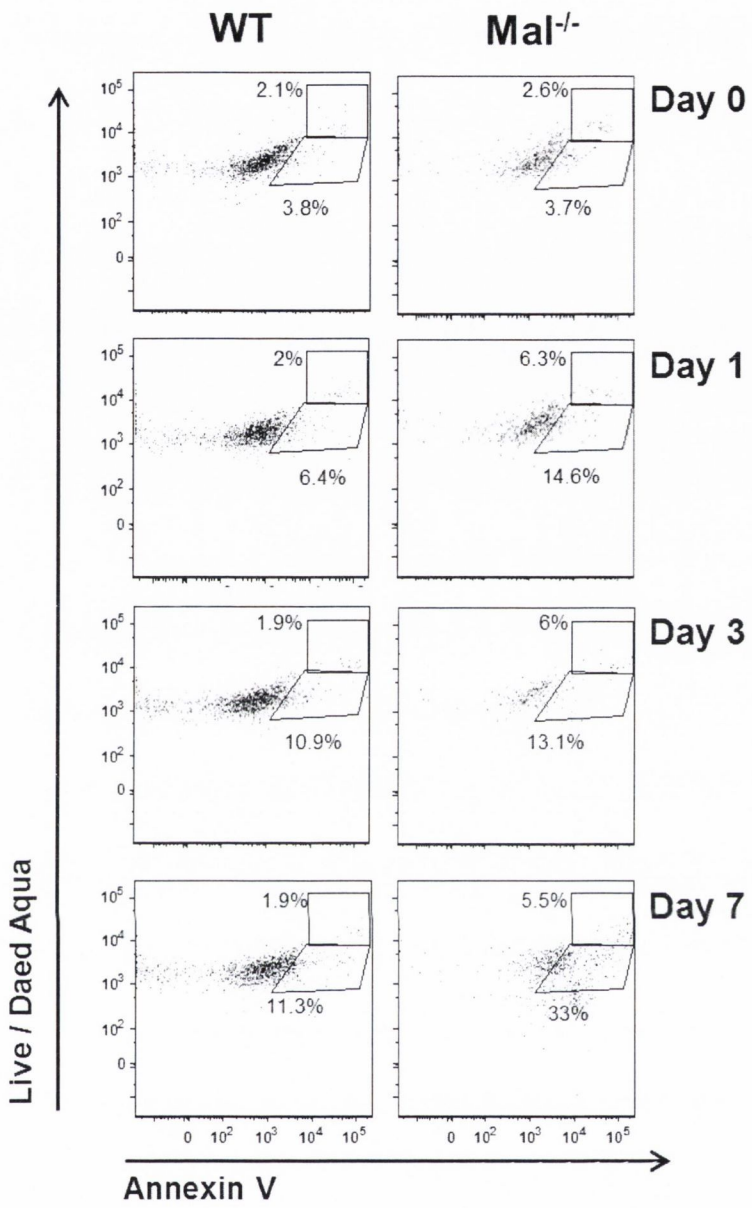


Figure 5.4A AMs in lungs of Mal$^{-/-}$ mice undergo apoptosis following infection with *B. pertussis*

WT and Mal$^{-/-}$ mice were exposed to a 20 minute aerosol challenge with 1×10^9 *B. pertussis* per ml. At various times post-infection BAL-F was collected and FACS analysed for Live/Dead Aqua and Annexin V positive AMs. Data are the mean ± SEM from *n* = 3 mice, from one experiment that is representative of two separate experiments.

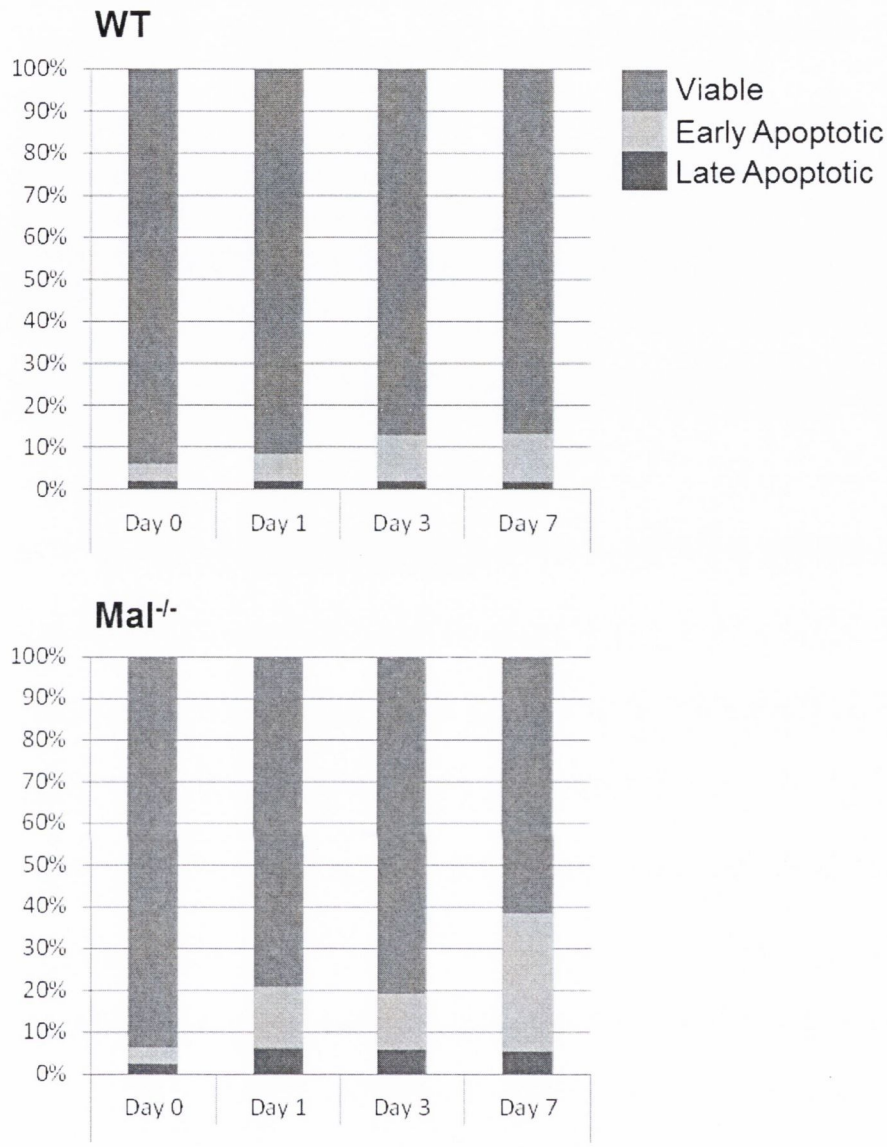


Figure 5.4B *B. pertussis*-induced AM apoptosis and death

WT and Mal$^{-/-}$ mice were exposed to a 20 minute aerosol challenge with 1×10^9 *B. pertussis* per ml. At various times post-infection BAL-F was collected and FACS analysed for Live/Dead Aqua and Annexin V positive AMs. Data are the mean ± SD from *n* = 3 mice, from one experiment that is representative of two separate experiments

was most significant 7 days after infection (approximately, WT: 13%, Mal: 38%). Most apoptotic cells were identified as 'early apoptotic' (Live/Dead$^-$ AnnexinV$^+$). These data supports an apoptotic mechanism of AM disappearance from the lungs of *B. pertussis*-infected Mal$^{-/-}$ mice, but does not exclude other mechanisms.

Consistent with previous experiments, it was also found that AMs comprised a smaller percentage of lung cells from uninfected Mal$^{-/-}$ mice compared with WT mice, possibly indicating a generally lower homeostatic replenishment of their numbers. However, the percentage of viable, early apoptotic and late apoptotic AMs in WT and Mal$^{-/-}$ uninfected mice was equivalent. Approximately 95% of AMs from uninfected WT and Mal$^{-/-}$ mice were viable and not apoptotic.

5.2.3 AM lung emigration

Emigration was the other potential mechanism of AM disappearance from *B. pertussis*-infected Mal$^{-/-}$ mice to be investigated. Because there were more *B. pertussis* CFUs in the lungs of Mal$^{-/-}$ mice compared with WT mice, it is possible that AMs from Mal$^{-/-}$ mice might be more activated, and thereby more likely to emigrate to the draining lymph nodes (LN) to present antigen. To assess this possibility, lung draining LNs and attached lymphatics from *B. pertussis*-infected mice were FACS analysed to identify autofluorescent MHC II^{int}CD11b$^+$F4/80$^+$CD11c$^-$Ly6G$^-$Siglec-F$^+$ cells. Anti-B220 and anti-CD3 antibodies were also included in the FACS gating strategy to ensure that isolated tissue was lymphatic **(Figure 5.6)**.

Consistent with published data,[144] FACS analysis identified a small population of AMs in the LNs of uninfected WT mice, and also Mal$^{-/-}$ mice **(Figure 5.7)**. 3 days after *B. pertussis* infection there was an increase in the frequency of AMs, and a further increase by 7 days in both genotypes.

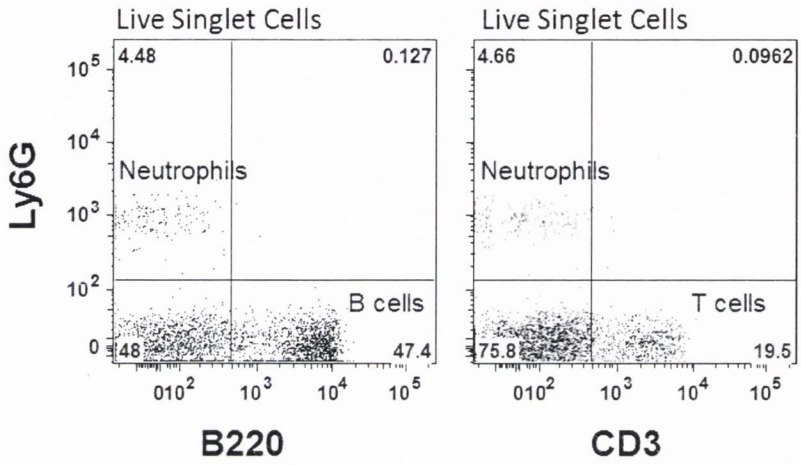


Figure 5.6 Validation of lymphatic pulmonary tissue isolation by identification of B and T cells

WT and Mal$^{-/-}$ mice were exposed to a 20 minute aerosol challenge with 1×10^9 *B. pertussis* per ml. At various times post-infection mediastinal lymph nodes and attached lymphatic vessels were isolated. Tissue was filtered to create single cell suspensions and analysed by FACS for CD3$^+$ T cells and B220$^+$ B cells to determine if tissue samples were lymphatic, thymic or pulmonary. Data are representative of all samples from one experiment.

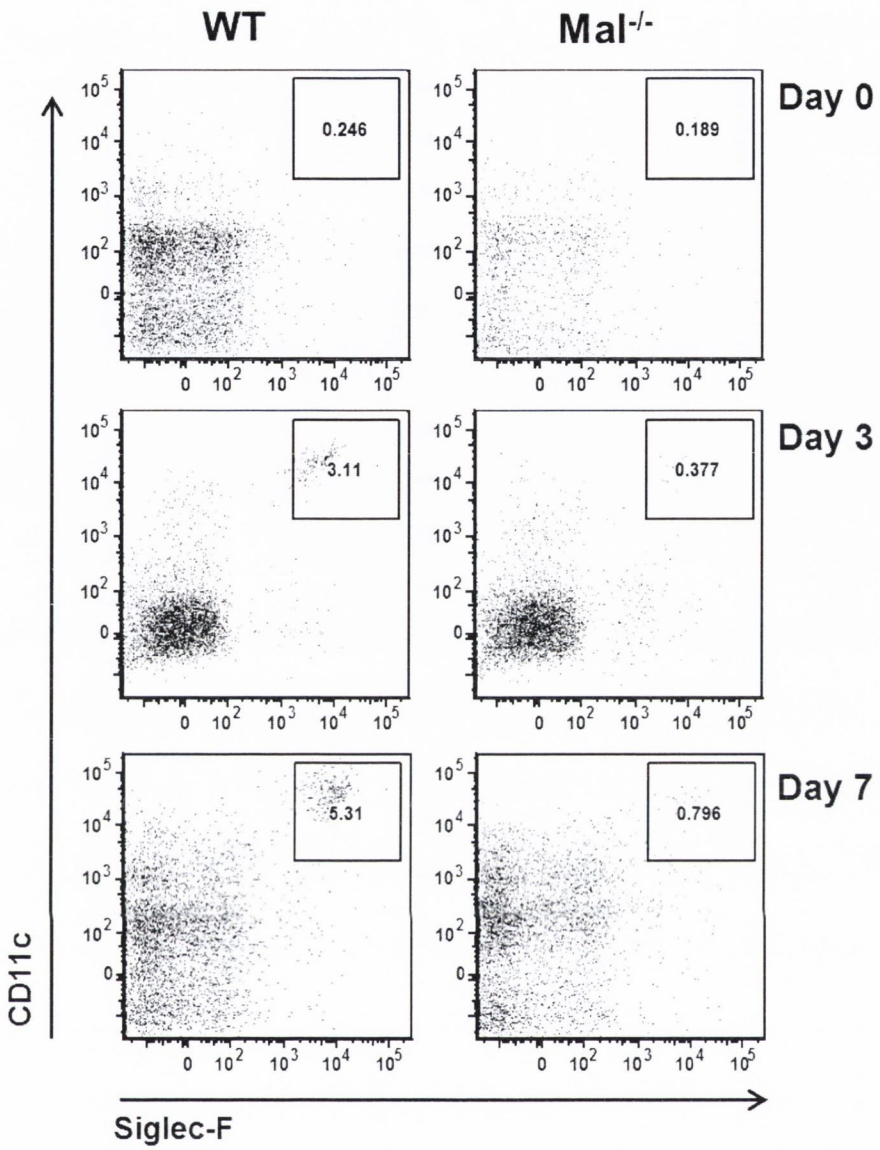


Figure 5.7 Fewer AMs emigrate to the LNs in *B. pertussis*-infected Mal$^{-/-}$ mice

WT and Mal$^{-/-}$ mice were exposed to a 20 minute aerosol challenge with 1×10^9 *B. pertussis* per ml. At various times post-infection mediastinal lymph nodes and attached lymphatic vessels were removed. Red blood cells were lysed and leukocytes were analysed by FACS for alveolar macrophages. Data are the mean from $n = 3$ mice, representative of two experiments.

However, the frequency was much higher in LNs from infected WT mice (approx. 3% at day 3, 5% at day 7) compared with $Mal^{-/-}$ mice (approx. 0.4% at day 3, 0.8% at day 7). These data indicated that fewer AMs emigrate out of the lungs of *B. pertussis*-infected $Mal^{-/-}$ mice compared with infected WT mice, suggesting that emigration alone cannot account for the disappearance of AMs from the lungs of $Mal^{-/-}$ mice. In case AMs from *B. pertussis*-infected $Mal^{-/-}$ mice were trafficking to a site other than the lung draining LNs, blood was also analysed. Unlike the isolated LNs, there was no risk of contamination with lung tissue.

As expected, there were no AMs detected in the blood of uninfected mice. However, consistent with the FACS analysis of the LNs, there were AMs in the blood of *B. pertussis*-infected mice **(Figure 5.8)**. This discovery is the first known account of AMs in blood. The percentage of cells in the blood that were of AM-phenotype was very small (<1%), as would be expected. AMs were consistently detected in the blood 7 days after *B. pertussis* infection. AMs constituted approximately 0.7% of white blood cells in WT mice, and 0.5% of white blood cells in $Mal^{-/-}$ mice.

A comparison of the appearance of AM-like cells in blood and pulmonary lymphatics is presented in **figure 5.9**. These data indicate that AMs appear earlier and at a higher frequency in the lymphatics than they do in the blood, as would be expected, and that AMs comprise a greater percentage of the blood and lymphatic vessels in *B. pertussis*-infected WT mice compared to $Mal^{-/-}$ mice. This is not consistent with the hypothesis that emigration is a mechanism for the disappearance of AMs from the lungs of $Mal^{-/-}$ mice.

5.2.4 *B. pertussis* in the blood

Because AMs were found in the blood and the lymphatic vessels of *B. pertussis*-infected mice, and macrophages from $Mal^{-/-}$ mice were defective at killing intracellular bacteria, it was decided to analyse the blood for viable bacteria, as evidence for a disseminating infection.

At various stages of *B. pertussis* infection cardiac blood was taken from WT and $Mal^{-/-}$ mice. After 7 days infection there were no CFUs detected in blood from infected WT mice, but in the cardiac blood of one $Mal^{-/-}$ mouse

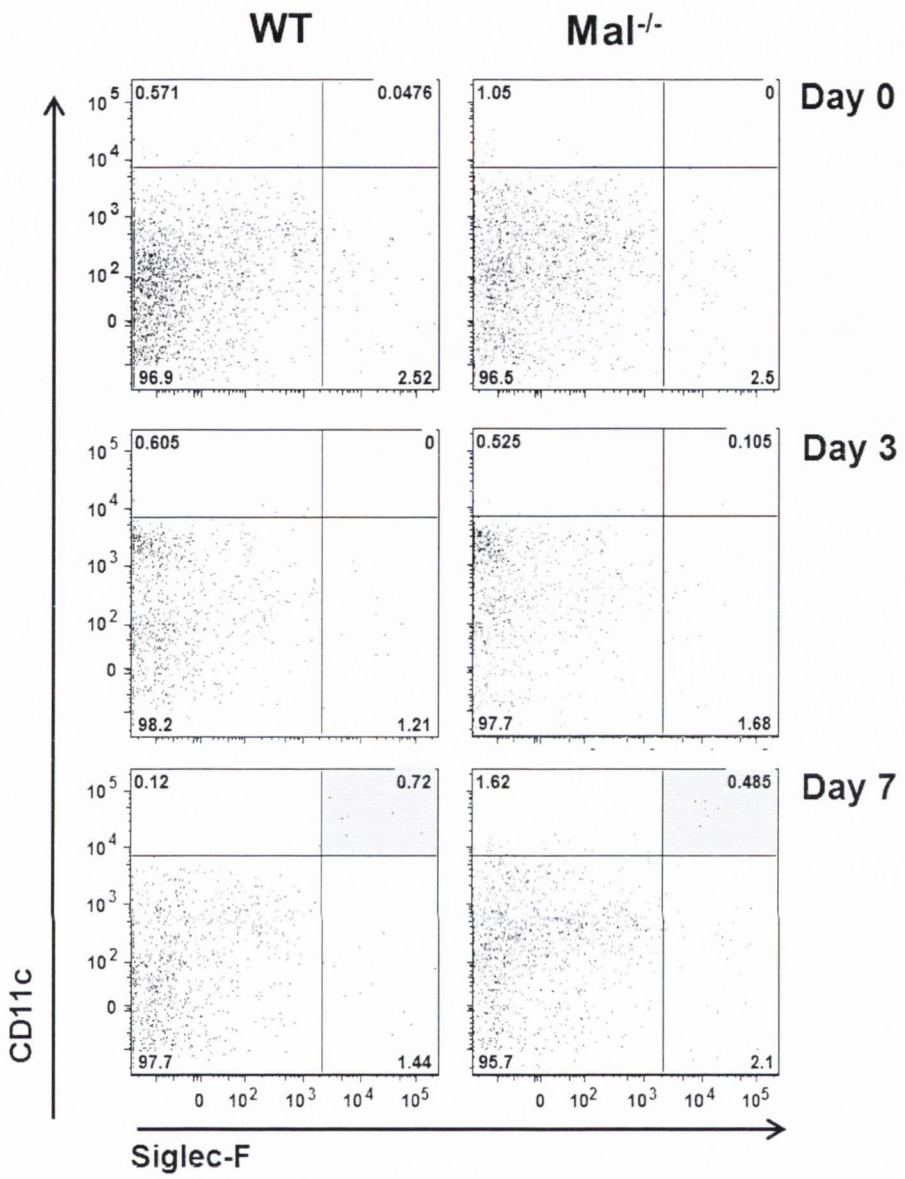


Figure 5.8 AMs are detected in the blood of *B. pertussis* infected mice

WT and Mal$^{-/-}$ mice were exposed to a 20 minute aerosol challenge with 1×10^9 *B. pertussis* per ml. At various times post-infection blood was collected and FACS analysed for alveolar macrophages. Data are the mean from n = 3 mice, from one experiment that is representative of two separate experiments. Shaded panels indicate significance relative to uninfected mice $P<0.01$ by Student's 2-tailed T test.

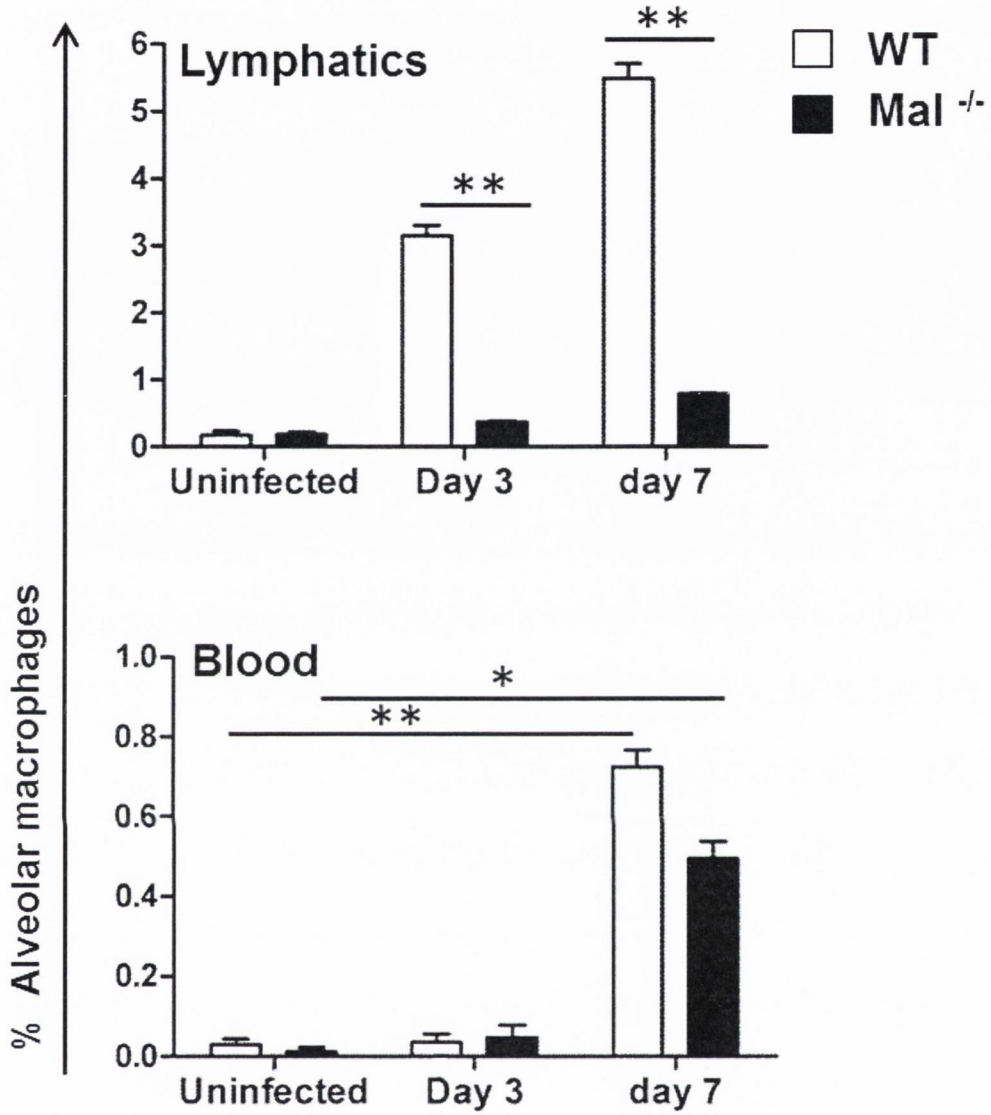


Figure 5.9 Emigrant alveolar macrophages in the lymphatics and blood

WT and Mal$^{-/-}$ mice were exposed to a 20 minute aerosol challenge with 1×10^9 *B. pertussis* per ml. At various times post-infection blood or lymph nodes were analysed by FACS. Data are the mean ± SEM from n = 3 mice, from one experiment. *P<0.05, **P<0.01, ***P<0.001 statistical analysis by 2-tailed unpaired Student's T test.

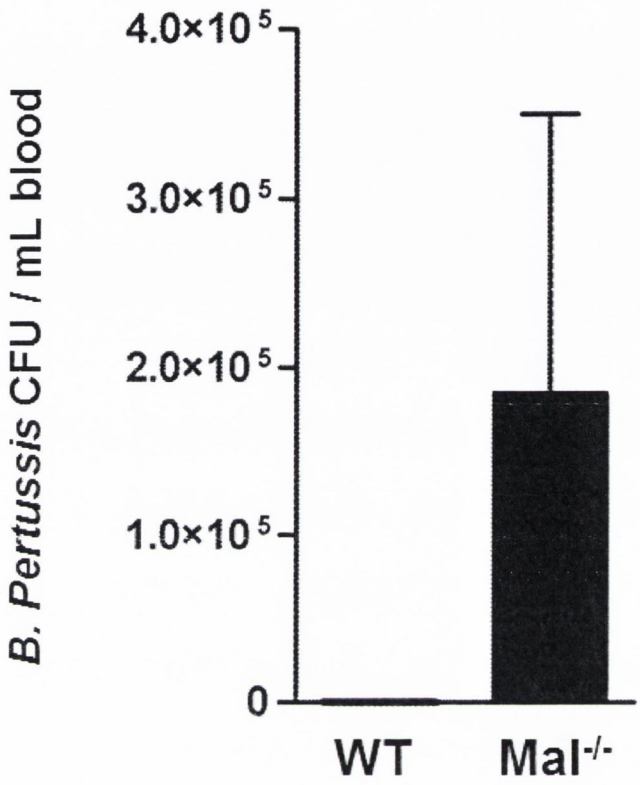


Figure 5.10 Viable *B. pertussis* in the blood of Mal$^{-/-}$ mice

WT and Mal$^{-/-}$ mice were exposed to a 20 minute aerosol challenge with 1×10^9 *B. pertussis* per ml. 7 days after infection cardiac blood was drawn, diluted and plated onto BG blood agar to enumerate *B. pertussis* CFUs.

there were as many as 500,000 CFUs/ml **(Figure 5.10)**. Given the variability of results between mice, and the spatial context of cardiac blood and the high CFUs in the lungs of $Mal^{-/-}$ mice, this extremely high number indicated that contamination was causing false positive identification of CFUs in the cardiac blood from $Mal^{-/-}$ mice. Thereby, this experiment was not repeated.

Other means of isolating blood such as neck bleeds were also deemed to be at substantial risk of producing false positives. Eye bleeds were not ethically approved, and tail bleeds require heating of the mice, which could have unknown effects on other systems such as the bacterial colonisation in the lungs. Thereby, it was decided to analyse the livers of *B. pertussis*-infected mice.

5.2.5 Mal prevents dissemination of *B. pertussis*

Viable *B. pertussis* was not detected in the livers of infected WT mice **(Figure 5.11)**. However, 7 days after infection, there were estimated to be an average of 4,000 viable bacteria per liver lobe, and by day 14 there was an average of 5,000 viable bacteria per liver lobe in infected $Mal^{-/-}$ mice. As livers were taken by dissecting the abdominal cavity, without exposing the thoracic cavity, there was little risk of contaminating samples with lung bacteria. These data indicate that Mal plays a novel role in prevention of dissemination of *B. pertussis* out of the primary site of infection in the airways.

5.2.6 Mal prevents *B. pertussis*-induced liver inflammation

Livers were taken at various times post-infection and fixed in 10% formalin, sectioned, mounted and stained with H&E for histologic analysis of liver pathology that might be associated with the dissemination of *B. pertussis*.

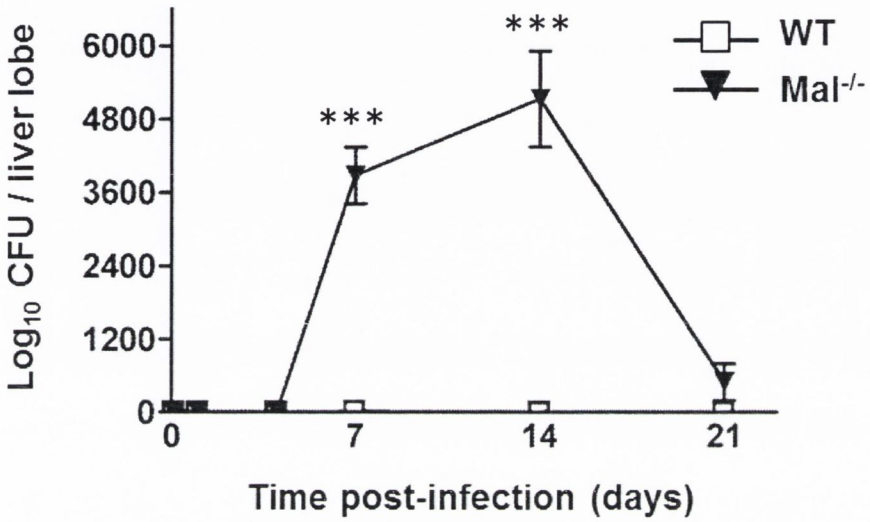


Figure 5.11 Mal prevents dissemination of *B. pertussis* out of the lungs

WT and $Mal^{-/-}$ mice were exposed to a 20 minute aerosol challenge with 1×10^9 *B. pertussis* per ml. At various times post-infection livers were homogenised, diluted and cultured on BG blood agar plates with ciproflaxin to determine CFUs per liver lobe. Data are the mean ± SD from at least three biological replicates from one representative experiment of three separate experiments. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ statistical analysis by 2-way ANOVA with Bonferroni test.

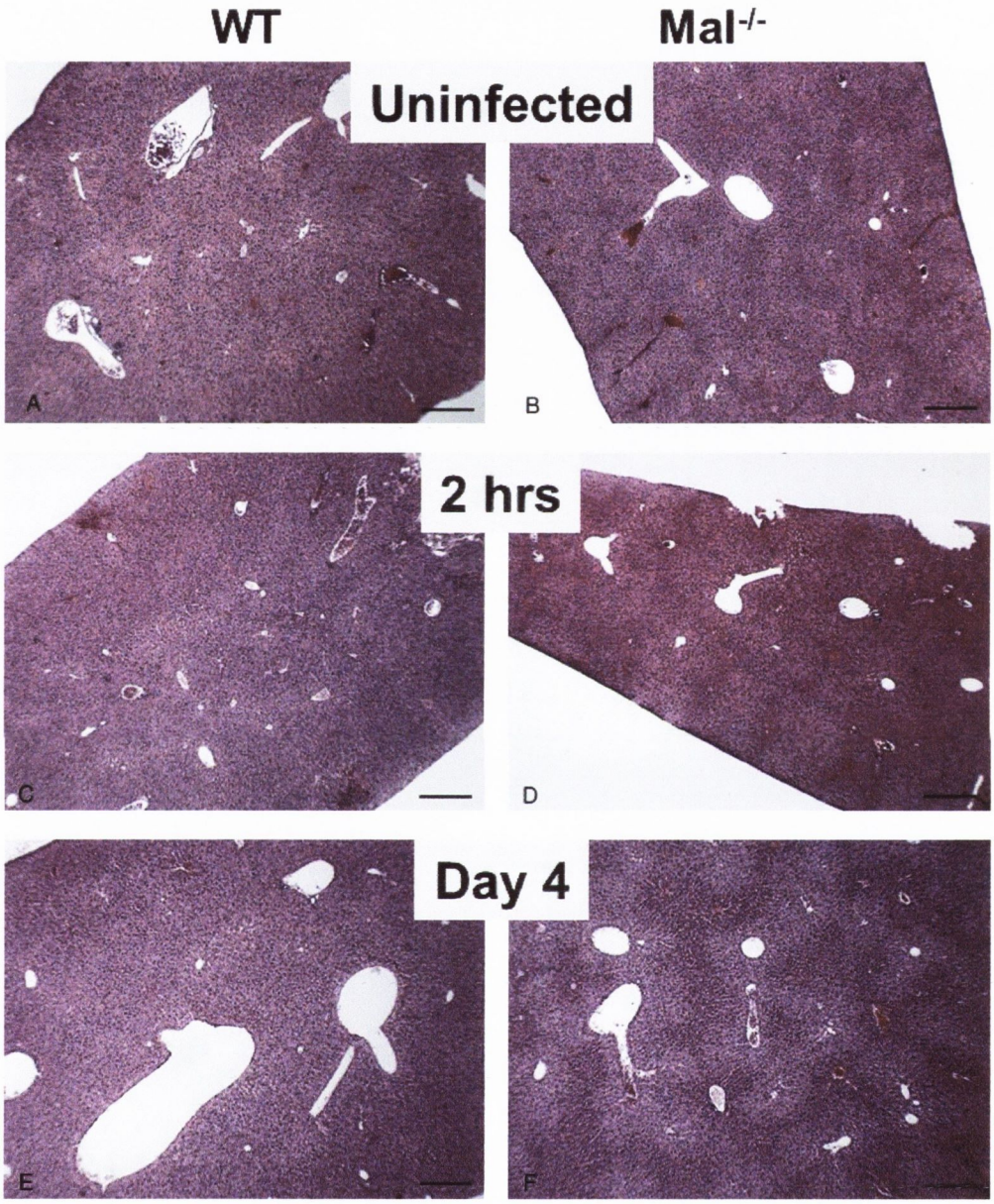


Figure 5.12 A–F No liver pathology after 4 days

Low magnification photomicrographs illustrating the lack of multifocal inflammation at early stages of infection in the liver of $Mal^{-/-}$ (B,D,F) and WT (A,C,E) mice. Haematoxylin and eosin stain. Scale bars = 250 μm.

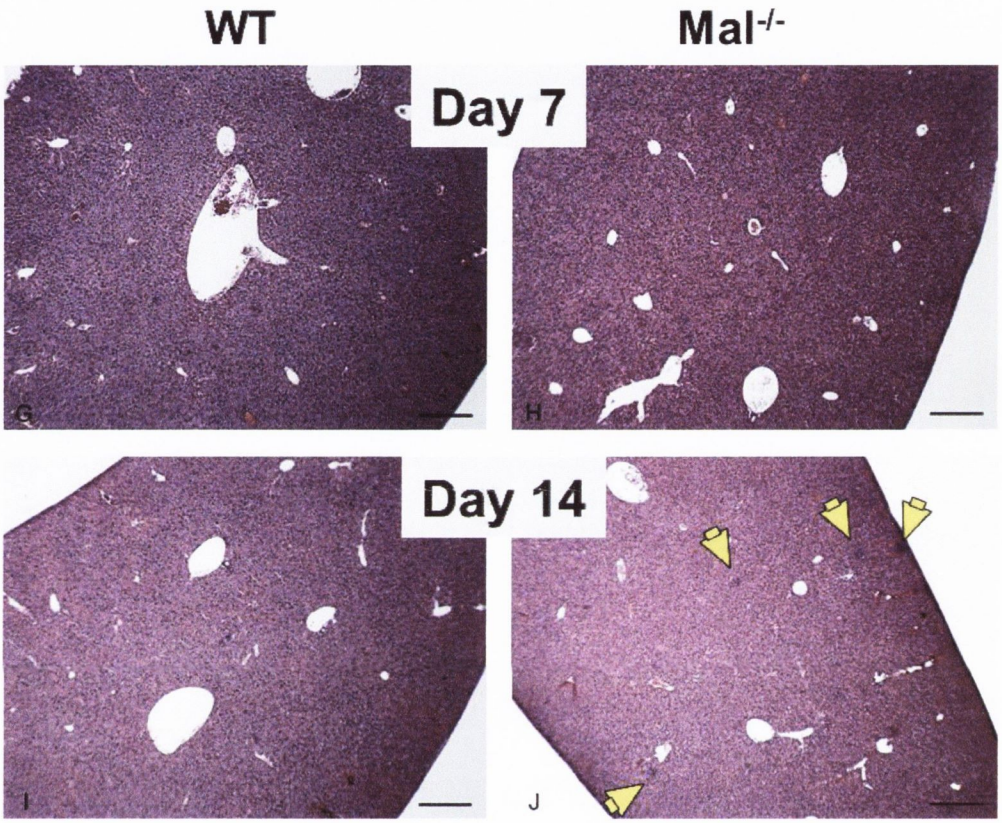


Figure 5.12 G–H Mal prevents *B. pertussis*-induced liver inflammatory foci

Low magnification photomicrographs illustrating the development of multifocal inflammation (yellow arrows; subtle aggregates of admixed neutrophils and macrophages) in the liver of $Mal^{-/-}$ (H,J) and not WT (G,I) mice at day 14 post-infection. Haematoxylin and eosin stain. Scale bars = 250 µm.

7 days after *B. pertussis* infection there was evidence of inflammatory foci in the livers of $Mal^{-/-}$ mice **(Figure 5.12)**. After 14 days of infection there were a substantial number of foci in the livers of $Mal^{-/-}$ mice. At high magnification the foci appeared as tiny granuloma-type structures of cells that were morphologically consistent with neutrophils and macrophages **(Figure 5.13)**. There were no foci detected in livers from uninfected mice or from infected WT mice. These data support the evidence that *B. pertussis* disseminates out of the lungs of $Mal^{-/-}$ mice, and that CFUs from liver homogenates (Figure 5.11) were not cross-contaminants.

5.2.7 Mal prevents severe *B. pertussis*-induced lung damage

The combination of observations that **1)** *B. pertussis* can disseminate, **2)** AMs are found in the blood of infected mice, and **3)** macrophages from $Mal^{-/-}$ mice are defective at killing intracellular *B. pertussis,* indicated a possible mechanism of dissemination out of the airways. Infected AMs from the lungs of $Mal^{-/-}$ mice might carry viable intracellular bacteria into the vasculature. To test this, experiments were designed to examine the mechanism of dissemination of GFP-tagged *B. pertussis*. However, transfection of a GFP tag from *E. coli* to *B. pertussis* failed due to poor plasmid quality and insufficient quantity.

Because damage to the lung barrier is a potential mechanism of bacterial escape into the vasculature, mice were infected with *B. pertussis* and lungs were taken for H&E staining. 7 and 14 days after *B. pertussis* infection, coincident with dissemination to the livers of $Mal^{-/-}$ mice, there was significant lung damage in infected $Mal^{-/-}$ mice. The damage included severe pleurisy in the lungs of infected $Mal^{-/-}$ mice, whereas pleural membrane inflammation was not detected in the lungs of WT mice at any stage of infection **(Figure. 5.14 A–D)**. There was also more damage to the alveolar membranes of $Mal^{-/-}$ mice compared with WT mice as early as

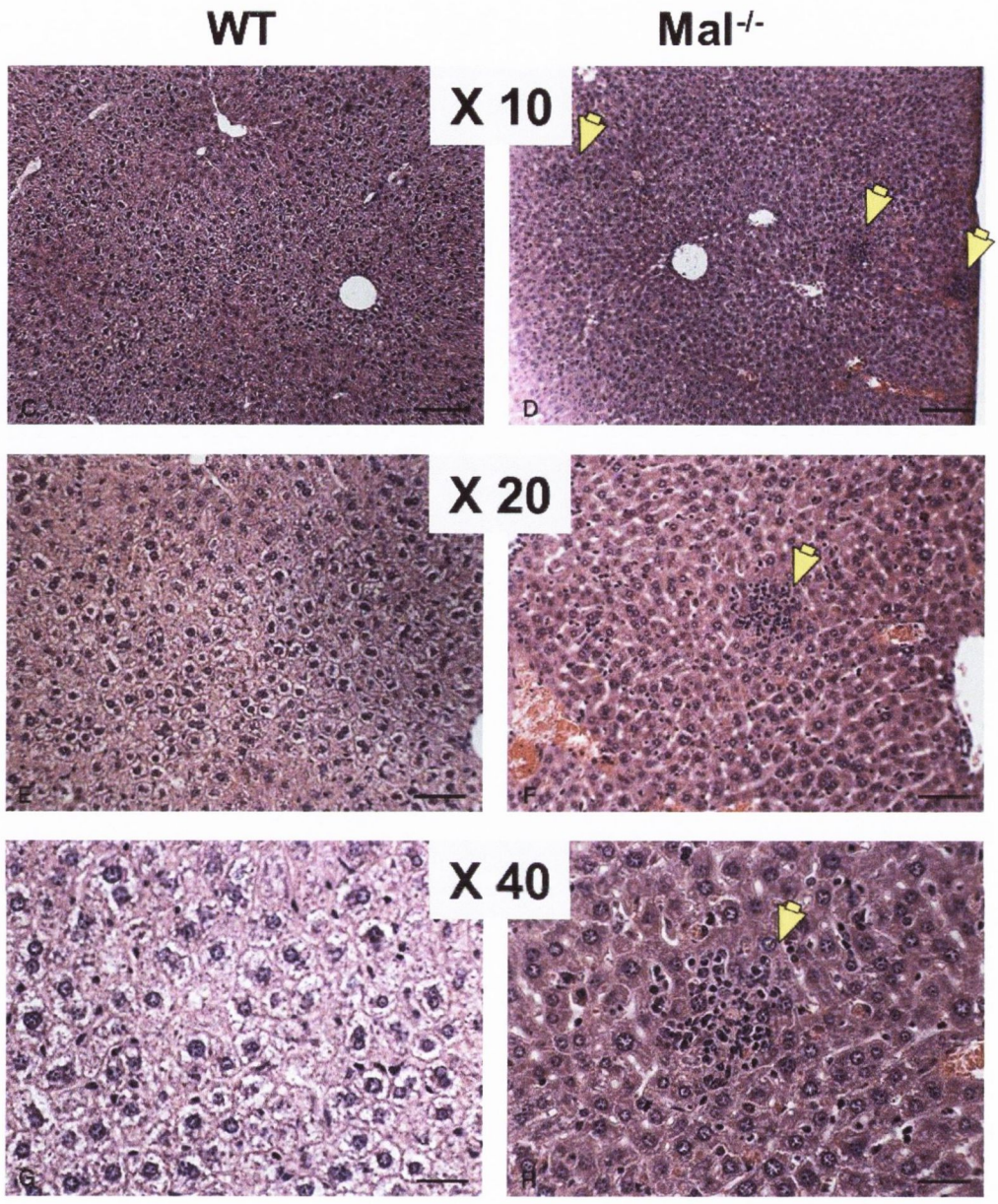


Figure 5.13 Mal prevents *B. pertussis*-induced liver inflammatory foci

Photomicrographs illustrating multifocal inflammation (yellow arrows; subtle aggregates of admixed neutrophils and macrophages) in the liver of $Mal^{-/-}$ (D,F,H), and the absence of such inflammation in their WT counterparts (C,E,G) at day 14 post-infection. Haematoxylin and eosin stain. Scale bars: C,D = 100 µm; E,F = 50 µm; and G,H = 25 µm.

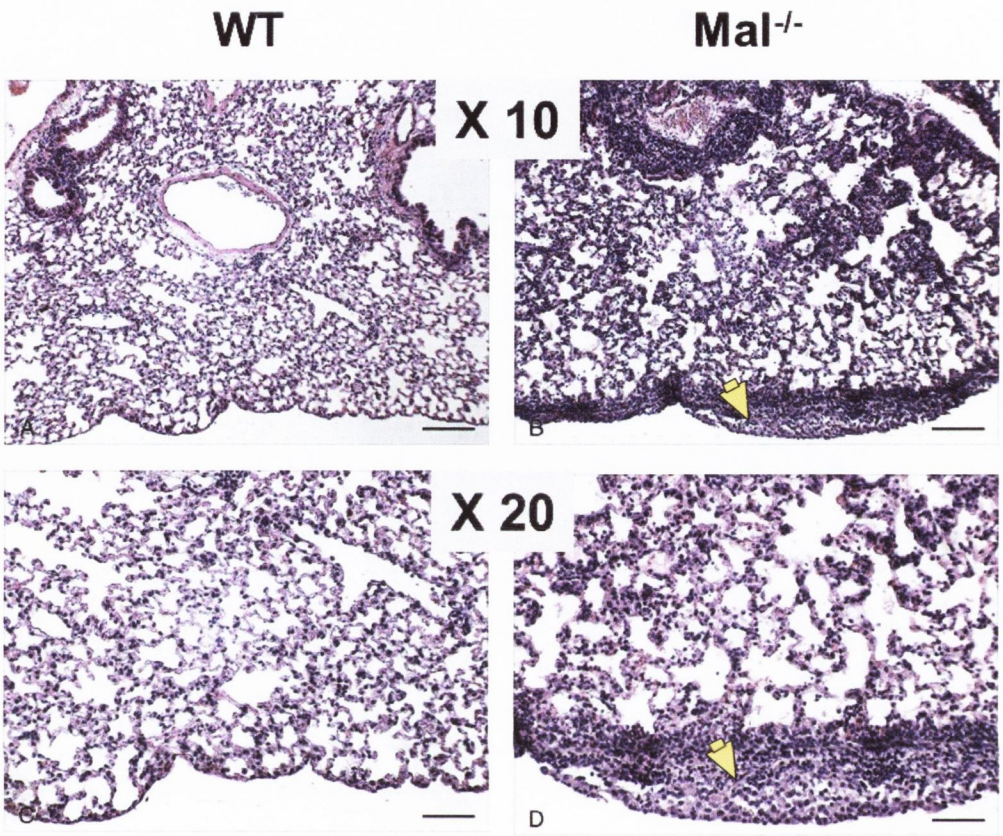


Figure 5.14 A–D Mal prevents *B. pertussis*-mediated lung damage

Photomicrographs illustrating pleurisy (yellow arrows) of lungs from $Mal^{-/-}$ (B,D) and not from WT (A,C) mice at day 14 post-infection. Haematoxylin and eosin stain. Scale bars: A,B = 100 µm; C,D = 50 µm

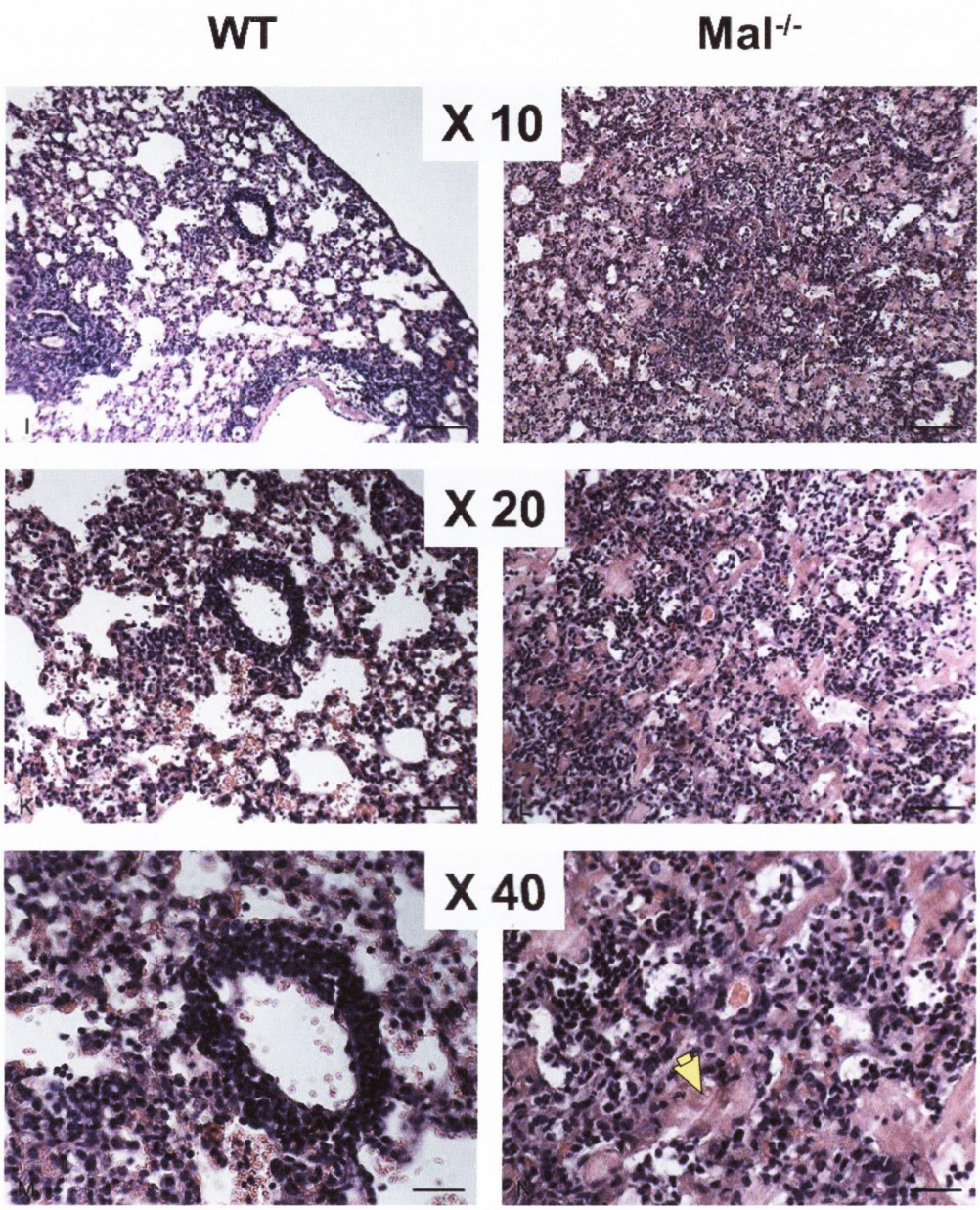


Figure 5.14 I–N Mal prevents *B. pertussis*-mediated lung damage

Photomicrographs illustrating: neutrophils, macrophages and proteinaceous fluid (yellow arrow) filling the alveoli of $Mal^{-/-}$ (J,L,N) mice at day 14 post-infection. At this time-point WT mice exhibit perivascular and peri-airway accumulations of admixed lymphocytes and plasma cells (I,K,M) suggesting resolving inflammation. Haematoxylin and eosin stain. Scale bars: I,J = 100 µm, K,L = 50 µm, M,N = 25 µm.

4 days following *B. pertussis* infection (Figure 3.5 H). After 14 days *B. pertussis* infection, inflammation in the lungs of WT mice was resolving whereas in the lungs of $Mal^{-/-}$ mice there was severe proteinaceous oedema of the alveoli (Figure 5.14 I–N), further evidence of alveolar permeability and a possible means of dissemination of bacteria to the liver.

5.3 DISCUSSION

Influenza can deplete AMs and thereby contribute to secondary pneumonia by *S. pneumoniae*.[330] This is strong evidence that AMs are vital for the prevention of bacterial pneumonias. In chapter three it was discovered that *B. pertussis* infection can cause pneumonia-like histopathology in the lungs of $Mal^{-/-}$ mice, and this correlates with the disappearance of AMs from alveolar spaces. In chapter four the host-protective function of AMs was described, thereby linking susceptibility to function. In this chapter the mechanism of AM disappearance from the lungs of $Mal^{-/-}$ mice was investigated, and this led to the discovery that Mal prevents dissemination of *B. pertussis* airway infection.

In vitro it was discovered that Mal is required for *B. pertussis*-induced activation of macrophages and that such activation may be responsible for killing of *B. pertussis* and for host cell death. It was also found that Mal prevents apoptosis and cell death of AMs in *B. pertussis*-infected lungs, and it was discovered that emigration is not involved in the Mal-dependent maintenance of AMs in the lungs. However, emigration might be responsible for dissemination of *B. pertussis* out of the airways and into the livers of infected $Mal^{-/-}$ mice. These data indicate that Mal plays a vital role in prevention of dissemination of *B. pertussis* infection, whether by maintenance of the lung barrier or by preventing cell-mediated transfer of viable bacteria.

5.3.1 Depletion of AMs from the lungs

The discovery that there is more frequent AM death and apoptosis in *B. pertussis*-infected $Mal^{-/-}$ mice compared with WT mice may not account for the complete ablation of the resident AM population in $Mal^{-/-}$ lungs. Mal-dependent homeostatic factors may also contribute to replenishment of the population. Like other cells, replenishment of AMs is probably driven by various growth factors, cytokines and chemokines, including TNF, GM-CSF, M-CSF, and MIP-2α. While differentiation of macrophages *in vitro* requires the use of CSFs, two studies revealed that MIP-2α is superior to G-CSF in the mobilisation of murine stem cells *in vivo*, and that it is synergistic with G-CSF,[328,343] perhaps indicating that chemokines are vital to the homeostatic replenishment of AMs.

In chapter three a burst of AM-derived Mal-dependent cytokines and chemokines was discovered to be a protective host response to *B. pertussis* infection. The production of MIP-2α by AMs from infected $Mal^{-/-}$ mice *in vitro,* and also in the lungs, was found to be completely defective. This indicated that early burst chemokines, such as MIP-2α, might be vital for the homeostatic replenishment of resident AMs that were depleted from the lungs of *B. pertussis*-infected mice.

Depletion of AMs from the lungs could occur by cell death mechanisms, including apoptosis, or by emigration of AMs. Both of these phenomena were detected in WT and $Mal^{-/-}$ mice after *B. pertussis* infection. However, there was no evidence to indicate that there were more emigrants in the $Mal^{-/-}$ mice relative to WT, and there was equally no evidence to indicate that AMs from $Mal^{-/-}$ mice are more susceptible to cell death in response to *B. pertussis* infection *in vitro*. Following infection *in vivo,* however, it was found that a greater proportion of resident AMs from $Mal^{-/-}$ mice were dead or apoptotic compared to AMs from WT mice.

These data indicate that emigration was not the singular mechanism of AM depletion in *B. pertussis*-infected $Mal^{-/-}$ mice. Equivalent rates of emigration of AMs in WT and $Mal^{-/-}$ mice could account for the disappearance of AMs from $Mal^{-/-}$ lungs if there was also defective replenishment of their numbers in the lungs of $Mal^{-/-}$ mice compared with WT mice.

It is also unlikely that the higher incidence of apoptosis and cell death in the lungs of infected $Mal^{-/-}$ mice, compared with WT mice, could entirely account for the lack of AMs. Although less significant than in $Mal^{-/-}$ mice, there was considerable AM death and apoptosis even in the lungs of WT mice. Despite this, there was no ablation of the WT population, indicating that replenishment by local progenitors must counterbalance cell death and emigration in WT mice, and that this might not occur in $Mal^{-/-}$ mice.

The data indicate that WT macrophages were more susceptible to cell death *in vitro*, whereas AMs from $Mal^{-/-}$ mice were more susceptible *in vivo*. Despite appearances, this is not a contradiction. *B. pertussis* can initiate apoptosis of macrophages;[33] therefore, it makes sense that WT macrophages program cell death *in vitro* more rapidly than macrophages from $Mal^{-/-}$ mice. However, TLR–Mal is not the only apoptogenic pathway.[364] Lacking Mal could slow down apoptosis, but not prevent it. 4 days after *B. pertussis* infection there were more than 10-fold more bacteria in the lungs of $Mal^{-/-}$ mice compared with WT mice. This is coincident with the disappearance of AMs in those mice. Thereby, the simple explanation for the higher incidence of early apoptotic cells *in vivo* could be that there is superior colonisation of the lungs of $Mal^{-/-}$ mice by *B. pertussis*.

5.3.2 *B. pertussis* dissemination

The discovery that AMs traffic out of the lungs into the draining lymphatics, and even into the blood, indicates that susceptibility to *B. pertussis* infection might relate to another phenomenon, dissemination. In chapter four it was discovered that $Mal^{-/-}$ macrophages have a defect in their ability to kill intracellular *B. pertussis*. The combination of AM emigration and an inability of macrophages from $Mal^{-/-}$ mice to kill intracellular bacteria could allow *B. pertussis* to disseminate out of the lungs and into the vasculature.

B. pertussis does not disseminate beyond the airways of C57Bl/6 mice. However, viable *B. pertussis* has been cultured from the livers and blood of airway-infected IFN-$\gamma R^{-/-}$ mice[110] and NK cell-depleted mice[137] Including $Mal^{-/-}$ mice, these three mouse models share defective macrophage activation in common. NK cells produce innate early IFN-γ, which activates macrophages, and in the present study it was shown that Mal is vital for activation of AMs. These data indicate that AMs, or optimal AM effector functions, are vital for prevention of dissemination. The cellular or molecular mechanism of *B. pertussis* dissemination in IFN-$\gamma R^{-/-}$ mice and NK cell-depleted mice was not explored. The two potential mechanisms of dissemination are **1)** active cell-mediated dissemination by so-called 'Trojan horse', and **2)** passive dissemination due to lung barrier permeability. In the present study we attempted to assess the potential of both of these mechanisms.

5.3.3 The Trojan horse

The concept of a Trojan horse mechanism of pulmonary bacterial dissemination was first proposed by Guidi-Rontani in reference to *Bacillus anthracis.*[344] Anthrax virulence in humans is dependent on the facilitated

trafficking of live spores inside emigrating pulmonary cells that enter the vasculature and deposit bacteria throughout the body.

The consequences of bacterial dissemination from any localised site of infection can be varied. The most obvious pathology is bacteraemia-driven sepsis that can cause a 'cytokine storm' in the blood, potentially followed by shock and fatality. Alternatively, bacteria can circulate and colonise highly vascularised organs such as the liver or the spleen. In those organs specialised tissue resident macrophages should phagocytose disseminated bacteria and prevent pathology, but in certain cases granulomas can form and contribute to organ failure.

The mechanism of dissemination of anthrax spores might be different to non-sporate bacteria. Dissemination of *S. pneumoniae* is thought to be enhanced by pulmonary DC as conditional deletion of DC was protective.[345] In another study, host cell-enhanced virulence was found; GFP-tagged *S. pneumoniae* were visualised inside emigrant AMs.[144] These data suggest that it is not the lack of pulmonary immune cells that leads to bacterial dissemination, rather, the cause is defective function of pulmonary cells, or the ability of bacteria to subvert their function. If this is the case, then the disappearance of AMs from the lungs of *B. pertussis*-infected $Mal^{-/-}$ mice, which was temporally consistent with bacterial dissemination, might be coincident rather than causal. The present study identified that Mal is needed for cytokine production by AMs and for activation of oxidative burst and intracellular *B. pertussis* killing. It is possible that these defective AMs were direct mediators of *B. pertussis* dissemination. Indeed, AMs were found in the lung-draining lymph nodes and in the blood, temporally consistent with the discovery of viable *B. pertussis* in the blood and liver. However, without GFP-tagged *B. pertussis* to track bacteria inside cells, the present study cannot definitively identify this as a mechanism of *B. pertussis* dissemination.

5.3.4 The lung barrier

The principal role of the mucosae of the lungs is to allow gas exchange at the same time as preventing the entry of pathogens into the vasculature. For this reason epithelial and endothelial layers are often referred to as the 'lung barrier'. A simple explanation for the dissemination of *B. pertussis* out of the airways of infected $Mal^{-/-}$ mice is that Mal is required for effective lung barrier function. Permeability of the barrier can be affected by pathogen-derived toxins, physical damage, or inflammatory immunopathology. Whatever the cause, the effect is interference of the tight junctions that bind epithelial cells, and the potential transmigration of molecules, fluids, and whole micro-organisms.

There is significant evidence that PRRs, including TLR2 and TLR4, can affect the permeability of epithelial and endothelial barriers. LPS can interfere with intercellular gap formation by causing tyrosine phosphorylation of zonula adherens proteins, permeabilising the barrier to paracellular transmigration,[275,276,277] and TLR2 ligation of Gram positive bacterial lipoteichoic acid can perturb pulmonary microvessel endothelial monolayers.[278] Mal has been implicated in barrier function by association with TRAF6,[279] and by inducing PKC,[274] which can tighten the barrier[280,281] *in vitro* between cultured human nasal epithelial cells,[282] and protect against bacterial infections, such as enteric *S. typhimurium.*[274]

In the present study Mal was found to prevent dissemination of *B. pertussis*. Dissemination of *B. pertussis* to the livers of $Mal^{-/-}$ mice was discovered to occur at the same time as severe damage to the lungs. Pleurisy and damage to alveolar membranes were found in the lungs of infected $Mal^{-/-}$ mice and not in the lungs of WT mice. Temporal coincidence cannot be used as evidence of a causal relationship, but lung damage is the most obvious and simplest explanation for the dissemination of *B. pertussis* in $Mal^{-/-}$ mice. Despite this, it should be noted that AMs were detected in the lymphatics and vasculature of WT

and $Mal^{-/-}$ mice as early as 4 days after infection. This indicates that Mal-dependent barrier dysfunction is not a factor involved in the emigration of AMs. Both barrier permeability and Trojan horse dissemination may occur in infected $Mal^{-/-}$ mice. It is clear that neither mechanism occurs in infected WT mice.

In summary, this chapter has **1)** discovered a Mal-dependent correlation between intracellular bacterial killing and AM death, **2)** discovered that Mal limits *B. pertussis*-induced AM death and apoptosis *in vivo,* **3)** identified emigrant AMs in the lymphatic tissue and blood of *B. pertussis*-infected mice, **4)** discovered that *B. pertussis* can disseminate and cause inflammatory foci in the liver of $Mal^{-/-}$ mice, and **5)** found that there is severe damage to the lung barrier of infected $Mal^{-/-}$ mice.

Chapter Six

General Discussion

6.1 DISCUSSION

B. pertussis, the causative agent of whooping cough, is resurgent on a global scale. The precise nature of this resurgence is unknown, but it is suspected that suboptimal immunity provided by widely-used P_a vaccines is a major contributing factor; new vaccines might be required. The questions that have been raised in relation to the efficacy of P_a vaccines, compared with the older P_w vaccines, highlight the fact that our understanding of the pathogenesis of *B. pertussis* airway-infection is limited. In this study, it was discovered that Mal is vital for prevention of severe, disseminating, fatal *B. pertussis* infection, and that alveolar macrophages are one of the principal effectors of that anti-*B. pertussis* immunity.

When *B. pertussis* first invade the deep lungs, AMs detect *B. pertussis* LPS via TLR4, and detect other PAMPs via other PRRs, including TLR2. TLR2 and TLR4 are dependent on Mal for the activation of various AM effector functions. In the present study it was found that Mal-dependent effector functions included the production of proinflammatory and chemotactic cytokines, and the killing of phagocytosed *B. pertussis* by activation of the oxidative burst and apoptosis. This study is the first to implicate a PRR pathway in the maintenance of resident AMs in the lungs and prevention of bacterial dissemination out of the lungs.

These data suggest that in response to infection with *B. pertussis* a sequence of host-protective immunological events are coordinated by Mal. Initial recognition of PAMPs leads to an immediate burst of proinflammatory cytokines, including IL-1β, TNF and MIP-2α. These cytokines have multiple roles including the recruitment of inflammatory cells, the activation of phagocytic and intracellular killing mechanisms, and they are also proposed to be involved in the stimulation of alveolar progenitor cells. At the same time as this vital host-protective cytokine production, bacterial invasion of alveolar macrophages occurs. These data

show that optimal activation of intracellular killing mechanisms requires Mal signalling. In alveolar macrophages, in which intracellular killing of bacteria was insufficient to overcome infection, Mal-regulated apoptosis was also triggered as a means of deleting infected cells and preventing the uncontrolled growth and intra-disseminating spread of *B. pertussis*. This 'de-population' of alveolar macrophages is likely offset by replenishment from local progenitors that are activated by Mal-dependent cytokines.

Without these host-protective functions of Mal, in a $Mal^{-/-}$ mouse model of *B. pertussis* infection, there is sub-optimal production of proinflammatory cytokines and chemokines, inefficient activation of the oxidative burst and inefficient intracellular killing of bacteria in alveolar macrophages, leading to uncontrolled bacterial growth in the lungs, dissemination of bacteria into the vasculature, and ultimate fatality. Mal could, therefore, be understood as a counterbalance to the armoury of *B. pertussis* virulence factors, reacting specifically to LPS and activating host defence responses to BvgA-BvgS regulated genes.

These data, although directly indicating the importance of Mal, also suggest that alveolar macrophages are vital for protection against *B. pertussis* infection of the lungs. Depletion of alveolar macrophages from the lungs of infected $Mal^{-/-}$ mice was coincident with fatality and thereby corroborates reports that influenza virus infection primes infected individuals for subsequent bacterial pneumonia by deleting alveolar macrophages.

The role of Mal in the homeostasis of alveolar macrophages remains to be established, but it is likely that Mal plays two connected and vital host-protective roles. Firstly, these data show that initial Mal-dependent recognition of lung bacteria by alveolar macrophages causes cytokine release. Studies suggest that these cytokines can activate and mobilise alveolar macrophage progenitors. Secondly, the data show that Mal is

required for optimal *B. pertussis*-induced apoptosis of infected alveolar macrophages. Therefore, Mal is required for the coordinated cell death and cell replenishment of alveolar macrophages. These two roles might appear to be opposing—ontogeny versus cell death—but they are both vital for balancing cell numbers, and importantly, for ensuring that fresh healthy alveolar macrophages are available to replace those that have undergone programmed cell death as a mechanism for prevention of bacterial proliferation.

TLR4 is required for protective immunity to *B. pertussis* infection.[115,177] The kinetics of *B. pertussis* growth in the lungs is similar in TLR4-defective C3HeJ mice and $Mal^{-/-}$ mice. Dissemination of *B. pertussis* into the vasculature is almost uniquely characteristic of these two mouse strains. The present study has expanded on the knowledge of the TLR4–Mal signalling pathway to include the above-described role in maintenance of alveolar macrophages and the activation of host defensive functions.

Some clinicians believe that mouse models of *B. pertussis* infection are too different to *B. pertussis* infection in humans to be worth studying. A particular source of concern is that the histopathology of pulmonary infection appears to be substantially different in mice and humans.[3,7] The accumulation of neutrophils in the lungs that is characteristic of *B. pertussis* infection of mice, as characterised here and in other mouse studies, is not a feature *detected* in human *B. pertussis* infection of the lungs. However, the knowledge of the pathology and human immune responses to *B. pertussis* are not detailed enough to warrant exclusion of valuable mouse data, including the data in this study. Current knowledge of the accumulation of neutrophils and other cells in human lungs in response to *B. pertussis* infection is limited by the fact that human lung sections of *B. pertussis* infected patients, for histological analysis, are extremely rare and for obvious reasons are limited to samples taken from individuals after fatality. These patients could be 'outliers', or the cellularity

of lung inflammation at the point of fatality might be substantially different to the cellularity of lung inflammation at earlier stages of infection. More to the point, these fatalities are cases in which there has been a catastrophic failure of the normal immune response. Proper analysis of the cell populations present in *B. pertussis*-infected human lungs requires FACS analysis of BAL-F, as done in this mouse study, but such an analysis of humans is not ethically or practically viable.

Another potential criticism of the findings in this study, related to the previous point, is that the role of *TIRAP* in humans is not clear from the current published data. Interesting analysis of the role of MyD88 in human resistance to bacterial infection by von Bernuth *et al.* found nine autosomal recessive MyD88-defective children who were highly susceptible to a range of pyogenic bacteria, but not to other pathogens.[346] This work suggests that, in humans, MyD88 might only be vital for protection from pyogenic bacterial infection, and redundant in adults. A similar redundancy in the function of human Mal might exist, thereby putting the role of Mal in immunity to *B. pertussis* (which is non-suppurative) into question. However, without a larger sample size and analysis of MyD88-defective individuals from a range of geographic locations, with and without a prior history of vaccination, which was lacking in the study by von Bernuth *et al.*, a non-redundant role for human MyD88 in protection from other non-pyogenic bacterial pathogens cannot be excluded. Redundancy of MyD88 might occur in those MyD88-defective children, but not necessarily in the general population. The same argument can be made for Mal. Furthermore, from a molecular perspective, it is not clear why MyD88-defective individuals would be susceptible to pyogenic bacteria and not to non-suppurative bacterial species.

Furthermore, MyD88 is known to be important not only for IL-1 signalling,[182,183,184,185] but also for signalling downstream of other TIR domain-containing cytokine receptors including IL-18R,[347] and IL-33R,[348]

and possibly also the IFN-γ receptor.[187] Also, Mal participates in signalling via MyD88-independent pathways. The differences in the downstream signalling pathways of Mal and MyD88 are significant enough that even if MyD88 is proven to be redundant for resistance to non-suppurative bacteria, the same will not necessarily apply to human Mal. The importance and validity of the interesting studies by von Bernuth *et al.* remain to be validated, but if it is discovered that MyD88 is redundant, and Mal is not redundant, for human immunity to *B. pertussis* and other non-suppurative bacteria, this would clearly position Mal as a central target for therapeutic modulation, and would warrant closer examination of the MyD88-independent Mal signalling pathways that are activated by bacterial PAMPs and bacterial infections in mouse models.

6.1.1 Future work

One of the most interesting findings from this project was that *B. pertussis* infection 'depleted' alveolar macrophages *in vivo* and that this temporally correlated with increasing *B. pertussis* growth and exacerbation of illness. An exciting translational project would therefore be to identify endogenous and pharmaceutical factors that can maintain and regenerate alveolar macrophage numbers. If such therapies could be developed then secondary pneumonias resulting from primary infections could be minimized and lives could be saved.

However, understanding the homeostasis of alveolar macrophages is a complicated task. Most likely this would require an understanding of a combination of effects summating survival signals, programmed cell death signals, lysis of infected cells, cellular trafficking and cellular differentiation from macrophage progenitors. While some of these processes are now beginning to be understood there is much yet to be learned.

As a starting point for such a project, future experiments should further characterise the depletion of alveolar macrophages from the lungs of

infected $Mal^{-/-}$ mice. Bioluminescent tagging of *B. pertussis* would be useful for both *in vitro* and *in vivo* analysis of bacterial localisation within macrophages and could be used to track dissemination of bacteria out of the lungs. Such a tool would help to understand if dissemination is passive and related to lung damage, or if it is actively related to a Trojan horse mechanism in which bacteria escape the lungs inside emigrant lung cells.

Also of importance would be to better understand the role that Mal plays in programmed cell death pathways by close analysis of infected BMDMs *in vitro* for autophagy, apoptosis, pyroptosis, necroptosis and other programmed mechanisms of cell death. The difficulty of such experimentation is in understanding whether these mechanisms function as host-protective antibacterial functions or if they are part of the pathology of *B. pertussis* infection.

Although only preliminary and not yet corroborated, data showing that IFN-γ signalling might require Mal, are now supported by work in press showing that Mal has a role in IFNγR signalling and that it is critical for macrophage responses to *M. tuberculosis* infection.[366]

6.1.2 Conclusions

In the complex mouse and human immune systems there is undoubtedly a degree of redundancy in the role of Mal. Other TLR pathways, utilising other adaptors, as well as other non-TLR PRR pathways, can all account for Mal-independent antibacterial immune function. But the fact that components of 'Toll-like' signalling pathways are so conserved between species as disparate as sea anemones, fruit flies and humans indicates their importance. To date the majority of knowledge of TLR adaptor functions relates to the effect these molecules have on NF-κB activity. Although NF-κB is vital for inflammation and immunity, there are many

other transcription factors that are regulated in parallel with NF-κB. It is clear that both MyD88 and Mal activate NF-κB, but as we now know, Mal is more than a bridge. The future of research into the function of Mal in disease should focus on these alternative signalling pathways.

The data presented in this study, based on mouse models of *B. pertussis* infection of the lungs, provide vital information about the role of Mal in protection against fatal *B. pertussis* infection. A better understanding of these Mal-dependent host-protective signalling pathways and their cellular effects could provide a framework for the production of next-generation human vaccines against *B. pertussis* infection to counter the resurgence in this deadly disease.

The discovery of *Drosophila* Toll led to our knowledge of human TLRs. But knowledge of Mal has now led to the discovery of a *Drosophila* Mal functional homolog, dMyD88, complete with a phosphoinositide binding domain.[349] This historical reversal of the cross-species flow of knowledge is testament to the fact that Mal is now one of the best studied TLR signalling molecules, a complex multifaceted molecule that plays a vital conserved role at the forefront of our immunological response to microbial infection

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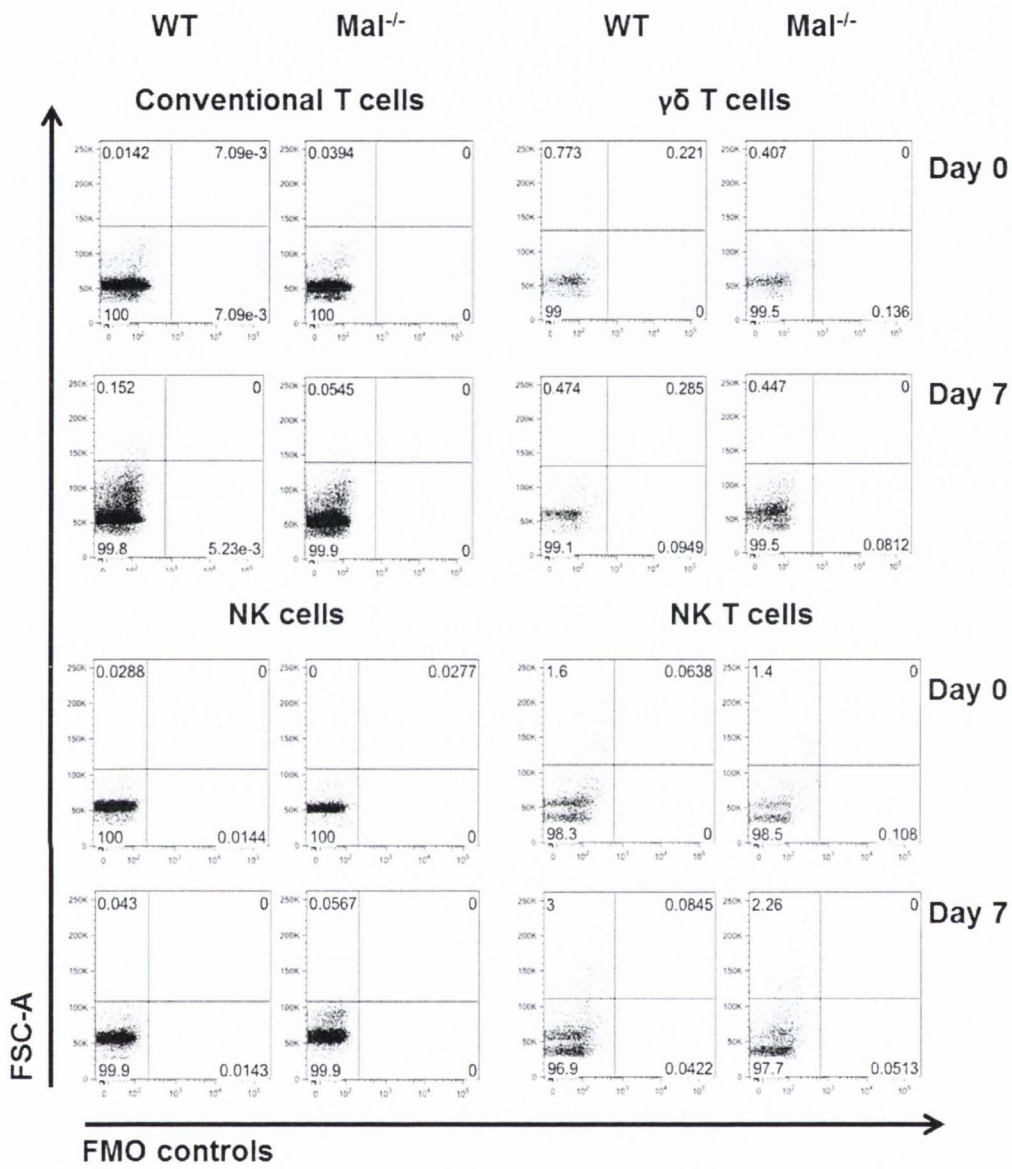
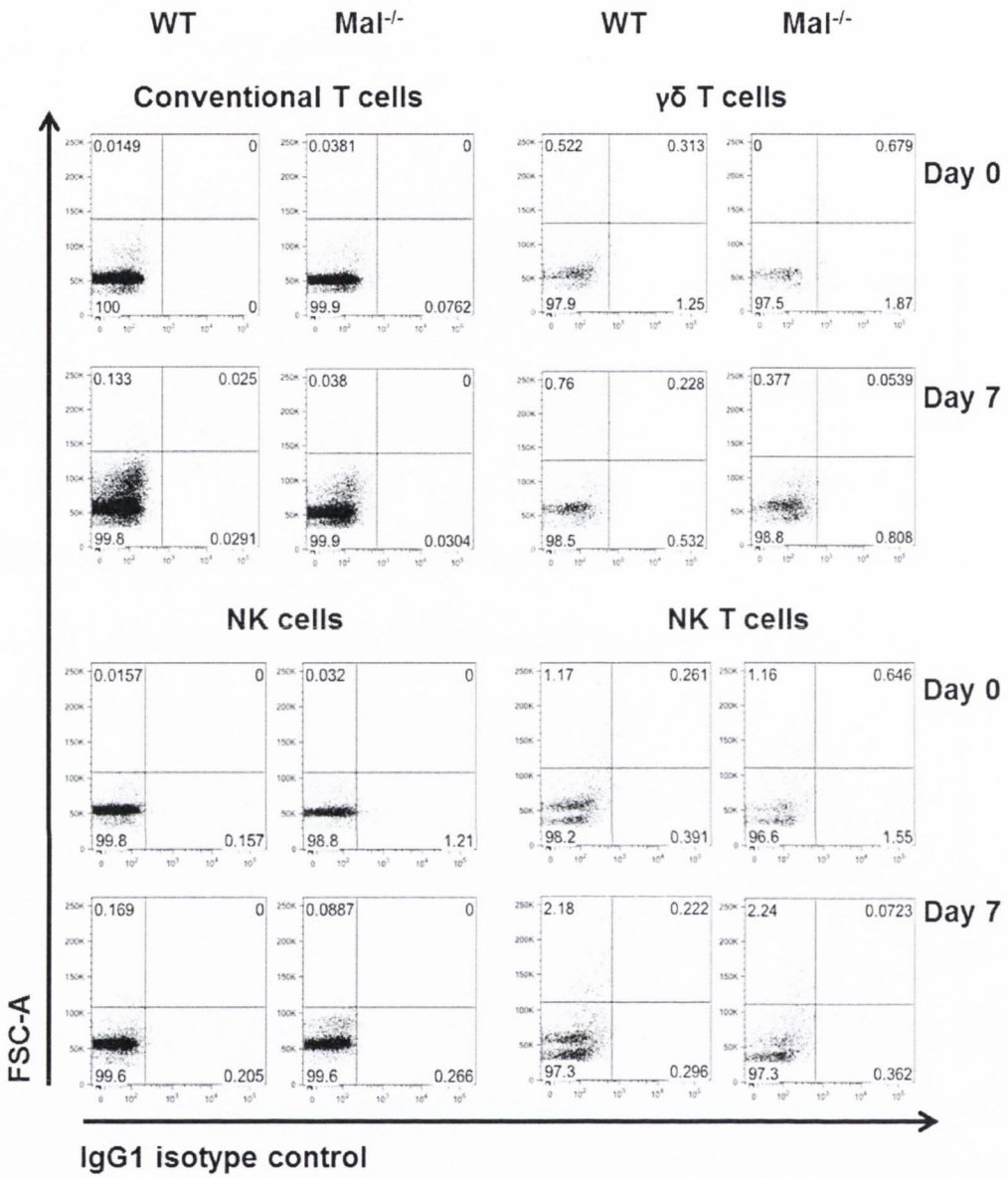


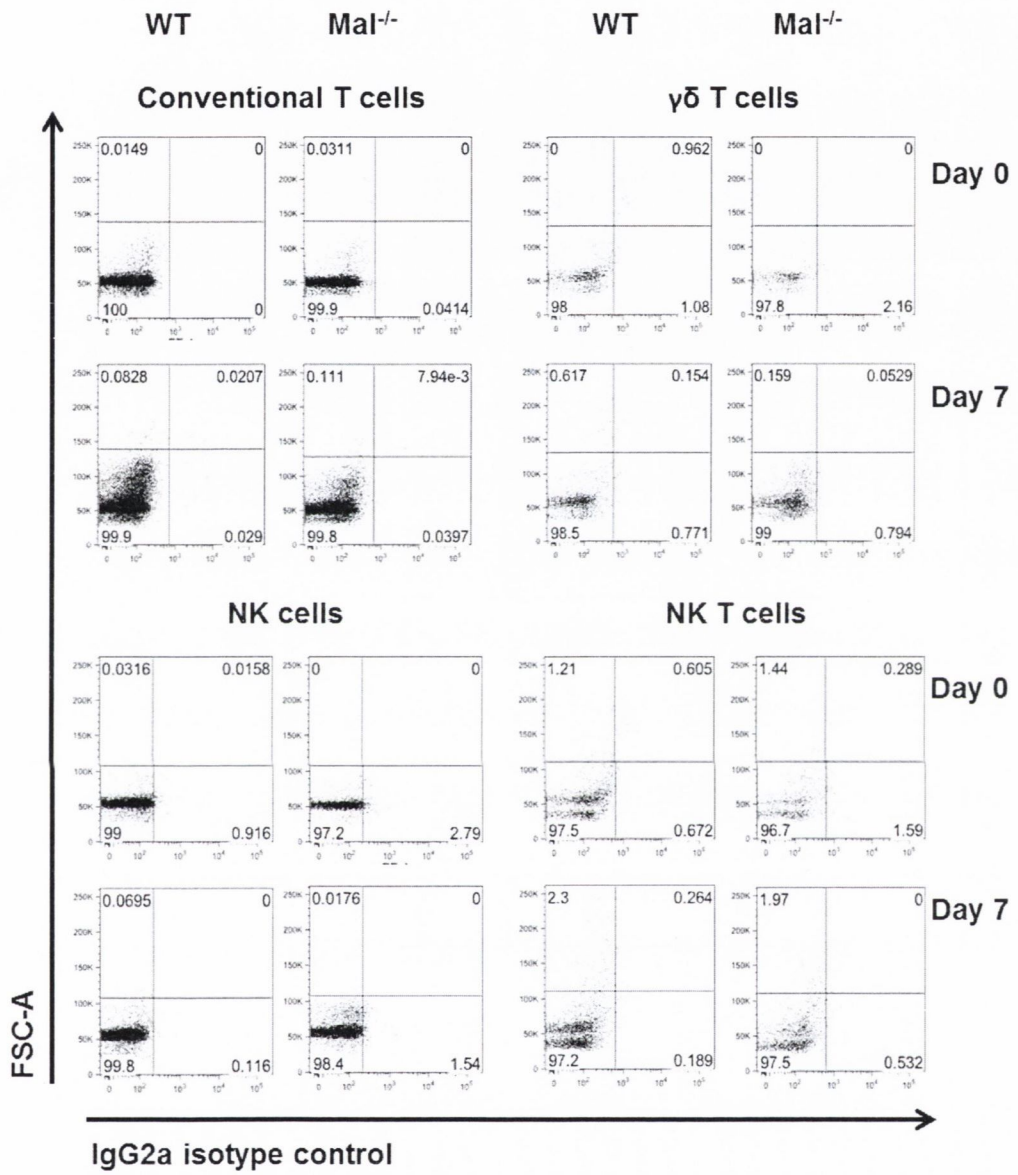
Supp. figure 1 FMO controls

WT and Mal$^{-/-}$ mice were exposed to a 20 mins aerosol challenge with 1×10^9 *B. pertussis* per ml. At day 7 post-infection lungs were mechanically and enzymatically digested. RBC cells were lysed and leukocytes were permeabilised and fixed for intracellular FACS analysis. Cells were not stained with anti-IFN-γ FACS antibody.



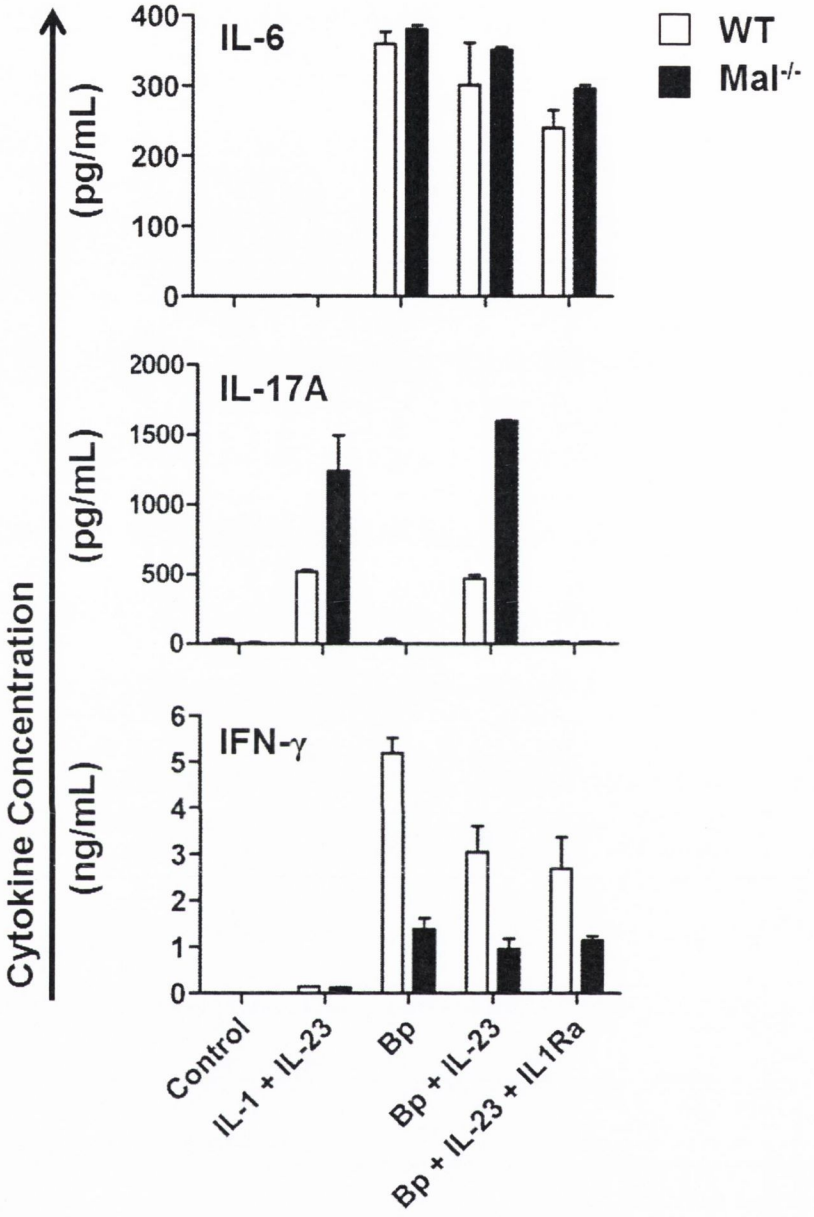
Supp. figure 2 IL-17A isotype controls

WT and $Mal^{-/-}$ mice were exposed to a 20 mins aerosol challenge with 1×10^9 *B. pertussis* per ml. At day 7 post-infection lungs were mechanically and enzymatically digested. Red blood cells were lysed and leukocytes were permeabilised and fixed for intracellular FACS analysis. Cells were stained with an IgG_1 isotype control PE antibody



Supp. figure 3 IFN-γ isotype controls

WT and $Mal^{-/-}$ mice were exposed to a 20 mins aerosol challenge with 1×10^9 *B. pertussis* per ml. At day 7 post-infection lungs were mechanically and enzymatically digested. Red blood cells were lysed and leukocytes were permeabilised and fixed for intracellular FACS analysis. Cells were stained with an IgG_{2a} isotype control PE antibody



Supp. figure 4 Heat killed *B. pertussis* skews WT splenocytes to T_H1, but $Mal^{-/-}$ splenocytes to T_H17

Spleens were removed from uninfected WT and $Mal^{-/-}$ mice. Splenocytes were isolated and co-cultured with heat killed *B. pertussis* and/or IL-1β, IL-23 and IL-1Ra for 48 hours. Culture supernatants were used for cytokine ELISA.