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Characterisation of host immune responses to *Burkholderia mallei*.

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A thesis submitted to the Open University for the degree of Ph.D.
in the Faculty of Science.

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Abstract

Burkholderia mallei is an intracellular, gram-negative bacterium which causes the disease Glanders in humans and mice. Little research has been performed to examine host immune responses to this organism. Therefore, the purpose of this work was to characterise host immunity to *B. mallei* by analysing splenic cellular and cytokine responses and systemic cytokine responses during intra-peritoneal infection in BALB/c mice. In summary, chronic *B. mallei* infection was characterised by an initial period of bacterial colonisation which was limited by the host immune system. This was followed by extensive bacterial replication within abscessed spleens. IFN-γ was essential for controlling early infection and was accompanied by expression of type 1 cytokines and macrophage and neutrophil activation within 24 hours post-infection (p.i.). The IFN-γ response was induced predominantly in NK cells by IL-12 and IL-18 *in vitro*, further demonstrating the requirement for type 1 immunity to *B. mallei* infection. TNFα and Gr-1^{+} cells (predominantly neutrophils) were also important in innate host immune responses. T cells were not important during this phase but played an important role in adaptive immune responses to infection. Downregulation of immune responses occurred 72h p.i. followed by an additional period of macrophage and neutrophil activation 5-7 days p.i.. A heterogeneous infection developed 14-36 days p.i., with mice either forming abscesses or apparently clearing the infection. Abscesses were associated with high bacterial loads and inflammation: TNFα and T cells were involved in controlling the infection, although this did not prevent bacterial colonisation or eventual lethality 8 weeks p.i.. In conclusion, this data shows that early, type 1 immune responses are ultimately overcome leading to extensive replication of *B. mallei* within abscessed tissues. Maintenance of the type 1 cell-mediated immune responses observed during early infection is likely to be crucial for the development of effective therapies and vaccines against *B. mallei*.

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Declaration

I hereby declare that the material contained within this report has not previously been submitted for any other qualification. The work contained within this report was performed by myself, unless otherwise stated and all sources of reference and information have been appropriately acknowledged.

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Dedication

This thesis is dedicated to my family.

To my little flower Florence for her love, and providing me with many happy distractions from writing. To Rob, without whose love, support and endless visits to the 'animals' I wouldn't have been able to complete this thesis – Thank you. To my precious bump, who has been my constant companion throughout the final writing of this thesis, becoming my little cherub Charlotte before final completion of this thesis.

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Publications

Open literature

The critical role of Type 1 cytokines in controlling initial infection with *Burkholderia mallei*. Caroline A. Rowland, Ganjana Lertmemongkolchai, Alison Bancroft, Ashraful Haque, M. Stephen Lever, Kate F. Griffin, Matthew C. Jackson, Michelle Nelson, Anne O'Garra, Richard Grencis, Gregory J. Bancroft, Roman A. Lukaszewski. *Infection and Immunity 74 (9): 5333-40; September 2006.*

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The Inflammatory response in mice following *Burkholderia mallei* infection. Caroline A. Rowland, K.F. Griffin, M.S. Lever & Roman A. Lukaszewski. BSI Annual congress, Harrogate, December 2002.

Early IFN-γ-mediated control of *Burkholderia mallei* infection coincides with increased expression of IL-6 and IL-27. Caroline A. Rowland, K.F. Griffin, M.S. Lever & Roman A. Lukaszewski. BSI Annual congress, Harrogate, December 2003.

The Role of Ly6G$^+$ neutrophils during *Burkholderia mallei* infection. Caroline A. Rowland & Roman A. Lukaszewski. BSI Annual congress, Harrogate, December 2004.

Abbreviations

Ab - Antibody

ACDP - Advisory Committee on Dangerous Pathogens

ANOVA - Analysis Of Variance

AoD - Assay on Demand

ATCC - American Tissue Culture Collection

B.m. - Heat killed *Burkholderia mallei*

CARS - Compensatory Anti-inflammatory Response Syndrome

CBA - Cytometric Bead Array

CD - Complimentary Determining

cDNA - complementary DNA

cfu - Colony forming unit

CpG - Cytosine linked to a Guanine by a Phosphate bond

CR3 - Receptor for Complement component 3

Ct - Threshold cycle

DC - Dendritic cell

DEPC - Diethyl-pyruvate carbonate

DNA - Deoxyribosenucleic acid

FCS - Foetal calf serum

FITC - Fluorescein isothyiocyanate

FS - Forward Scatter

GM-CSF - Granulocyte Macrophage Colony Stimulating Factor

ICAM - Intercellular Adhesion Molecule

IFN - Interferon

Ig - Immunoglobulin

IKK - IkB kinase

IL - Interleukin

IL-18 R - IL-18 Receptor

i.p. - Intraperitoneal

IRAK - Interleukin-1 receptor associated kinase

KO - Knockout

LBP - Lipopolysaccahride Binding Protein

LPS - Lipopolysaccharide

MCP-1 - Macrophage Chemoattractant Protein-1

MHC - Major Histocompatability Complex

MOPS - Morpholinopropane Sulphonic acid

mRNA - messenger RNA

NADPH - Nicotinamde Adenine Dinucleotide Phosphate Hydrogen

NK - Natural Killer

NOD - Nucleotide-binding Oligomerisation Domain

NOS - Nitric Oxide Synthetase

NO - Nitric Oxide

PAMPs - Pathogen-associated Molecular Patterns

PBS - Phosphate Buffered Saline

PDAR - Predetermined Assay Reagents

PE-Cy5 - Phycoerythrin-cyanin 5

PCR - Polymerase Chain Reaction

pDC - Plasmacytoid Dendritic Cell

PE - Phycoerythrin

p.i. - Post-infection

PMN - Polymorphnuclear

PRRs - Pattern Recognition Receptors

ROI - Reactive Oxygen Intermediate

ROS - Reactive Oxygen Species

RNA - Ribose Nucleic Acid

RNS - Reactive Nitrogen Species

RT - Room Temperature

RT-PCR - Reverse Transcriptase Polymerase Chain Reaction

SIRS - Systemic Inflammatory Response Syndrome

SOCS - Suppressor of Cytokine Signalling

SS - Side Scatter

STAT - Signal Transducing Activator of Transcription

Th cell - T helper cell

Tc cell - Cytotoxic T cell

TGF - Tumour Growth Factor

TIR - Toll-IL-1-Receptor

TLR - Toll-like receptor

TNF - Tumour Necrosis Factor

Chapter 1 Introduction

1.1 *Burkholderia mallei*

1.1.1 Introduction

Burkholderia mallei is a Gram-negative, non-motile, aerobic, non-spore forming bacillus and an obligate animal parasite (Figure 1.1). In humans, the nasal and pulmonary form of the disease caused by *B. mallei* is known as glanders whereas a cutaneous form of the disease affecting the lymphatic system is known as Farcy (McGilvray, 1944a). Glanders is principally a disease of solipeds (horses, donkeys and mules) but can infect humans (Bernstein & Carling, 1909; Bristow & White, 1910; Howe & Miller, 1947; Srinivasan *et al*, 2001) and other animal species such as cats and dogs (Blancou, 1994). Experimental animal models exist including monkeys (Kalachev *et al*, 1997; Khomyakov *et al*, 1998) guinea pigs and ferrets (Miller *et al*, 1948a), hamsters (Fritz *et al*, 1999), mice (Fritz *et al*, 2000) and the invertebrate species *Caenorhabditis elegans* (O' Quinn *et al*, 2001). Other animals, including cattle, sheep, swine (McGilvray, 1944a), rabbits and white rats (Miller *et al*, 1948a) are not susceptible to *B. mallei* infection. Limited studies have been undertaken to study *B. mallei* infection, possibly due to the few natural cases now observed in the Western world and the dangerous nature of working with this pathogen.

The *Burkholderia* genus contains a number of bacteria including the human pathogens *B. mallei, B. pseudomallei, B. cepacia, B. gladioli* and *B. pickettii. Burkholderia mallei* and *B. pseudomallei* are close phylogenetic relatives and some strains of *B. mallei* are antigenically related to *B. pseudomallei* (Anuntagool & Sirisinha, 2002). Examination of population genetics suggests that *B. mallei* and *B. pseudomallei* share a large proportion of

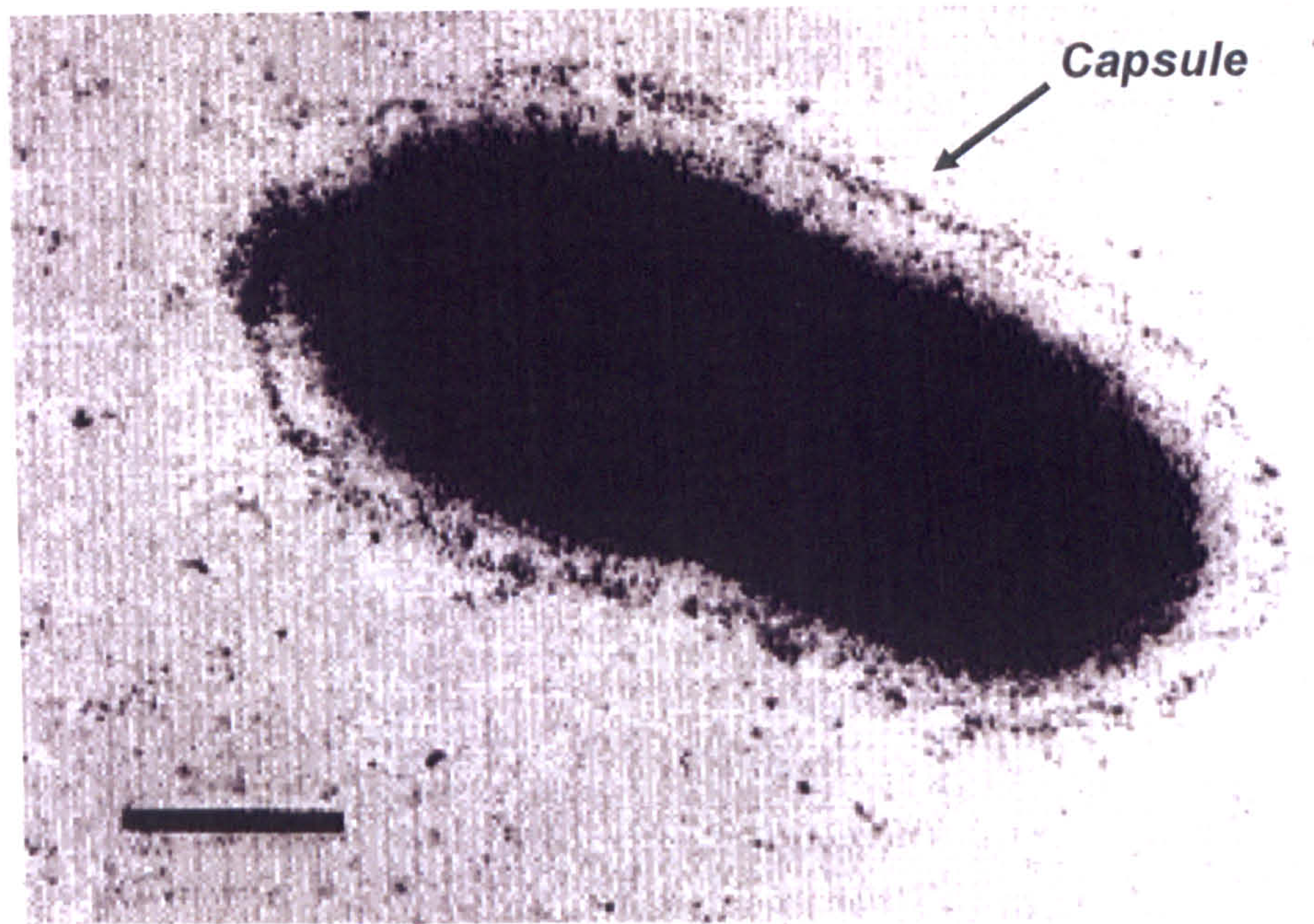


Figure 1.1: **Immunogold electron microscopy of a *B. mallei* bacterium (strain ATCC23344).** The capsule of the bacterium is visible and surrounds the cell. The bar represents 500nm. Reproduced from DeShazer *et al*, 2001.

allelic loci and that on this basis, both bacteria belong to the same species despite their differing disease profiles (Godoy *et al*, 2003).

1.1.2 Epidemiology

Glanders has been eradicated from most of the Western World due to a policy to slaughter all infected animals or potentially exposed ones and strict quarantine controls. Glanders was eradicated from Canada by 1915 (McGilvray, 1944a) and the USA by 1934 (Sharrer, 1995). It is a notifiable disease in most countries (including the UK) due to its pathogenicity in humans and animals (Muhammad *et al*, 1998). During the 1990s glanders was reported to be endemic in Asia and Africa (Dance, 1990) and prevalent in developing countries such as India (Verma, 1981), Turkey (Arun *et al*, 1999) and Pakistan (Muhammad *et al*, 1998) where it is mainly associated with disease in horses although human cases are occasionally reported (Arun *et al*, 1999). The latest report showing *B. mallei* cases was produced in 1999 and countries reporting the disease were Brazil, Pakistan and Mongolia (Derbyshire, 2002).

1.1.3 B. mallei and Biological Warfare

Horses were the main form of civilian and military transport in the 19th and early 20th centuries and infection of horses with *B. mallei* caused serious tactical and strategic problems during the American Civil war (Sharrer, 1995). It has also been suggested that during the 1st and 2nd World Wars glanders was used as a form of biological warfare to infect military horses thus depleting military capability (Christopher *et al*, 1997; Wheelis, 1998 [communication]). Samples obtained from the German Legation in 1916 contained *B. mallei* cultures and Romanian sheep to be transported to Russia during World War I were infected with *B. mallei* (Christopher *et al*, 1997). *Burkholderia mallei* is currently considered to be a potential biological warfare agent (Rosenbloom *et al*, 2002).

1.1.4 Route of infection

Burkholderia mallei may be transmitted via a number of routes to cause disease. Accidental inhalation of aerosolised *B. mallei* produced during laboratory procedures was the most likely source of infection in 6 human cases of laboratory acquired glanders (Howe & Miller, 1947). Transmission via the inhalation route has also been demonstrated experimentally in hamsters (Miller *et al*, 1948a) and in BALB/c mice (Lever *et al*, 2003) following aerosolisation of *B. mallei*. Direct contamination of broken skin or wounds with the bacterium has been demonstrated in laboratory infection (Bernstein & Carling, 1909) and was thought to be the most likely mode of infection in a recent laboratory acquired case of glanders (Srinivasan *et al*, 2001). Inoculation of pus from infected animals or humans into another animal experimentally also allows transfer of the disease (Blancou, 1994). A mortality rate of 56% was reported in acute human cases of the disease following wound inoculation with further deaths occurring within 5 years in chronic forms of the disease (Bristow & White, 1910).

Natural transmission of glanders in horses may occur via ingestion from shared feeding troughs and water supplies (Verma, 1981; Sharrer, 1995). Transmission of the disease via nasal discharge from infected horses rubbing noses with each other has also been reported (Blancou, 1994; Sharrer, 1995). *Burkholderia mallei* infection is also naturally acquired by humans via transmission from horses (Bernstein & Carling, 1909; Gaiger, 1913; McGilvray, 1944a; McGilvray, 1944b; Arun *et al*, 1999) and cases of Glanders have been reported in people who worked closely with horses including grooms, stable workers and veterinarians (Bernstein & Carling, 1909; Blancou, 1994). Human to human transmission may occur with carers and family members of patients with glanders contracting the disease. In some cases the disease was sexually transmitted, with wives of sufferers becoming infected *per vaginum* (Bristow & White, 1910).

1.1.5 Incubation period

Varying incubation periods for the disease exist and months or years may elapse between the time of infection and the onset of disease (Bernstein & Carling, 1909). An incubation period of about 10-14 days has been recorded in some cases of laboratory acquired infection although the exact time of exposure of the infected individuals was not known (Howe & Miller, 1947). Following exposure of one individual to an infected horse an incubation time of 2 days was recorded (McGilvray, 1944b). The likelihood of infection with *B. mallei* is increased by factors such as overcrowding and poor housing of horses and immunosuppression due to other infections (Verma, 1981; Sharrer, 1995). Climatic conditions also affect the spread of glanders. *B. mallei* is easily destroyed by heat and dessication (Verma, 1981) but can remain viable in tap water for at least 4 weeks (Miller *et al*, 1948b). Therefore, rainy seasons in countries like India enhance the spread of disease with warmth and moisture allowing bacterial survival (Verma, 1981). Diabetes has also been implicated in increasing susceptibility to infection with *Burkholderia spp.* (Simpson, 2000) and the most recent case of human glanders occurred in an individual with *Diabetes mellitus* (Srinivasan *et al*, 2001).

Latency of infection has been recognised in human cases. Chronic cases may develop into the acute form of the disease from weeks, months or up to 10 years following infection (Berstein & Carling, 1909). Pathological signs of the disease have also been discovered during autopsies of people who worked with infected horses but did not die as a result of glanders (Bernstein & Carling, 1909) suggesting a much higher prevalence of human infection than previously recorded. This latency may result from *B. mallei* acting as an intracellular pathogen (Arun *et al*, 1999).

1.1.6 Glanders: characteristics of the disease

1.1.6.1 Natural infection

1.1.6.1.1 Human cases

Glanders is a rare disease in humans and most reported cases have been due to working with infected animals, particularly horses, or from laboratory acquired infections. However, the number of cases reported is likely to be only a small proportion of the number of infected people due to difficulties in diagnosing the disease. Cases of human glanders have been misdiagnosed and patients treated for other diseases including syphilis, typhus, tuberculosis and typhoid before correct diagnosis (Bristow & White, 1910). In the acute form of the disease general symptoms include high fever, mucopurulent nasal discharge, overwhelming exhaustion (Bernstein & Carling, 1909) and respiratory distress (Srinivasan *et al*, 2001) leading to pneumonia and/or septicaemia and death in up to 100% of cases if left untreated (Howe & Miller, 1947). Other symptoms observed include subcutaneous and intra-muscular abscesses, nodules and abscesses on the limbs, pus-filled abscesses and nodules in the lungs and ulceration of mucosal surfaces (Bernstein & Carling, 1909). Swollen lymph nodes, chest pain, cough, fatigue, general malaise and night sweats have also been reported (Howe & Miller, 1947; Srivinasan *et al*, 2001). Severe pain can occur following the development of skin ulcers, bone infections and abscesses at multiple sites within the body and the continual development of new lesions may occur for many months (Gaiger, 1913). Abscesses in the liver and spleen may also be present. In the last recorded human case, glanders was diagnosed in a US Defence laboratory worker following a systemic febrile illness (Srinivasan, 2001; Anon, 2001). In this case, a chest radiograph revealed diffuse airspace infiltrates in the lung. In the liver and spleen cystic lesions were revealed using a computer topography (CT) scan suggesting the presence of abscesses that were more pronounced in the spleen (Georgiades & Fishman, 2001).

1.1.6.1.2 Equine cases

Glanders in equine species occurs in an acute or chronic form. In mules and asses the disease is principally acute whereas in horses the disease is often chronic (Verma, 1981), although an acute phase may develop following chronic disease. Symptoms commonly found in glanderous horses with the acute form of the disease include a high fever, and thick nasal discharge followed by death after a few days (Sharrer, 1995). In the chronic form of the disease nasal, pulmonary and cutaneous forms exist (Sharrer, 1995). Other symptoms include bloody, mucopurulent nasal discharge, nodules and ulceration of nasal tissue, sinuses and lungs (Arun *et al*, 1999). Enlargement of the lymph nodes, cough, debilitation and loss of stamina in horses whilst working are also features of the disease (Muhammad *et al*, 1998). An intratracheal experimental model of acute equine glanders exists to study pathogenesis of disease and to assess the efficacy of potential vaccine candidates for *B. mallei* infection. Similar disease characteristics to natural equine infection occur in this model of infection and neurological degeneration was observed suggesting that *B. mallei* may invade the brainstem or spinal cord (Lopez *et al*, 2003).

1.1.6.2 Experimental infection

Experimental animal models of infection allow in-depth characterisation of the disease where parameters such as dose, route of infection and time course of infection can be controlled. This information can be used to allow development of therapies against *B. mallei*, diagnosis of disease and predicting the outcome of infection.

1.1.6.2.1 Non-human primate

In experimental non-human primate models of glanders, variation in individual susceptibility to the disease is evidently similar to human forms of the disease (Manzeniuk *et al*, 1997). Several outcomes to the infection are observed, with some animals having no

external signs of disease, others having obvious signs of disease associated with pyrexia (>39.5°C) and in other individuals the infection is fatal.

Following subcutaneous and intravenous infection (2.5 x 10^8 cfu) of non-human primates (baboon-hamadryads), severe glanders develops within 1-2 days. This was characterised by fever, apathy, anorexia, multiple abscesses and cutaneous ulcers on the head and extremities followed by death 5 days post-infection (p.i.) (Khomyakov *et al*, 1998). Post-mortem investigation of monkeys with lethal outcome of disease showed suppurative inflammation of local lymph nodes, oedema and haemorrhage in remote lymph nodes, enlarged liver and kidneys and oedema in the lungs, pleural space and spleen. Destruction of lymphoid tissues prevented control of the infection leading to bacteraemia and septic shock. In the chronic form of the disease, extensive abscesses were infiltrated by lymphocytes which developed in the soft tissues of upper and lower extremities. Progressive damage to kidneys, liver, spleen and lungs occurred, characterised by substitution of functional structures with connective tissue (Manzeniuk *et al*, 1997).

1.1.6.2.2 Experimental Rodent Models

Both outbred (Porton) and inbred (BALB/c and C57BL/6) mouse strains (Fritz *et al*, 2000; Amemiya *et al*, 2002) have been used to study glanders following intraperitoneal challenge and more recently an aerosol model of infection in BALB/c mice has been developed (Lever *et al*, 2003).

Intraperitoneal infection of BALB/c mice with 10^6 *B. mallei* organisms induces a systemic infection characterised by histopathological changes in the lymph nodes, bone marrow, liver and spleen (Fritz *et al*, 2000). Livers and spleens of mice contain high numbers of bacteria and mild splenomegaly (increasing over the time course of infection) and thymic

atrophy are observed from day 2 p.i.. Varying numbers of bacteria are detected in the blood and lungs in different mice but by day 3 post-infection, bacteria are not found in the blood. During the first 4 days of infection mice are overtly ill but begin to recover after 5 days and appear normal at day 7 even though bacterial numbers remain high in the liver and spleen. Intraperitoneal challenge with 10^7 *B. mallei* organisms causes an acute disease and 75% of mice die by day 3 post-infection. Splenomegaly, pathological changes to the liver and bacterial colonisation of the blood, lung, liver and spleen occurs in most animals (Fritz *et al*, 2000). Aerosol infection of BALB/c mice with *B. mallei* causes a disease similar to acute human glanders with highest bacterial numbers detected in the lungs. Mice are more susceptible to the disease following challenge via the aerosol route with a 1000-fold lower median lethal dose in comparison to intraperitoneal challenge (Lever *et al*, 2003).

Following aerosol infection of mice, hamsters and guinea pigs the pathogen is localised in the respiratory organs leading to haemorrhage 3-12 hours post-infection. Inflammation and bronchopneumonia develop in the lungs within 24 hours. Rapid dissemination of the infection follows, possibly via alveolar macrophages spreading bacteria to local lymph nodes. This leads to bacteraemia, bacteriuria and colonisation of the liver and spleen 24 hours p.i. where metastatic inflammation develops. In the terminal stages of the disease, colonisation and necrosis of the internal organs occurs followed by septicaemia and death within 3-14 days, depending on species (Alekseev *et al*, 2000).

1.1.7 Diagnosis

The wide range of symptoms and variability in outcome between individuals leads to difficulties in diagnosing glanders definitively (Bernstein & Carling, 1909; McGilvray, 1944; Howe & Miller, 1947; Srinivasan *et al*, 2001). Granulomatous lesions which develop in chronic glanders are hard to distinguish from those produced during infection with *M. tuberculosis* or syphilis (Bernstein & Carling, 1909). The Mallein test is used for the

diagnosis of glanders in horses and has been used in the diagnosis of human glanders (Bernstein & Carling, 1909; Howe & Miller, 1947). It involves giving autoclaved *B. mallei* sub-cutaneously to an animal or human suspected of carrying the bacillus either chronically or in a latent form. A positive reaction in both humans and horses may cause local swelling and/or pain at the site of injection, rigors and increases in temperature (Bernstein & Carling, 1909; McGilvray, 1944a). Modern methods in molecular biology allow more definitive identification of bacterial species. Gas-liquid chromatography of cellular fatty acids and 16S ribosomal RNA gene-sequence analysis on bacteria isolated from an infected individual allowed clinical identification of *B. mallei* (Srinivasan *et al*, 2001). However, these methods rely on the isolation of bacteria. This may delay identification as bacterial blood cultures often prove negative even when acute abscesses are present (Bernstein & Carling, 1909; Howe & Miller, 1947) and bacteria may not enter the bloodstream until the final stages of the disease.

Detection of *B. mallei* antigen in experimental models of infection is possible by chemiluminescent immunoanalysis and solid phase immunoenzyme analysis. Identification of *B. mallei* antigen in the nasopharynx, blood and urine of mice, guinea pigs and hamsters 3-12 hours post-infection allows the isolation of sick animals. Further identification of antigen in the blood is predictive of bacteraemia with levels decreasing 1-2 days before the death of the animal (Alekseev *et al*, 2000).

1.1.8 Current treatments and prophylaxis

1.1.8.1 Pre-antibiotic treatments

Early suggestions for the treatment of glanders in horses in eighteenth century Europe included administering an extract made from "boiling two new-born puppies in eight pints of white wine with an ounce of ginger and pepper and four ounces of sugar..." (Sharrer, 1995). Human cases of glanders during the late 19th century and early 20th century were treated using severe forms of medicine including continual operations or placing phenol into infected cavities following drainage of pus from abscesses. This was often supplemented with doses of morphine and heroin to suppress the severe pain caused by the disease (and the treatment) (Gaiger, 1913). Currently no vaccine is available for the treatment of glanders although an autogenous vaccine was trialled using *B. mallei* bacilli isolated from the patient's own infection. This involved a treatment regimen of 15 injections over 4 months with doses rising from $1x10^7$ to $3x10^8$ heat-killed *B. mallei* bacilli leading to relief of symptoms for several months, although the disease was not shown to have been cured (Bristow & White, 1910).

1.1.8.2 Antibiotic therapy

Little information exists regarding the antibiotic treatment of human glanders which is partly due to the small number of cases definitively diagnosed and recorded since the development and widespread availability of antibiotics - only one case of human glanders has been reported in the English language medical literature since 1949 (Srinivasan *et al*, 2001). Several antibiotic treatments have been trialled *in vitro* and, in the case of laboratory-acquired infection, clinically. Sulfadiazine, a bacteriostatic sulphonamide, was used to treat patients with *B. mallei* infection due to its previous proven efficacy in treating animal models with *B. mallei* infection (Miller *et al*, 1948c). This antibiotic was clinically

effective in some of the 6 patients treated but this depended on the stage of the disease in which the antibiotics were delivered and the time course of treatment (Howe & Miller, 1947). Penicillin B is ineffective on the outcome of experimental glanders (Miller *et al*, 1948c) and penicillin G resistance in *B. mallei* occurs naturally (Al-Izzi & Al-Bassam, 1989; Muhammad *et al*, 1999). Natural resistance of *B. mallei* isolated from infected horses to other antibiotics including ampicillin, colistin (Al-Izzi & Al-Bassam, 1989) and metronizadole (Muhammad *et al*, 1998) also occurs. Tetracycline-resistant strains have also been reported (Manzenyuk *et al*, 1995). However, *B. mallei* is sensitive *in vitro* to chloramphenicol, danofloxacin, norfloxacin, co-trizamole (Muhammad *et al*, 1998) and streptomycin (Miller *et al*, 1948c; Al-Izzi & Al-Bassam, 1989). Antibiotic susceptibilities of *B. mallei* in a mouse model showed that doxycycline had some bactericidal action against *B. mallei* (Russell *et al*, 2000). Doxycycline in combination with imipenem, followed by azithromycin and doxycycline treatment, was successful in keeping the most recent recorded human case of glanders under control following initial ineffective treatment with a first generation cephalosporin and later clarithromycin (Srinivasan *et al*, 2001). Treatment normally has to be continued for several months even after apparent recovery from the disease as *B. mallei* may remain latent for many years (Bernstein & Carling, 1909; Russell *et al*, 2000).

1.1.8.3 Alternative Treatments

Treatment of glanders without the use of antibiotics has been reported in a non-human primate model of infection: circulating blood from animals with severe forms of glanders was detoxified by dialysis and haemosorption on carbon columns. This improved the outcome of the disease, reduced the number of *B. mallei* organisms in the circulation and improved the outcome of septic shock (Khomyakov *et al*, 1998).

1.1.9 Host responses to B. mallei infection

B. mallei interacts with the host immune system leading to activation of general inflammatory responses. A range of non-specific immune factors such as β_2-microglobulin (part of the major histocompatiblity complex (MHC) on antigen presenting cells), prostaglandin-E_2 (PGE_2) - an inflammatory mediator involved in production of acute phase proteins, proliferation of lymphoid tissue and migration of cells - and complement factor C3a, are raised during infection in non-human primates demonstrating the presence of an inflammatory response (Manzeniuk *et al*, 1997). *B. mallei* causes disruption of homeostatic mechanisms and hormonal balance affecting the hosts' innate protection against infection (Manzeniuk *et al*, 1997). Although the host is able to mount an inflammatory response, *B. mallei* may be able to evade this response using its extracellular capsule which has immunosuppressive actions and is important for the virulence of this pathogen (DeShazer *et al*, 2001; Popov *et al*, 2002). The type III secretion system of *B. mallei* is also important in its virulence and allows it to replicate within macrophages (Ulrich & DeShazer, 2004). Macrophages normally engulf and kill bacteria following infection. However, following aerosol challenge in the guinea pig and hamster, *B. mallei* interacts with alveolar macrophages to downregulate their bactericidal mechanisms and transfer the infection to regional lymph nodes (Alekseev *et al*, 2000). Nitric oxide, a bactericidal product of macrophages, is not essential for protection against infection as the absence of iNOS2 (inducible Nitric Oxide Synthetase-2) in knockout mouse studies did not change the outcome of infection (Lukaszewski, 2004). Specific antibodies formed during infection do not appear to be essential for immunity to *B. mallei* infection. Humoral immunity was not found to be protective in monkeys developing high titres of antibody during infection and then reinfected with *B. mallei* (Manzeniuk *et al*, 1997). Genetic knockout mice lacking B cells or CD28 (a T cell co-stimulatory molecule) survive infection demonstrating that

antibody mediated immunity is not essential during *B. mallei* infection (Lukaszewski, 2004).

Although little is known about the immune response to *B. mallei* infection, immune responses to other intracellular pathogens have been well characterised. Innate and type 1 immune responses commonly observed during infection with intracellular bacteria are described below to illustrate the immune interactions which may be important in immunity to *B. mallei* infection.

1.2 Innate immune responses and inflammation

1.2.1 Bacterial initiation of innate responses

Innate inflammatory responses are triggered in response to pathogen invasion and aim to protect the host against infection. Innate immune activity is stimulated directly by the pathogen to create an immediate non-specific response to infection. This is in contrast to adaptive immune responses where specific, long-lived responses are initiated and become effective several days after infection. Innate responses are instigated during bacterial infection by bacterial components that may either be membrane bound, secreted or released following pathogen destruction. Some bacterial components are known initiators of inflammation including lipopolysaccharide (LPS), bacterial lipoprotein (BLP), peptidoglycan, porins, lipoproteins, lipopeptides, lipid-A associated proteins and pili (Heumann *et al*, 1998a). Other immuno-stimulatory molecules are specific to particular bacterial species. For example, flagellin is present only in motile bacteria such as *B. pseudomallei* (Hersh *et al*, 1998).

Neutrophils, monocytes, macrophages and dendritic cells (DCs) express specific receptors known as pattern recognition receptors (PRRs) which recognise the conserved structures of these bacterial components known as pathogen associated molecular patterns (PAMPs) (Medzhitov & Janeway, 2000). Toll-like receptors (TLRs) are PRRs that recognise conserved fragments from pathogens including bacteria, viruses and fungi (Akira & Takeda, 2004). TLRs (1-10) are expressed on the surface or within the cytoplasm of innate immune effector cells including macrophages, neutrophils and DCs. Several TLRs exist which recognise specific bacterial components. TLR2 recognises the cell wall polysaccharide peptidoglycan from gram-positive bacteria eg. *S. aureus* (Yoshimura *et al*, 1999). TLR5 recognises flagellin from motile bacteria eg. *S. typhimurium* (Hayashi *et al*, 2001). TLR4, in combination with an adaptor molecule MD2, recognises lipopolysaccharide (LPS), a component of the gram-negative bacterial cell wall. LPS is initially bound by CD14 on the surface of monocytes, macrophages and neutrophils. This complex associates with TLR4 following LPS binding to enable signal transduction via the intracellular portion of TLR4 (Heumann *et al*, 1998a). TLR9 recognises and associates with conserved bacterial (and viral) DNA sequences, which contain unmethylated dinucleotide motifs containing cytosine and guanine residues (CpGs), within the endosomal compartment (Hemmi *et al*, 2000). TLR9 is present in macrophages, DCs and B cells in mice and plasmacytoid DCs (pDCs), B cells, monocytes (Takeshita *et al*, 2001) and NK cells (Roda *et al*, 2005) in humans. TLR9 is not expressed on the cell surface but resides within the endoplasmic reticulum and moves into the endosomal membrane and lysosomal systems following internalisation of DNA. Direct association between TLR9 and DNA from intracellular bacteria may occur in the endosomal compartment as bacteria are phagocytosed into the endosome and destroyed releasing DNA fragments in close proximity to TLR9 (Latz *et al*, 2004).

Interaction of TLRs with their specific ligand leads to induction of common signal transduction cascades via the cytoplasmic tail of the receptor - the Toll-IL-1-Receptor (TIR) domain. This interacts with the intracellular signal transducing molecule MyD88 and interleukin-1 receptor associated kinase (IRAK) initiating phosphorylation cascades and leading ultimately to the transcription of immune response genes and the production of inflammatory mediators via the nuclear transcription factor NFκB (Figure1.2) (Hawiger, 2001). In addition to common pathways of activation by TLRs, alternative signal transduction pathways may be utilised by a specific TLR and its ligand. Alternative adaptor molecules and transcription factors are utilised to allow the transcription of genes specific for the TLR. This leads to expression of specific gene profiles and the production of inflammatory mediators which allow fine-tuned immune responses to develop which are appropriate for the infective pathogen (Boldrick *et al*, 2002; Feezor *et al*, 2003).

Intracellular pathogens are also recognised within the cytoplasm by a set of recently recognised receptors known as nucleotide-binding oligomerisation domain (NOD) proteins. These recognise components of peptidoglycan presented to the receptors directly within the cytoplasm. Gram-positive and gram-negative bacteria contain different types of peptidoglycan and these differences are recognised by NOD1 and NOD2 respectively leading to responses tuned to the pathogen (Girardin *et al*, 2003). Although these receptors appear to recognise intracellular pathogens, their role in infection has yet to be elucidated (Murray, 2005).

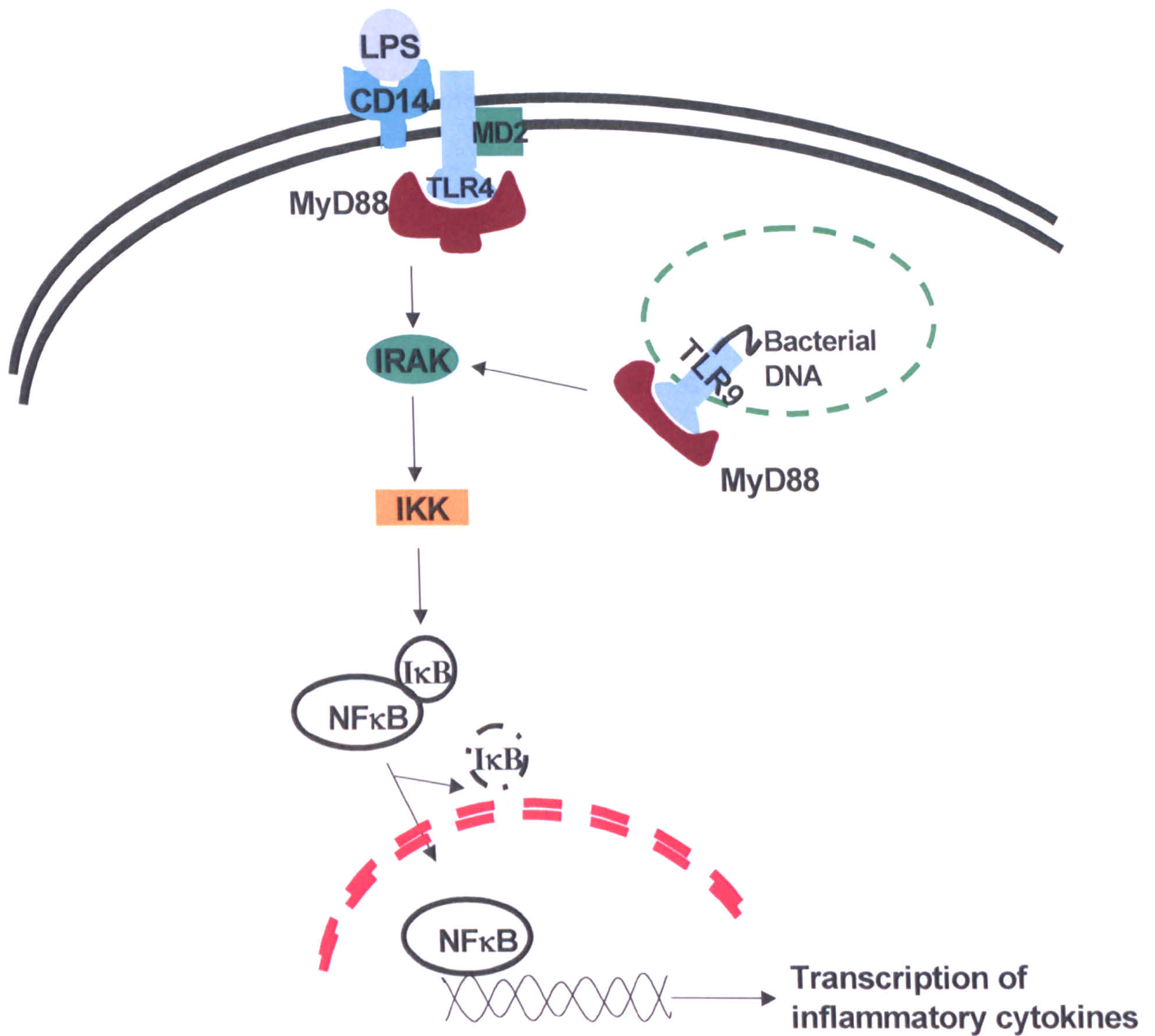


Figure 1.2: Diagram showing a common pathway of activation of inflammatory mediators via Toll-like receptors (TLR)4 and TLR9 and the nuclear transcription factor NFκB. TLRs activated following ligand binding (LPS/CD14:TLR4; DNA:TLR9) recruit the adaptor protein MyD88 which interacts with the serine-threonine protein kinase IRAK. Following a series of intermediate steps IκB kinase (IKK) phosphorylates the NFκB inhibitor IκB releasing NFκB and allowing it to translocate into the nucleus where it interacts with the NFκB binding motif leading to transcription of immune response genes and inflammatory cytokines. Alternative adaptor molecules are used by TLR4 in MyD88-independent pathways and alternative transcriptions factors eg. IRF3 may be used to induce different patterns of gene expression including IFN-inducible genes.

1.2.2 The Inflammatory response: Cells

The inflammatory responses instigated in response to bacterial infection in the spleen are summarised in Figure 1.3. The main cellular components of the innate immune system are neutrophils, macrophages, natural killer (NK) cells and DCs. These cells enable innate, non-specific killing of pathogens and are able to respond rapidly to infection due to the pathogen-recognition systems that they express. In addition to their role in innate immunity, the responses elicited by these cells during the early phase of infection may influence and determine the development of adaptive responses.

1.2.2.1 Neutrophils

Neutrophils are blood-borne phagocytic, granulocytic, polymorphonuclear (PMN) cells and are important in the innate response to infection. They are a major component of the innate immune system and are the most numerous white blood cell. Neutrophils migrate into infected tissues in response to chemotactic factors particularly beta-chemokines and peptides eg. fMLP (Burg & Pillinger, 2001). Neutrophils are activated following recognition of pathogen components (PAMPs) via PRRs and also by pro-inflammatory mediators, such as TNFα and IL-6. Neutrophils ingest pathogens by phagocytosis which is enhanced by neutrophil receptors that recognise bacteria coated with antibody (Fc receptors) or complement components (eg. iC3R; CD11b) allowing opsonisation to occur (Hellwig *et al*, 2001). Neutrophils effect pathogen killing using anti-bacterial and pro-phagocytic molecules including proteinases and reactive oxygen species produced following activation of NADPH oxidases and myeloperoxidases (Hersh *et al*, 1998; Gregory & Wing, 2002). Neutrophils also secrete a range of pro-inflammatory mediators such as TNF-α (Bliss *et al*, 1999) and IL-6 to propagate inflammatory processes and also secrete cytokines involved in the development of Type 1 immune responses including IL-12 (Bliss *et al*, 2000).

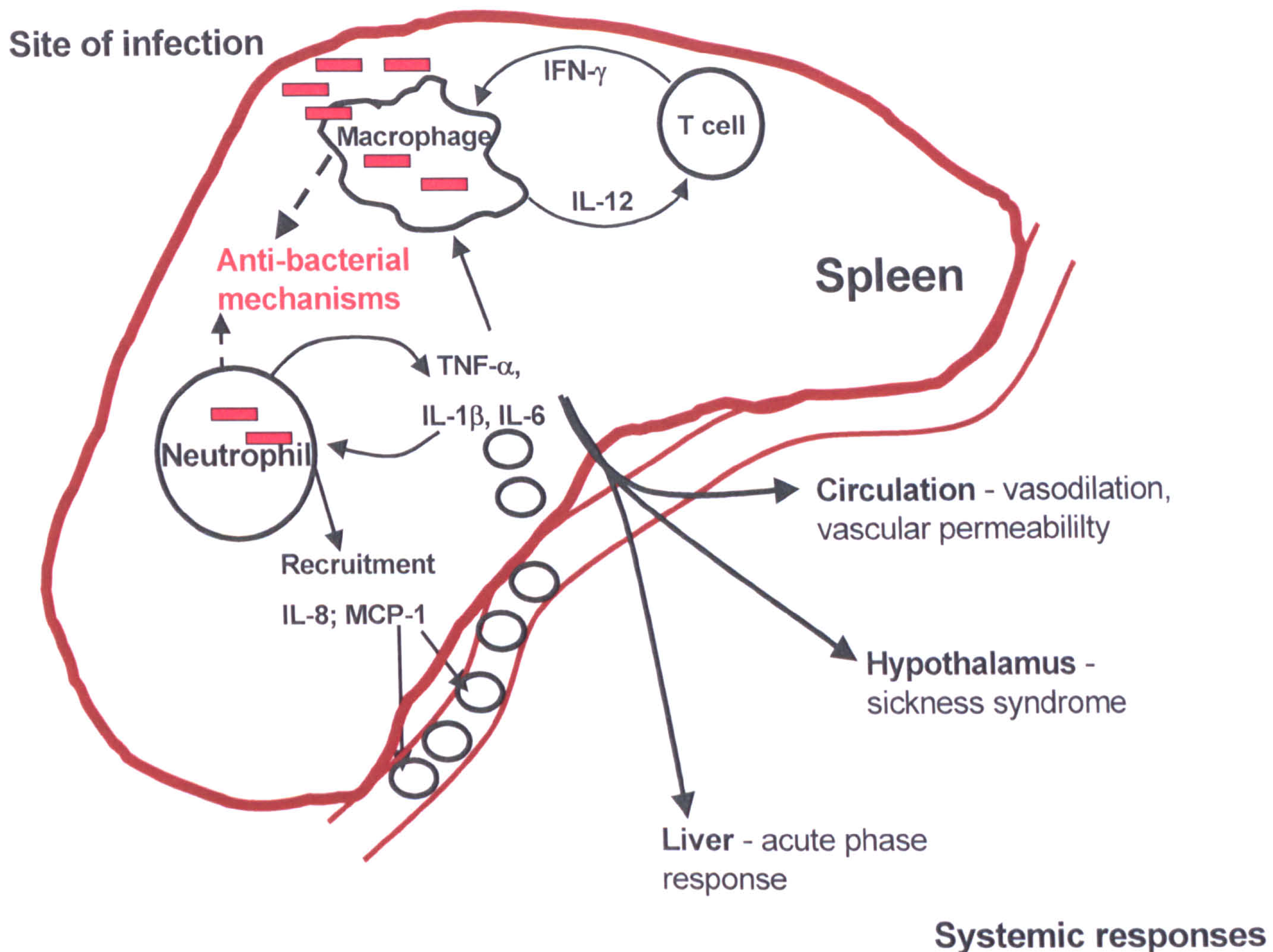


Figure 1.3: Representation of the inflammatory response following bacterial infection of the spleen. Macrophages and neutrophils are activated by bacteria and their components and release a range of cytokines in response. Release of pro-inflammatory cytokines such as TNFα, IL-1β, IL-6 and IFN-γ causes local induction of anti-bacterial mechanisms by innate immune cells. Systemic effects on the hypothalamus leads to the development of symptoms associated with infection and effects on the liver increase protein synthesis to stimulate host defences. Release of chemokines (IL-8, MCP-1) recruit neutrophils and macrophages into the site of infection aided by increased blood flow and vascular permeability to allow cellular influx.

1.2.2.2 Macrophages

Macrophages are phagocytic cells which reside in the tissues and mature from blood-borne monocytes which continually migrate into the tissues. During infection, monocyte migration and macrophage maturation occurs to enable further bacterial killing (Gonzalez-Juarrero *et al*, 2003). Resting macrophages express a range of TLRs enabling them to respond rapidly to infection and are further activated by interferon-γ (IFNγ) released by T cell subsets and NK cells during infection (Berg *et al*, 2005). Macrophages engulf pathogens by phagocytosis using cell surface scavenger receptors eg. MARCO or receptors for complement components including C3 which, when bound to bacteria eg. *F. tularensis*, enhance uptake (Clemens *et al*, 2005). Macrophages kill bacteria by a number of intracellular mechanisms including degradation by lysosomal enzymes, release of toxic reactive oxygen and nitrogen species (Farrar & Schreiber, 1993) and apoptosis of infected macrophages (Sousa-Franco *et al*, 2005; Ulett *et al*, 2005). Prior to their activation during infection, resting macrophages may be infected by bacteria including *M. tuberculosis* (Leemans *et al*, 2005) and *B. pseudomallei* (Utaisincharoen *et al*, 2001). These bacteria are able to survive, multiply and propagate the infection leading to a complex interplay between host cells and the infective pathogen.

1.2.2.3 Dendritic cells

Dendritic cells are important in initiation of innate and adaptive responses due to their efficient recognition of pathogens and constitutive expression of markers involved in antigen presentation. DCs are a heterogeneous family of cell populations distinguished by their differential expression of cell surface markers. These include the differential expression of TLRs on different subsets of DCs allowing them to respond appropriately to PAMPs (Sousa *et al*, 2004). Tissue-resident DCs sample their environment continually until they recognise PAMPs via PRRs (Foti *et al*, 2004). DCs then migrate to the lymph

nodes where they are able to present processed antigen to T cells leading to the development of adaptive immune responses. MHCII and co-stimulatory molecules, including CD80 and CD86, are expressed on the cell surface to allow efficient antigen presentation to T cells (Warncke *et al*, 2005). Activated DCs secrete pro-inflammatory cytokines involved in the induction of Type 1 responses including IL-12, IFN-γ and IFN-α, depending on the DC subset involved (Hochrein *et al*, 2001). DCs can also stimulate the development of type 2 responses important for fighting extracellular pathogens (Boonstra *et al*, 2003). In addition to their role in instigating adaptive immune responses, DCs also play an integral role in controlling innate immune responses in a reciprocal relationship with NK cells.

1.2.2.4 Natural Killer (NK) cells

NK cells are lymphocytes traditionally known for cytolytic killing of infected host cells or tumour cells. NK cells play an important role directly in innate responses to infection and by influencing the development of appropriate adaptive immune responses. The cytolytic activity of NK cells is enhanced by IFN-α production by DCs (Gerosa *et al*, 2005) and DCs also stimulate NK cells through secretion of IL-2. In turn, NK cells induce the maturation of DCs thus stimulating the development of type 1 immunity to intracellular pathogens (Mailliard *et al*, 2003). Human NK cells express a range of TLRs involved in recognition of pathogens early in the innate immune response leading to their activation (Hart *et al*, 2005). Following their activation, NK cells secrete cytokines, e.g. IFN-γ and TNF-α, in a non-antigen-specific manner (Zingoni *et al*, 2005). NK cells are a major source of innate IFN-γ during infection with pathogens such as *B. pseudomallei* (Lertmemongkolchai *et al*, 2001; Haque *et al*, 2005) and *F. tularensis* (Lopez *et al*, 2004). NK cells also secrete IFN–γ in response to IL-12 and IL-18 (Baratin *et al*, 2005). This allows promotion of Type 1 cell-mediated immune responses and IFN-γ dependent activation of macrophages.

1.2.2.5 *T cells*

T cells are lymphocytes predominantly associated with the development of adaptive immunity although certain T cell subsets have important roles during innate responses to infection. $CD8^+$ T cells, expressing markers characteristic of memory T cells, can secrete IFN-γ non-specifically in response to cytokines early in infection (eg. IL-12 and IL-18) and are involved in protection against infection with *L. monocytogenes* (Berg *et al*, 2005) and *B. pseudomallei* (Haque *et al*, 2005). Other T cell populations involved in innate immune responses include γδ T cells and NKT cells which appear to have pathogen-specific roles in immunity to infection (Kawakami, 2004).

Specific effector and memory T cell subsets are generated following presentation of antigen to naïve T cells with the aid of co-stimulatory molecules on the cell surface. $CD4^+$ T cells recognise peptide associated with MHCII molecules on the surface of antigen presenting cells (Neild *et al*, 2005). Appropriate polarising conditions allow the differentiation of naïve T cells into Th1 (IFN-γ; IL-12) or Th2 (IL-4; IL-5) subsets with specific effector functions (Abbas *et al*, 1996). $CD4^+$ Th1 cells activate macrophage responses through IFN-γ secretion (Murray, 1990) and also induce production of opsonising antibody by B cells enabling targeted uptake of pathogens (e.g. *Candida albicans*) into phagocytic cells (Kozel *et al*, 2004). $CD4^+$ Th2 cells stimulate B cells to generate antigen-specific antibody responses important in protective immunity to extracellular pathogens (Coffman *et al*, 1988). Effector $CD8^+$ T cells can also differentiate into Tc1 and Tc2 subsets that secrete cytokines and are cytotoxic for infected host cells that are presenting peptide associated with MHC I molecules (Li *et al*, 1997).

1.2.3 The Inflammatory response: Inflammatory mediators

Binding of bacterial components to cellular receptors triggers the expression of immune response genes leading to the production of inflammatory mediators which are released in order to propagate and control host defences.

1.2.3.1 Pro-inflammatory mediators

A range of mediators which stimulate inflammatory responses are released during infection. Pro-inflammatory cytokines are released by activated neutrophils, monocytes, DCs and macrophages (Zhan & Cheers, 1995) in response to recognition of bacterial components early in infection. Cytokines, including Tumour Necrosis Factor (TNF)α, Interleukin (IL)-1β and IL-6, act locally by activating cellular responses and have diverse roles in protection against infection with pathogens including *M. tuberculosis* (Mohan *et al*, 2001) and *B. pseudomallei* (Santanirand *et al*, 1999).

Systemic release of TNFα, IL-1β and IL-6 leads to the transcription of immune response genes in the liver causing an acute phase response (Deutschman, 1998). This involves the production of proteins such as LPS-binding protein (LBP), fibrinogen, C3, serum amyloid P (van der Poll *et al*, 1996) and affects blood coagulation cascades (Esmon *et al*, 1999). These acute phase proteins can bind to bacterial molecules and activate complement or phagocytosis (Oberholzer *et al*, 2001). For example, LBP enhances the binding of LPS or whole bacteria to CD14 on monocytes or macrophages propagating inflammatory responses (Stelter, 1998) and thus plays an important role in host inflammation (Heinrich *et al*, 2001). These cytokines also act on the hypothalamus and cause the release of prostaglandins leading to fever, pyrexia and behavioural changes known as "sickness syndrome" (Oberholzer *et al*, 2001).

Chemokines, including IL-8 and CCL-2 alternatively known as macrophage chemoattractant protein (MCP)-1, also propagate inflammatory responses by recruiting neutrophils, monocytes and macrophages to the site of inflammation. This allows bacterial killing and repair at the site of infection. Colony stimulating factors, including macrophage-colony stimulating factor (M-CSF), granulocyte-CSF (G-CSF) and granulocyte/macrophage-CSF (GM-CSF), stimulate the development and proliferation of phagocytic cells eg. macrophages and neutrophils (Rollins, 1997; Beishuizen *et al*, 1999).

1.2.3.2 Anti-inflammatory mediators

Anti-inflammatory cytokines, including IL-10, IL-4 and TGF-β, are also secreted during the inflammatory process and act antagonistically to control pro-inflammatory processes, preventing tissue damage by mediators released during inflammation (van der Poll, 1999). Interleukin-10 (IL-10) is an anti-inflammatory cytokine released by T cells, B cells, monocytes and macrophages following activation with bacterial products that downregulates inflammatory immune responses by inhibiting macrophage functions (Beishuizen *et al*, 1999). It causes the upregulation of anti-inflammatory molecules by increasing Suppresser Of Cytokine Signalling (SOCS)-3 expression. SOCS-3 is a cytoplasmic protein that negatively regulates responses to proinflammatory cytokines and bacterial products (Hanada & Yoshimura, 2002). Other natural anti-inflammatory molecules exist in the serum and act by mopping up specific inflammatory mediators, preventing their action on target cells. Soluble CD14 binds to LPS in the circulation preventing it from activating cells expressing the CD14/TLR4 complex (Stelter, 1998) and soluble TNFα Receptors bind excess TNFα to prevent further propagation of inflammatory responses (Goetz *et al*, 2002). Release of soluble IL-1β binding protein,

inhibits the binding of IL-1β to its receptor and is modulated by IL-10, thus negatively regulating inflammatory responses (Crepaldi *et al,* 2002).

1.2.3 Cell migration during inflammation

The inflammatory response is dependent on cell trafficking from the blood into the tissues in order to overcome the infection. This requires a complex series of events involving cell recruitment by chemokines and changes to the local vascular system and cell surface receptors. Nitric Oxide synthetase (iNOS) is induced on endothelial blood vessels by proinflammatory cytokines and bacterial components (eg. LPS) (Ciornei *et al,* 2002) leading to synthesis of NO. This results in vasodilation of blood vessels, increasing blood flow and cellular infiltration into infected areas. The vascular permeability of blood vessels also increases in response to proinflammatory cytokines allowing cells to move from the blood into the tissues (Panas *et al,* 1998). Adhesion molecules on the surface of cells are upregulated to allow neutrophil, monocyte and T cell infiltration into tissues. Intercellular Adhesion Molecules (ICAMs) in particular ICAM-1 (CD54) are upregulated on macrophages, neutrophils (Wang *et al,* 1998) and endothelial cells during inflammation in response to TNF-α (Krakauer, 2000). CD54 is important in neutrophil infiltration and when expressed on endothelial cells interacts with Mac-1 (CD11b) on the surface of neutrophils and macrophages (Kishimoto *et al,* 1989). CD11b, a receptor for the C3a component of the complement system (CR3), is upregulated following neutrophil activation with bacterial lipoprotein (Power *et al,* 2001). Both CD54 and CD11b are important in neutrophil recruitment and accumulation at sites of inflammation (Goncalves & Appelberg, 2000). L-selectin (CD62L) is also involved in neutrophil and mononuclear leukocyte infiltration into areas of inflammation through the vascular endothelium (Pizcueta & Luscinskas, 1994). However, CD62L is shed once neutrophils and other leukocytes have entered into tissues possibly to prevent them from escaping from areas of inflammation (Kishimoto *et al,* 1989).

1.2.5 Dysregulation of inflammatory responses: sepsis

Regulation of immune responses is crucial to provide a balance between pathogen killing and damage to the host. Inflammatory responses provide an environment suitable for the destruction of bacteria that is beneficial to the host. However, in the presence of severe infection or in immunocompromised individuals a massive, unregulated systemic inflammatory response can occur with the overproduction and release of potentially damaging inflammatory mediators. This systemic inflammatory response syndrome (SIRS) is known as sepsis following bacterial infection (Bone, 1993). During sepsis, dysregulation of both pro- and anti-inflammatory processes occurs leading to serious tissue damage and is of clinical relevance in intensive care units (ICU) as a major cause of death and morbidity each year (Bone, 1993).

Dysregulation of the pro-inflammatory response leads to massive overproduction of inflammatory mediators eg. TNFα, IL1β and IL-6. High levels of TNFα (Supputtamongkol *et al*, 1992; Terregino *et al*, 2000; Gogos *et al*, 2000), IL-1β (Berner *et al*, 1998) and IL-6 (Gallagher *et al*, 2001; Terregino *et al*, 2000; Groeneveld *et al*, 2001) are implicated in poor outcome and severity of sepsis in man. Overproduction of these inflammatory mediators can cause symptoms which contribute to the development of sepsis. Hypovolaemia, low blood volume, commonly occurs during sepsis and develops as a result of systemic vasodilation of blood vessels. The continual production of nitric oxide by endothelial cells following unregulated TNFα production also contributes by increasing vascular permeability (Fujii *et al*, 2000). This overproduction of nitric oxide, TNF-α and IL-1β may also decrease myocardial contraction (Panas *et al*, 1998; Kumar *et al*, 1996) leading to hypotension. Increased expression of adhesion molecules (eg. CD54) on vascular endothelium (mediated by TNFα) leads to an increase in adherence of leukocytes to endothelial cells (Zeng *et al*, 2002). Coagulation cascades, also induced by TNFα, cause

the release of pro-coagulant factors such as thromboxane and inhibition of anti-coagulant mechanisms (Esmon *et al*, 1999). This can lead to micro-vascular thrombi restricting organ blood perfusion and contributing to organ failure. Neutrophils are also implicated in organ pathogenesis and damage by the uncontrolled release of large amounts of necrotic inflammatory products including proteinases and free radicals causing widespread cell death and tissue necrosis. Increased production of prostaglandins mediated by TNFα, IL-1β and IL-6 production leads to fever, rigors, anorexia, hyper metabolism and altered metabolism of fat and glucose (Davies & Hagen, 1997).

Unregulated production of pro-inflammatory molecules can be accompanied by overproduction of anti-inflammatory compounds to downregulate the pro-inflammatory response known as compensatory anti-inflammatory response syndrome (CARS). This can lead to macrophage and monocyte deactivation by increasing serum levels of soluble CD14 (Gluck *et al*, 2001), reduction of monocyte membrane-bound CD14 and decreased TNFα production (de Werra *et al*, 2001). High serum levels of IL-10 and a high IL-10:TNFα ratio are indicators of severe sepsis and poor outcome (Gogos *et al*, 2000). Abnormalities and reductions in neutrophil function (Rothe *et al*, 1990; Terregino *et al*, 1997) may also be present, leading to immunosuppression, and allowing secondary infections to develop (Steinhauser *et al*, 1999a).

1.3 Development of immune responses during intracellular infection

Intracellular bacteria are pathogens that grow and replicate within host cells enabling them to survive and colonise the host organism. In addition to causing acute infections, chronic or latent infection may develop which can reactivate after several years following initial infection with pathogens such as *M. tuberculosis* and *B. pseudomallei.*

Immune responses targeting intracellular pathogens are generally characterised as type 1 immunity. Polarisation of the immune response towards type 1 immunity during these early innate responses ensures the development of appropriate, antigen-specific, memory responses. In general, type 1 responses are characterised by the induction, production, and interaction of IFN-γ and other cytokines including IL-12, IL-18 and IL-27, with macrophages, NK cells and T cells allowing the destruction of sequestered intracellular bacteria (Figure 1.4). This is in contrast to type 2 responses which target the extracellular stages of infection by production of antibody to neutralise or opsonise extracellular pathogens and toxins. Type 2 responses are generally mediated by IL-4 and other cytokines including IL-5, IL-6 and IL-10 are involved in propagation of these responses (Mosmann & Coffman, 1989).

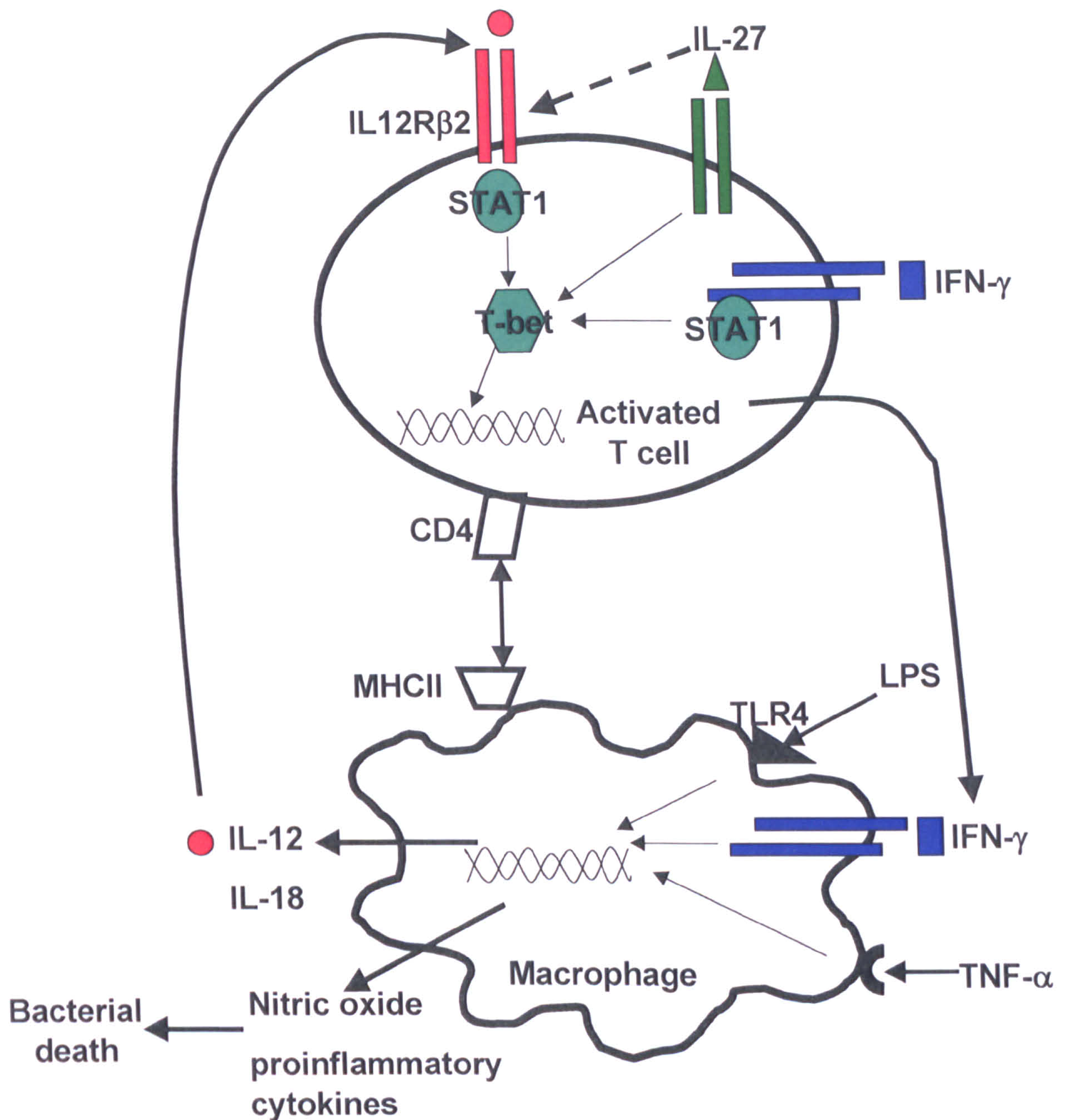


Figure 1.4: Representation of the interactions occurring between cytokines in type 1 immune responses during intracellular infection. Following infection, macrophages and other innate cells are activated to produce type 1 cytokines IL-12 and IL-18 by bacterial components e.g. lipopolysaccharide (LPS) or pro-inflammatory cytokines eg. TNF-α. IL-12 acts on activated T cells expressing IL-12Rβ2, whose expression is controlled by IL-27 and T-bet (type 1 transcription factor), leading to IFN-γ production via signal transducing activator of transcription (STAT)-1 signalling. IFN-γ further activates macrophages to express IFN-γ inducible genes leading to nitric oxide production, anti-bacterial mechanisms and upregulation of MHCII leading to presentation of antigen to T cells and the development of adaptive type 1 immunity.

1.3.1 Interferon-γ

IFN-γ is essential in protection against a number of intracellular pathogens including *B. pseudomallei* (Santanirand *et al*, 1999), *Leishmania major* (Wang *et al*, 1994), *Mycobacterium tuberculosis* (Shams *et al*, 2001) and *Listeria monocytogenes* (Portnoy *et al*, 1989). The importance of host resistance to *B. pseudomallei* was demonstrated following neutralisation of IFNγ with anti-IFN-γ antibodies during infection in BALB/c mice which caused acute sepsis and death (Santanirand *et al*, 1999). Studies with IFN-γ gene knockout mice have also demonstrated the importance of IFN-γ in protection against infection with *Bacille Calmette Guerin* (BCG) (Dalton *et al*, 1993), *L. major* (Wang *et al*, 1994) and *M. tuberculosis* (Cooper *et al*, 1993).

IFN-γ is secreted by activated type 1 T cells and NK cells in response to infection. During the early stages of infection with *B. pseudomallei*, NK cells and memory $CD8^+$ $CD44^{hi}$ T cells are stimulated to produce IFN-γ by cytokines in a non-specific manner in a 'bystander effect' (Lertmemongkolchai *et al*, 2001). This propagates the development of an IFN-γ-dependent immune response. IFN-γ acts on T cells and other cells expressing a functional IFN-γ-receptor (IFN-γ-R) (Th1 cells releasing IFN-γ do not express a functional receptor) and induces signal transducer and activator of transcription (STAT)–1 and IFN-γ dependent gene expression in Th2 cell subsets (Tau *et al*, 2000). This appears to have anti-proliferative and anti-metabolic effects on these subsets and downregulates inappropriate IL-4-mediated responses. This allows the proliferation of IFN-γ-secreting T cell subsets to develop a protective type 1 immune response to intracellular pathogens (Wang *et al*, 1994; Tau *et al*, 2000).

IFN-γ induces a range of IFN-γ-inducible genes following engagement with its receptor on macrophages and neutrophils which in turn stimulates anti-bacterial mechanisms and enhances non-specific bacterial killing of intracellular pathogens. These genes include inducible nitric oxide synthase (iNOS) leading to production of nitric oxide, P47 GTPases implicated in host defence against intracellular bacteria (Taylor *et al*, 2004) and the Th1 differentiation transcription factor T-bet, (Szabo *et al*, 2000). Cellular receptors including MHC II involved in antigen presentation (Dalton *et al*, 1993) and Fc Receptors involved in phagocytosis are also upregulated in response to IFN-γ.

IFN-γ is essential in inducing macrophage bactericidal mechanisms during infection with *M. tuberculosis* (Cooper *et al*, 1993), *B. pseudomallei* (Utaisincharoen *et al*, 2001) and *L. monocytogenes* (Portnoy *et al*, 1989). Macrophages engulf bacteria and cause death by a number of anti-microbial mechanisms including the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Miyagi *et al*, 1997). Nitric oxide (NO)-mediated killing of BCG infection in mice by macrophages is IFN-γ-dependent although production of reactive oxygen intermediates (ROIs) is only partially dependent on IFN-γ (Dalton *et al*, 1993). Macrophage-mediated killing of *B. pseudomallei* is also IFNγ-dependent. In the absence of IFN-γ, . *B. pseudomallei* is able to multiply within macrophages without increased expression of inducible nitric oxide synthetase (iNOS) (Utaisincharoen *et al*, 2001) and survives the respiratory burst in human phagocytes *in vitro* (Pruksachartvuthi *et al*, 1990). In the presence of IFN-γ, macrophages kill internalised *B. pseudomallei in vitro* using an iNOS-dependent pathway (Utaisincharoen *et al*, 2001), utilising both ROS and RNS, although RNS plays a greater role in bacterial death (Miyagi *et al*, 1997). IFN-γ also aids bacterial killing by preventing movement of intracellular bacteria eg. *L. monocytogenes* from the phagolysosome into the cytoplasm thus preventing cell-to-cell spread of the bacteria via the cytoplasm of adjacent cells (Portnoy *et al*, 1989).

IFN-γ also acts on NK cells to increase the cytolytic capability of these cells (Dalton *et al*, 1993). Control of IFN-γ production is crucial in regulating cell-mediated responses to intracellular infection and may be mediated by a number of cytokines during infection including IL-12, IL-18 and IL-27 (Robinson & O'Garra, 2002).

1.3.2 Type 1 inducing cytokines

Cytokines which induce type 1 responses directly or via inducing IFN-γ production include IL-12, IL-18 and the IL-12 related cytokines IL-23 and IL-27 (Figure1.4).

1.3.2.1 IL-12 and IL-23

Interleukin-12 (IL-12), a pro-inflammatory cytokine, is a heterodimer composed of IL-12p35 and IL-12p40 subunits. It is released by monocytes, macrophages and B cells during early infection following recognition of bacterial components via TLRs. IL-12 is also released by macrophages following activation by inflammatory cytokines eg. TNF-α which activates macrophages during early infection with *M. avium* to produce IL-12 and IL-18 (Danelishvilli & Bernudez, 2003). IL-12 is pivotal in polarising type 1 immune responses and ensuring Th1 cell differentiation. Engagement of IL-12 with the IL-12R causes activation of signalling pathways including STAT-1 which ensures the expression of T-bet and development of type 1 responses. IL-12 interacts with the IL-12R on NK cells and activated T cells inducing production of IFN-γ thus propagating macrophage activation and type 1 responses (Gazzinelli *et al*, 1994; Flesch *et al*, 1995). IL-12 is important in protection against infection with the intracellular pathogens *L. major* (Heinzel & Rerko, 1999) and *B. pseudomallei* (Lertmemongkolchai *et al*, 2001) by augmenting type 1 responses and IFN-γ production thus stimulating the development of Th1-mediated immune responses (Heinzel & Rerko, 1999).

The IL-12p40 subunit of IL-12 is shared with IL-23, another closely related cytokine. IL12p40 combines with an IL-12p35 related subunit p19 to form IL-23, a heterodimer similar to IL-12. Although IL-12 and IL-23 have similar structures, IL-23 has distinct roles in maintenance of Type 1 responses and acts on $CD4^+$ memory T cells to induce IFN-γ production whereas IL-12 exerts its effects on naïve T cells (Lankford & Frucht, 2003). IL-23 is important in immunity to intracellular infection which has been shown indirectly by comparing the susceptibility of IL12p35-/- and IL12p40-/- knockout mice to *Francisella tularensis* (Elkins *et al*, 2002).

1.3.2.2 IL-18

In addition to its independent role in Type 1 responses, IL-12 works in synergy with another cytokine, IL-18 which is produced by macrophages and DCs and acts on NK cells and T cells. An IL-12/IL-18 synergy is involved in the induction of IFN-γ during infection with a number of pathogens including *B. pseudomallei* (Lertmemongkolchai *et al*, 2001), *L. monocytogenes* (Neighbors *et al*, 2001) and *Mycobacterium leprae* (Garcia *et al*, 1999). IL-18 also has an essential role in host defence during *L. monocytogenes* infection in the absence of IL-12, through IFN-γ-independent induction of NO and TNF-α from macrophages (Neighbors *et al*, 2001). IL-18 plays an IL-12-independent regulatory role early in host defences during *Streptococcus pneumoniae* infection (Lauw *et al*, 2002) and is critical in host resistance to *L. major* infection playing a role in regulation of Th1/Th2 balance not mediated by IL-12 (Wei *et al*, 1999).

1.3.2.3 IL-27

Although IL-12 is important in polarisation of Type 1 responses, other cytokines are also involved as Th1 polarisation occurs in the absence of both IL-12 subunits and IL-12 receptor subunits during mouse knockout studies (Lankford & Frucht, 2003). IL-27 is a

cytokine structurally related to the IL-12 family of cytokines. IL-27 is independently involved in the early development of Th1 cells by inducing expression of T-bet. It is also involved in commitment to type 1 immunity by upregulating the IL-12Rβ2 on T cells, thus increasing and maintaining the responsiveness of T cells to IL-12 (Lucas *et al*, 2003; Hibbert *et al*, 2003). Initial studies demonstrated a role for IL-27 in IFN-γ production from naïve $CD4^+$ T cells *in vitro* (Pflanz *et al*, 2002). This finding was corroborated *in vivo* in a model of *L. major* infection where WSX-1 (IL-27 Receptor) knockout mice had impaired IFN-γ production and consequently increased susceptibility to infection (Yoshida *et al*, 2001). However, other *in vitro* studies (Lucas *et al*, 2003) and studies with other intracellular pathogens eg. *Toxoplasma gondii*, suggest that IL-27 is not required for the development of IFN-γ mediated immunity but is important in controlling the kinetics of Th1 responses later in infection (Villarino *et al*, 2003). The discrepancy between the requirement for IL-27 in development of IFN-γ mediated immunity appears to be due to the production of IL-4 during infection. High levels of IL-4 are produced early during *L. major* infection and in these circumstances IL-27 appears to be important in IFN-γ production. However, in infections with low IL-4 involvement (eg. *T. gondii*, *T. cruzii*) IL-27 is not important during the developmental phases of a Th1 response (Artis *et al*, 2004).

1.4 Aims of this research

The objective of this research is to characterise early host immune responses to intraperitoneal infection of BALB/c mice with *Burkholderia mallei.* This will be achieved by:

- Describing an intraperitoneal model of *B. mallei* infection by investigating bacterial colonisation of the spleen and peripheral effects by monitoring body temperature and signs of infection.
- Investigating the effect of *B. mallei,* in this intraperitoneal model of infection, on host neutrophil, macrophage T and B cell cellular responses by measuring the infiltration and activation profiles of these cells during infection. In addition to this, splenic and systemic type 1 and proinflammatory cytokine host responses will be measured during infection to provide a comparison with cytokine responses important during infection with other intracellular pathogens.
- Investigating the effectiveness of host neutrophil and T cell responses and the responses of the type 1 cytokine IFN-γ and proinflammatory cytokine TNF-α in protecting against *B. mallei* infection.
- Investigating the role of type 1 cytokines, upregulated early during *B. mallei* infection, in induction of IFN-γ *in vitro* in response to heat-killed *B. mallei*; and to investigate the role of T cells, NK cells and neutrophils (and other Gr-1$^+$ cells) in IFN-γ production *in vitro* in response to heat-killed *B. mallei.* This will provide a comparison with IFN-γ responses induced by other intracellular pathogens and determine whether a link exists between IFN-γ and Gr-1$^+$ cells, both important in protection during the early phases of *B. mallei* infection.

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 General Reagents

Distilled water and Dulbecco's phosphate buffered saline (D-PBS) were purchased from Gibco™, Invitrogen Corporation, UK. Sterile syringes (2 ml), nylon cell strainers, 15ml and 50ml conical Falcon tubes with caps and 1 ml insulin syringes were purchased from Becton Dickinson, UK. Sterile, flat-bottomed 96-well, 24-well and 6-well plates were purchased from Corning Incorporated. Foetal calf serum (FCS), RPMI 1640, L-glutamine (with penicillin and streptomycin), Trypan blue (0.4%), Red Blood Cell Lysis Buffer and *Salmonella typhimurium* lipopolysaccharide (LPS) were purchased from Sigma, Poole, UK. Nutrient broth and Congo Red agar plates for bacteriological analysis were made at, and ethanol was supplied by, Dstl, Porton Down. Paraformaldehyde (24%) was purchased from Pioneer Research Chemicals Ltd and O-ring sealed tubes were purchased from Sarstedt, UK. J774A.1 macrophage cell line was purchased from the European Cell And Culture Collection (ECACC), Health Protection Agency, Salisbury, UK.

2.1.2 Flow cytometry reagents

Fluorochrome labelled antibodies and isotype controls were purchased from Becton Dickinson, UK, Beckman Coulter, UK and Serotec, UK. OptiLyse™ C and Flow count™ beads were purchased from Beckman Coulter, UK. Flow cytometry tubes and Mouse Inflammation cytometric bead array™ (CBA) kits were purchased from Becton Dickinson, UK. For intracelullar staining, Leucoperm™ reagents were purchased from Serotec, UK and Brefeldin A (Golgi stop™) was purchased from Becton Dickinson, UK.

2.1.3 RNA extraction and removal of DNA contamination

Chloroform, isopropanol (>99%), molecular biology grade absolute ethanol (>99% purity), diethyl-pyruvate carbonate (DEPC) and RNA sample loading buffer were purchased from Sigma, Poole, UK. RNAseZap®, RNAse-free microfuge (1.5 ml) tubes, mouse spleen total

RNA from adult BALB/c mice and RNA millenium™ size markers were purchased from Ambion, UK. Removal of DNA was performed using DNA-free™ Kit (Ambion, UK). Nuclease-free water was purchased from Promega, UK, Trizol™ from Invitrogen, UK and micropestles and spectrophotometry cuvettes from Eppendorf, UK.

2.1.4. Gene Amp PCR system 9700

The Gene Amp PCR system 9700 machine (Perkin Elmer, UK) was used for reverse transcription of RNA using the Omniscript™ kit (Qiagen, UK). Random hexamers and recombinant RNAsin® ribonuclease inhibitor were purchased from Promega, UK. The Hotstart™ kit (Qiagen, UK) was used to perform PCR reactions to assess the effectiveness of DNA-free treatment on extracted RNA samples and to test RNA extraction methods. Primers used in this system were synthesised by MWG-biotech, UK. Thin wall-tube strip (200µl) PCR reaction tubes and thin wall-cap strips were purchased from BioRad, UK. High molecular weight DNA from BALB/c mouse spleen (Sigma, Poole, UK), DNA molecular weight markers (0.019-1.11 kbp) (Roche) and DNA loading buffer containing bromophenol blue (0.25%) and sucrose (40%) (Dstl, Porton Down) were also used.

2.1.5 Taqman 7000 PCR system

The TaqMan® 7000 PCR machine (Applied Biosystems, UK) was used to examine gene expression in experimental cDNA samples. Pre-Developed TaqMan® Assay Reagents (PDAR), Assay-on-demand primers and probes, TaqMan® Universal PCR master mix and optical 96 well reaction plates and optical caps and covers were purchased from Applied Biosystems, UK.

2.1.6 Agarose Gel Electrophoresis

Agarose powder and MOPS (Morpholinopropane Sulphonic acid) buffer purchased from Sigma, Poole, UK were used for making and running 1% agarose gels for RNA analysis.

Agarose (1.2% and 2%) E-gels™ for analysis of DNA and E-gel™ bases were purchased from Invitrogen, UK. All gels were run using a Biorad power pack 300 and gel products were detected by UV light using a Gel Doc™ system.

2.1.7 Depleting Antibodies and cytokines

Antibodies used during *in vivo* depletion studies were supplied by Cymbus Biotechnology, UK (Table 2.1). Antibodies were adjusted to the appropriate concentration by dilution with D-PBS under sterile conditions. Anti-IL18R antibodies (Clone: TC30-28E3) used during *in vitro* depletion studies were a kind gift from Anne O'Garra, NIMR, London, UK, [originally made at DNAX research Institute, Palo Alto, CA, USA (Neighbours *et al*, 2001)]. Recombinant murine IL-6, TNF-α, IL-12 and IL-18 were purchased from R&D Systems, UK.

2.1.8 Mice

Female BALB/c mice purchased from Charles River Ltd, UK aged 6-8 weeks, were used for *in vivo* experiments involving infection or treatment with antibodies. Eight to 14 week-old female IFN-γ (BALB/c) knockout (KO), C57BL/6 wild type, IFN-γ (C57BL/6) KO mice, IL12p35 (C57BL/6) KO and IL12p40 (C57BL/6) KO mice were obtained from the London School of Hygiene and Tropical Medicine (LSHTM, UK). WSX-1 (IL-27R) (C57BL/6) KO mice, C57BL/6 wild type and IL-6 (C57BL/6) KO mice were supplied and processed by Alison Bancroft at the University of Manchester, UK for *in vitro* stimulation studies with heat-killed *B. mallei*.

Antibody clone	Target	Isotype
RB6-8C5	Murine Gr-1 (Neutrophils)	IgG2b
MP6-XT22	Murine TNF-α	IgG1
YTS 169	Murine CD8	IgG2b
YTS 191	Murine CD4	IgG2b
Mac5 (control)	Chlamydomonas	IgG2b
Mac49 (control)	Avena sativa	IgG1

Table 2.1: Antibody clone, antibody target and antibody isotype for monoclonal antibodies used during depletion experiments. IgG: immunoglobulin G.

2.2 Methods

2.2.1 Summary of experiments to characterise host immune responses during intraperitoneal B. mallei infection

In order to characterise the host immune responses to an intraperitoneal (i.p.) model of *B. mallei* infection three experiments were performed in BALB/c mice to:

1) Monitor temperature and signs of disease non-invasively;

2) Analyse bacterial colonisation and host immune responses to infection by investigating cellular and cytokine responses in a 36-day time course experiment (Immune Analysis (1));

3) Analyse bacterial colonisation and improve and modify analysis of cellular and cytokine responses over the first 7 days of infection performed during Immune Analysis 1 (Immune Analysis (2)).

Splenic colonisation, cellular responses, splenic cytokine expression and systemic serum cytokine production were investigated during both immune analysis experiments. During Immune Analysis (2), investigation of cellular and cytokine responses 0-7 days p.i. performed in Immune Analysis (1) were repeated. An additional time-point 5 hours p.i. was undertaken to describe early immune events and higher animal numbers (n=8) were investigated in order to reduce variation in bacterial counts observed during Immune Analysis (1). Modifications to flow cytometric methods were also performed to improve analysis of cellular responses. Data retrieved over the 7 days of infection from the second immune analysis experiment is presented in detail in chapters 4-6. Observations of trends in cellular and cytokine responses and their relationship to bacterial colonisation from days 14 to 36 from Immune Analysis (1) were made to determine the progression of disease following abscess formation and are discussed in chapters 4-6. A summary of the experiments performed are described in Table 2.2.

<table>
<tr><th>Experimental Aim</th><th>Duration</th><th>Infection (i.p.)</th><th>Sample</th><th>Method</th><th>Sampling time-points</th></tr>
<tr><td>Temperature & signs</td><td>70 day</td><td>10^6 cfu B. mallei</td><td>n=10 (non-invasive)</td><td>Temperature probe
Signs</td><td>Diurnally</td></tr>
<tr><td rowspan="3">Immune Analysis (1)</td><td rowspan="3">36 day</td><td rowspan="3">10^6 cfu B. mallei</td><td>(n=5) Spleen</td><td>Flow cytometry
Colonisation</td><td rowspan="3">Days 0,1,3,5,7,14,21, 28, 36</td></tr>
<tr><td>(n=5) Spleen</td><td>RT-PCR</td></tr>
<tr><td>Serum</td><td>Systemic cytokine</td></tr>
<tr><td rowspan="2">Immune Analysis (2)</td><td rowspan="2">7 day</td><td rowspan="2">10^6 cfu B. mallei</td><td>(n=8) Spleen</td><td>Flow cytometry
RT-PCR
Colonisation</td><td rowspan="2">Days 0, 0.25, 1,3,5,7</td></tr>
<tr><td>Serum</td><td>Systemic cytokine</td></tr>
</table>

Table 2.2: Summary of experiments performed to characterise the disease and host immune responses following i.p. infection with *B. mallei* (ATCC 23344).

2.2.2 *In vivo procedures*

All experimental procedures on live animals were carried out within the requirements of the Animals (Scientific Procedures) Act 1986. A Home Office project license was approved and obtained following a stringent Ethical Review process at Dstl, Porton Down.

2.2.2.1 *Animal Welfare*

Mice infected with *B. mallei* and control uninfected mice were housed in a rigid wall isolator within an ACDP animal containment level 3 facility in groups of 5 or 6 and were acclimatised for at least 5 days prior to experimental procedure. Mice housed at both containment level 2 and 3 were provided with fresh food and water daily and environmental enrichment (eg. nesting boxes) were present in each cage. All mice were monitored daily for signs of illness. A humane endpoint for *B. mallei* infection was defined when mice developed hind leg paralysis and hunching. Mice were sacrificed by cervical dislocation when they reached this humane endpoint, or prior to this, to provide samples for experimental analysis at predefined timepoints.

2.2.2.2 *Intraperitoneal injection*

For intraperitoneal infection with *B. mallei* (Chapters 3-6), mice were scruffed and held firmly, but gently, by a trained operator within a containment level 3 isolator. A further trained operator injected the mice intraperitoneally using a 1 ml insulin needle with approximately 10^6 *B. mallei* colony forming units (cfu) in 100 μl of nutrient broth. For treatment with depleting antibodies at containment level 2 (Chapters 4-6), mice were held firmly and injected intraperitoneally using a 1 ml insulin needle with less than 200μl volume by a trained individual.

2.2.2.3 Temperature monitoring and signs of disease

Mice used during temperature monitoring experiments (Chapter 3) were supplied with implanted micro-chips (Datamars, Switzerland) to allow the identification of individual mice using a microchip reader. Peripheral body temperature was monitored using a temperature probe placed in the fold under the back leg. Mice were gently scruffed and the back leg folded and held over the temperature probe. Temperature was recorded once a steady measurement was observed on the monitor of the probe. Signs of disease were recorded objectively twice daily (morning and afternoon) for each mouse. Control animals not challenged with *B. mallei* were also monitored for temperature and signs of disease in the same environmental conditions within the isolator.

2.2.3 Bacterial culture, sample extraction and storage

2.2.3.1 Burkholderia mallei

Work involving live *B. mallei* was carried out under Advisory Committee on Dangerous Pathogens (ACDP) containment level 3. *Burkholderia mallei* (strain ATCC 23344) was used for all challenge experiments. Frozen *B. mallei* stock cultures were defrosted and cultured for 48 h in nutrient broth at 37°C to give a bacterial concentration of approximately 10^8/ml. Cells were harvested by centrifugation and washed three times in PBS before resuspending in one tenth the original volume of PBS.

2.2.3.2 Heat inactivation of B. mallei

For *in vitro* stimulation assays, *B. mallei* (ATCC 23344) was inactivated by heat treatment in a water bath at 70°C for 3 h, with occasional gentle shaking. After inactivation each suspension was checked for viability by inoculating 10 ml volumes of nutrient broth with 0.5 ml aliquots of the suspension or 10% volume and incubating at 37°C for seven days.

Nutrient agar plates were then inoculated with the total volume of the broth cultures to check for bacterial growth, and incubated for a further seven days at 37°C. If no growth occurred on agar plates, inactivation of the bacterial suspension was assumed to have occurred.

2.2.3.3 Removal of blood samples

Mice were terminally anaesthetised by exposure to halothane vapour in a glass bell jar and blood was removed by cardiac puncture using 1 ml insulin needles and allowed to clot. Mice were then sacrificed by cervical dislocation. Blood was placed into O-ring sealed (1.5 ml) tubes and centrifuged (13000 rpm, 5 min). Serum supernatant was removed and placed into O-ring sealed (1.5 ml) tubes which were stored at –20°C until analysis by cytometric bead array. Spleens were removed under sterile conditions and were processed immediately following excision.

2.2.3.4 Preparation of spleens for bacteriological analysis, flow cytometry and RT-PCR

For bacteriological analysis and flow cytometry, spleen sections were transferred into 2 μm nylon cell strainers with in the wells of sterile 6 well plates. Spleens were passed through sieves into the well below using the pestle from a sterile syringe into 3 ml of D-PBS. For bacteriological analysis, 2 ml of splenic cell suspension were removed from each well, placed into 15 ml tubes and stored on ice. For flow cytometric staining, 1 ml of D-PBS containing 5% FCS was added to the remaining 1 ml of spleen suspension in each well and flow cytometric staining was performed immediately. For RT-PCR analysis, Trizol™ (800 μl) was added to RNAse-free O-ring sealed tubes under sterile conditions. Spleen sections were added and broken down in Trizol™ using micropestles. Samples were stored at –20°C or –80°C for up to 8 months prior to RNA extraction.

2.2.4 *Flow cytometric analysis*

2.2.4.1 *Flow cytometric staining*

Aliquots of spleen suspension (100 µl) prepared for flow cytometric analysis were added to O-ring sealed tubes (Containment level 3) or flow cytometry tubes (Containment level 2) containing antibody stain combinations or isotype controls (Tables 2.3 & 2.4). Tubes were incubated in the dark for at least 20 min at room temperature (RT). OptiLyse C (400 µl) was added to each tube, mixed gently and then incubated for 15 min to allow erythrocyte lysis to occur. A 16% paraformaldehyde solution (diluted in PBS) (500 µl) was added to infected samples for cellular fixation and bacterial killing to give a final concentration of 8% paraformaldehyde. Samples were refrigerated for at least 24 h prior to analysis but for no more than one week. Samples were transferred into flow cytometry tubes immediately prior to acquisition. For non-infected samples, a 1% paraformaldehyde solution was used for fixation and samples were analysed immediately or within 48 h. Flow count™ beads (100 µl) were added to samples immediately prior to analysis to allow determination of absolute counts. Samples were analysed using an EPICS XL® flow cytometer (Beckman Coulter, UK).

2.2.4.2 *Flow cytometric analysis : Immune Analysis 1*

During an initial 36 day time course experiment (Immune Analysis 1), 5000 data events were collected within the lymphocyte, monocyte or granulocyte populations (Table 2.3). Samples were acquired uncompensated. Following acquisition, data was analysed using Winlist 4.0™ (Verity Software, Software House, Inc., USA) and compensation was performed using compensation controls acquired on the same day as sample acquisition. The positive population was determined from isotype controls and unstained samples appropriate for each stain (Table 2.3).

Cell Type	Fluorochrome		
	FITC	**PE**	**PE-Cy5**
Neutrophils	Neutrophil (7/4) (IgG2a)	CD54 (HIgG1)	
T Cells	CD3 (HIgG1κ)	CD54	
	CD3	CD25 (RIgG1λ)	

Table 2.3: Stains used for flow cytometric analysis of samples during Immune Analysis 1. Cell type lineage markers and cell surface activation markers were identified by specific antibodies conjugated to fluorochrome labels: FITC: Fluorescein Isothiocyanate; PE: Phycoerythrin; PE-Cy5: phycoerythrin-cychrome-5; CD: complimentarity determining. The isotypes of the antibodies are bracketed () next to the appropriate marker. H: hamster; R: rat; IgG: immunoglobulin G.

2.2.4.3 Flow cytometric analysis: Immune Analysis 2

In a further 7 day time course experiment (Immune Analysis 2), several amendments were made to the original protocol in order to enhance data capture. Several different markers were used in order to improve identification of lymphocyte, monocyte and neutrophil populations (Table 2.4). Data from samples investigating lymphocytes was collected for 60 secs and data from samples which identified monocyte or granulocyte populations was collected for 5 min. Samples were compensated during acquisition using compensation controls. Following acquisition, data was analysed using Winlist 4.0™. The positive population was determined from isotype controls and unstained samples appropriate for each stain (Table 2.4).

2.2.5 Bacteriological analysis

2.2.5.1 Bacterial counts

Spleen suspensions for bacteriological analysis were refrigerated for up to 48 hours prior to plating out onto Congo red agar. Nutrient broth media (900 µl) was aliquoted into 24 well plates. Neat spleen suspensions (100 µl) were added to nutrient broth for the initial dilution. From this dilution, 100 µl was removed and placed into the next well, mixed and doubling dilutions of spleen cell suspensions were made until an appropriate dilution series was completed. Spleen suspensions (250 µl) from the dilution series were spread onto Congo Red plates. Duplicate plates for each dilution sample were made. Plates were incubated for 48 h at 37°C and were counted immediately or refrigerated to stop bacterial growth and then counted. Colonies of *B. mallei* were identified and counted manually. Average bacterial counts on duplicate plates for each spleen were calculated. This method was also used to determine the number of bacterial counts injected into animals during challenge with *B. mallei*.

Cell Type	Fluorochrome		
	FITC	PE	PE-Cy5
Neutrophils	Gr-1 (IgG2bk)	CD54 (HIgG1)	CD69 (HIgG)
	Gr-1	CD62L (rIgG2aκ)	CD11b (rIgG2b)
Macrophages	F4/80 (rIgG2b)	CD54	CD11b
	F4/80	IA/IE (rIgG2aκ)	CD11b
B cells and NK cells	DX5 (CD49b) (rIgMk)	CD19	CD69
T Cells	CD8a (RIgG2aκ)	CD3 (HIgG1)	CD44 (RIgG2b)
	CD3 (HIgG1κ)	CD54	CD44
	CD3	CD4 (IgG2aκ)	CD44
	CD3	CD25 (RIgG1λ)	CD44

Table 2.4: Stains used for flow cytometric analysis of samples obtained during Immune Analysis 2. Cell type lineage markers and cell surface activation markers were identified by specific antibodies conjugated to fluorochrome labels: FITC: Fluorescein Isothiocyanate; PE: Phycoerythrin; PE-Cy5: phycoerythrin-cychrome-5; CD: complimentarity determining. The isotypes of the antibodies are bracketed () next to the appropriate marker. H: hamster; R: rat; IgG: immunoglobulin G.

2.2.5.2 Sterility Check

A sterility check was performed on spleen samples stored in Trizol™ for RT-PCR analysis prior to removal from containment level 3 isolation. From each of the spleen samples, 10% was removed and placed into nutrient broth (1 ml), shaken by hand and centrifuged (13,000 rpm, 15 min) to remove Trizol™ (which may inhibit bacterial growth). The remaining pellet was resuspended in nutrient broth (1ml) and added to a further 9 ml of nutrient broth. Samples were then incubated at 37°C for seven days. Following this, 250 µl were removed from the broth samples and plated onto Congo Red agar plates in duplicate. Plates were incubated for a further seven days and were then examined for bacterial growth. Plates with no bacterial growth were assumed to be sterile and were removed from containment level 3. Samples were then manipulated under ACDP containment level 2 conditions.

2.2.6 *Cytometric bead arrays*

Cytokine protein in serum and cell culture supernatants was measured using cytometric bead array™ (CBA) kits. Cytokine (10x) bulk standards were prepared according to manufacturer's instructions by reconstituting each vial of lyophilised standard with assay diluent (200 µl) provided in the kit. This was left to equilibrate for at least 15 minutes and then mixed thoroughly. The 10x bulk standard (100 µl) was then diluted in assay diluent (900 µl). The top standard (300 µl) was then added to assay diluent (300 µl) and mixed thoroughly. From this, 300 µl were removed and added to the next tube containing 300 µl of assay diluent and so on until a dilution range of 1:2 to 1:256 was completed. Assay diluent served as a negative control. Capture beads specific for each cytokine were mixed and 10 µl of each cytokine specific bead was added for each sample. Mixed capture beads (50 µl), sample (serum or supernatant) (50 µl) and PE detection reagent (50 µl) were then added to O-ring sealed tubes (containment level 3) or flow cytometry tubes (containment level 2). Tubes were incubated in the dark at room temperature for 2 h. Samples were washed using wash buffer provided in the kit, centrifuged (200 g; 5 min) and the supernatant discarded. For infected samples, the pellet was resuspended in 6% paraformaldehyde (500 µl) to allow bacterial killing and refrigerated for 24 h prior to analysis. For non-infected samples, the pellet was resuspended in wash buffer (300 µl) and analysed immediately. Samples were analysed using FACS scan flow cytometer (Becton Dickinson, UK) and BD CBA kit software was used for data analysis.

2.2.7 RNA isolation and preparation of cDNA

A diagram summarising the processes involved from RNA extraction to identifying changes in gene expression is presented in Figure 2.1.

2.2.7.1 Preparation of RNA-free tubes

O-ring sealed Sarstedt tubes were washed with RNAse Zap® and rinsed twice in DEPC-treated water and left to dry under sterile conditions. DEPC (1 ml) was added to distilled water (1 L) in an ACDP Class I cabinet, mixed thoroughly and incubated at room temperature for 2 h to prepare nuclease-free DEPC-treated water. DEPC was degraded following nuclease-free treatment by autoclaving.

2.2.7.2 RNA extraction of experimental samples

The method for RNA extraction was based on a guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski & Sacchi, 1987). Spleens stored in Trizol™ were defrosted and 200 µl Trizol™ was added to each sample which were then incubated at room temperature for 5 min. Chloroform (200 µl) was added to samples which were shaken vigorously by hand twice for 15 sec and incubated at room temperature for 5 min. Samples were centrifuged (12,000 g; 15 min) and the aqueous layer was transferred into a fresh RNAse free eppendorf tube and the pellet discarded. Isopropanol (500 µl) was added to the aqueous supernatant and samples were mixed and left at room temperature for 10 min. The supernatant was discarded and the pellet was washed in 1 ml of 75% molecular-biology grade ethanol diluted in nuclease free water. Samples were centrifuged (7,000 g; 5 min). Ethanol was removed carefully from the pellets by pipetting and samples were left to air dry for a few minutes. The RNA pellet was dissolved in nuclease free water (100 µl) and processed immediately or stored at –20°C or –80°C.

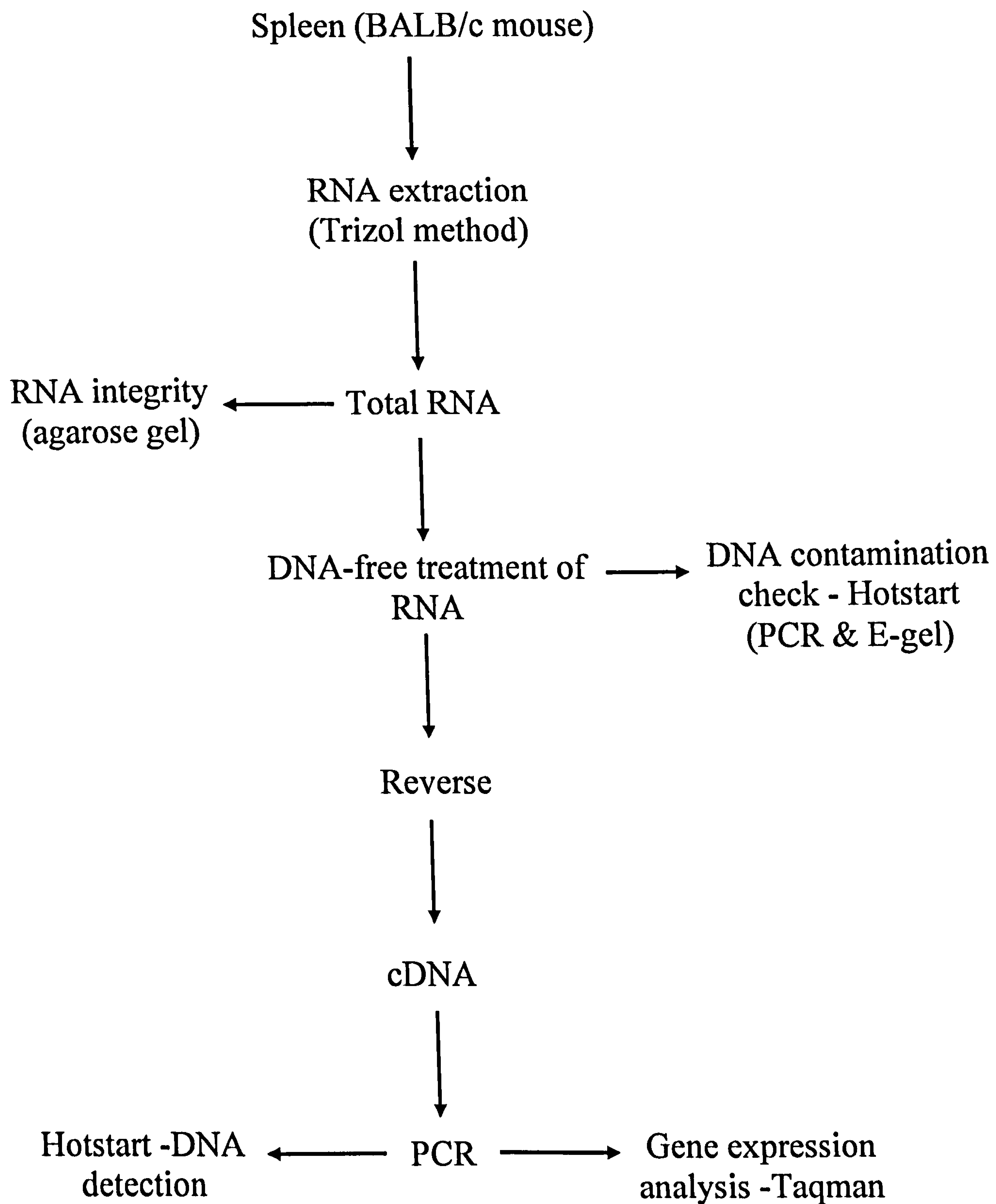


Figure 2.1: Summary of the experimental process from the removal of a spleen from a BALB/c mouse to the final PCR product.

2.2.7.3 *Removal of DNA contamination from RNA samples*

Following RNA extraction, DNA contamination was removed from samples using a DNA-free™ kit. In an RNAse free tube, DNAse I buffer (5 μl) and DNAse I (2 μl) were added to the aqueous RNA sample (50 μl) and incubated for 30 min at 37°C. The DNAse inactivation reagent was vortexed to ensure mixing and 5 μl was added to samples. Samples were vortexed gently and incubated (2min; RT). The sample was then centrifuged (10,000 rpm, 1 min) to pellet the DNAse inactivation reagent. The DNA-free treated RNA solution was then removed to a fresh RNAse free tube without disturbing the bead pellet and stored at –20°C or reverse transcribed immediately. DNA-free treatment was repeated on RNA samples if DNA contamination was identified on an E-gel™ following the initial treatment.

2.2.7.4 *Detection of RNA and DNA integrity and contamination*

To check the integrity of the RNA, a 1% agarose gel was prepared by adding agarose powder to a 1x dilution of MOPS buffer diluted in DEPC-treated water to a volume of approximately 75 ml. The agarose solution was heated until the agarose dissolved completely during microwaving. The solution was left to cool, poured into an 8-lane gel system and sealed with autoclave tape. RNA samples (5 μl) and RNA millennium markers™ (5 μl) were mixed with 15 μl of RNA loading buffer and denatured by heating for 10 min at 60°C and rapidly cooling to 4°C using a Gene Amp 9700 PCR machine. A positive control of Total RNA™ (Ambion, UK) was run on each gel. The gel was run at 70 volts (V) using MOPS buffer for approximately one hour. HotStart™ PCR kit was used during method development and to assess the effectiveness of DNA-free treatment on RNA samples. A reaction mix of components (Table 2.5) was prepared in an RNAse free

Component	Volume (μl)
Buffer (10X) (Hotstart)	**5**
dNTP's	**1**
Taq polymerase	**0.25**
Nuclease-free water	**31.75**
Histone-H3 forward primer	**4**
Histone-H3 reverse primer	**4**
Total Volume	**46**

Table 2.5: PCR reaction mixture using the Hotstart™ kit.

tube to provide enough mixture for all PCR samples for each run. This reaction mixture (total volume 46 µl) was then aliquotted into PCR reaction tubes and cDNA or RNA samples (4 µl) were added to tubes as appropriate. A negative control of nuclease free water and a positive DNA control were run with each PCR reaction. The reaction was performed using the Gene Amp PCR system 9700 on programme HotStart™ 36 (Figure2.2). DNA was detected using a 2% agarose E-gel™. Prior to loading sample onto an E-gel™, loading buffer was mixed with cDNA sample or molecular weight markers to a volume of 20 µl. A positive control of high molecular weight DNA was run on each gel. The gel was run at 70 V for 45 minutes.

2.2.7.5 Reverse Transcription

Reverse transcription of DNA-free treated RNA samples to produce cDNA for PCR analysis was performed using an Omniscript™ Kit. A reaction mixture of components (Table 2.6) was prepared in an RNAse free reaction tube to provide enough mixture for all PCR samples during each run. This reaction mix (47.5 µl) was then aliquoted into PCR reaction tubes and sample (2.5 µl) was added to tubes as appropriate to a total reaction volume of 50 µl. The reaction was performed using the reverse transcription programme recommended by Qiagen, UK (Figure 2.3) on the Gene Amp PCR System 9700. cDNA samples were stored at –20°C or used for PCR analysis.

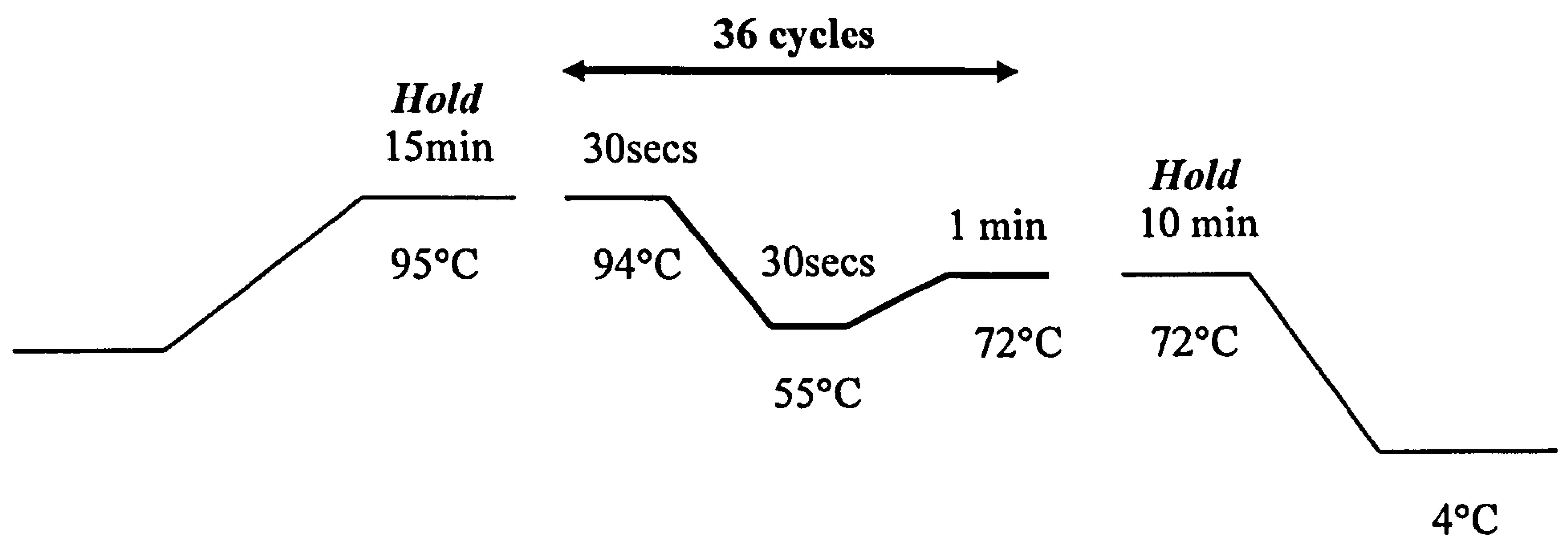


Figure 2.2: Hotstart 36 programme consisted of an initial Hotstart™Taq Polymerase activation (95°C; 15 min hold) followed by 36 cycles of denaturation (94°C; 30 secs), annealing (55°C; 30 secs) and extension (72°C; 1 min) followed by a final extension (72°C; 10 min) and rapid cooling to 4°C.

Component	Volume (μl)
Buffer (10X) (omniscript)	**5**
dNTP's	**1**
Reverse transcriptase	**2.5**
Nuclease-free water	**31.75**
Random Hexamers	**5**
Recombinant RNAsin Ribonuclease inhibitor	**1**
Total Volume	**47.5**

Table 2.6: Reaction mixture for reverse transcription using Omniscript™ kit.

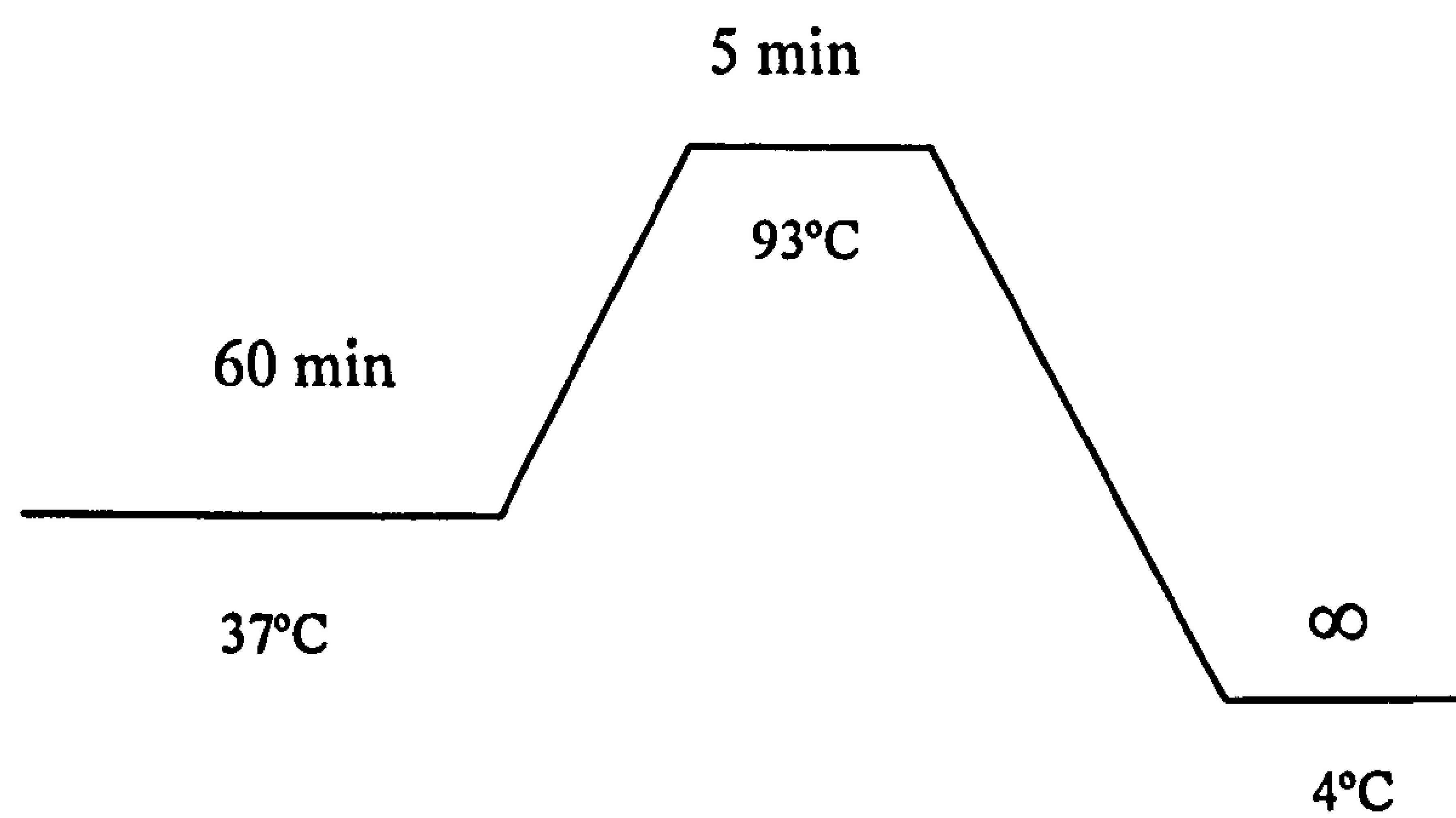


Figure 2.3: Reverse transcription reaction programme consisted of incubating the reaction mixture at 37°C for 60 min followed by inactivation of the reverse transcriptase by heating the mixture to 93°C for 5 min and rapid cooling to 4°C.

2.2.8 *Real Time Polymerase Chain Reaction (RT-PCR)*

2.2.8.1 *RT-PCR*

Real time PCR was performed using a Taqman® 7000 PCR instrument to detect cytokine or endogenous control gene (18sRNA) expression in cDNA samples. Pre-developed Assay Reagents (PDAR) and Assay-on-demand (AoD) reagents contained primers and probes specific for target cDNA (Table 2.7). All PDAR and AoD probes were fluorescently labelled with FAM™. The 18sRNA probe was labelled with the fluorescent label VIC® during initial experiments and with FAM™ in further experiments. The reaction mix for PCR as recommended by the manufacturer is detailed in Table 2.8. A mixture of all components was prepared for each target gene in RNAse free tubes under sterile conditions. The reaction mixture (total volume 23 μl/well) was added to 96 well reaction plates. cDNA samples (2 μl) were then added to wells which were sealed with optical caps or covers. Each sample was run in triplicate. Nuclease free water (negative controls) were run in triplicate for each target reaction to ensure that contaminating DNA was not present in the reaction mixtures. Plates were then centrifuged briefly to remove any air bubbles from the bottom of wells and to ensure no reaction mixture or sample was left on the side of wells. The plate was then run on the Taqman® 7000 PCR machine using thermal cycling conditions recommended by the manufacturer (Figure 2.4).

2.2.8.2 *Data analysis of Real time RT-PCR results*

Data analysis was performed using ABI PRISM 7000 Analysis Software (Applied Biosystems, UK) using the comparative threshold cycle (Ct) method outlined below. The amount of cDNA expressed in each sample relative to the total amount of cDNA

Product	Target
PDAR	IFN-γ, IL-1β, TNFα, IL-6, IL-12p35, IL-12p40, IL-10, GM-CSF, 18sRNA
Assay-on-demand	IL-18, IL-27

Table 2.7: Cytokine and endogenous control gene cDNA targets for primers and probes in PDAR and AoD products.

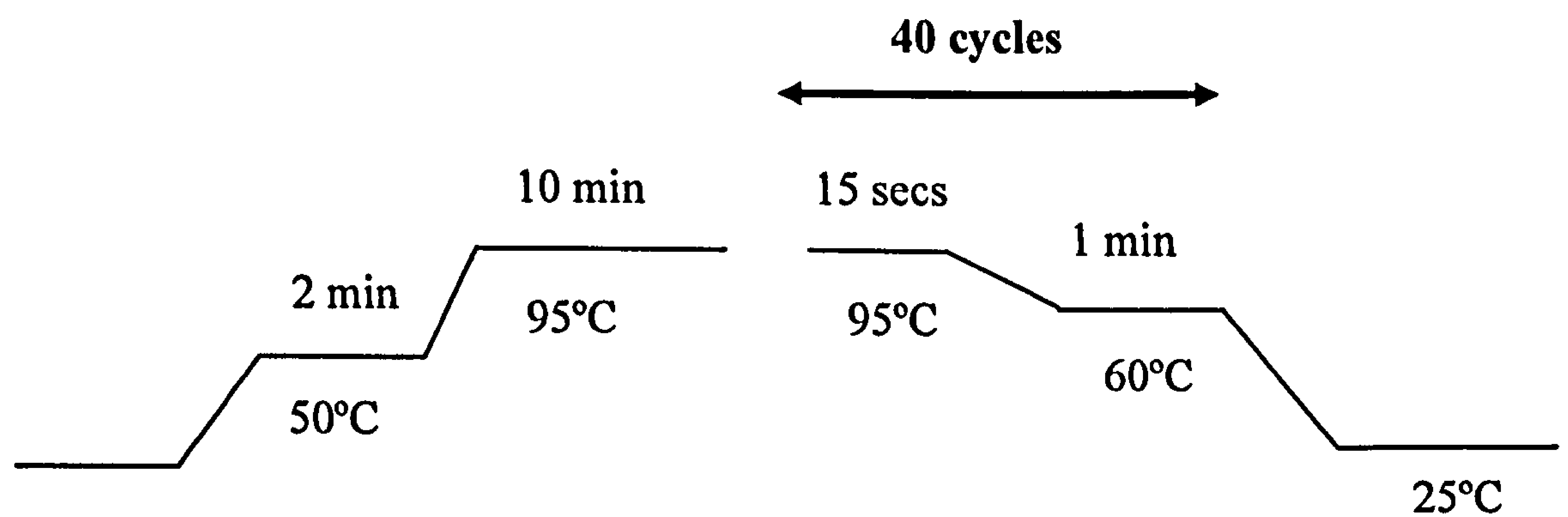


Figure 2.4: Taqman programme consisted of a step to ensure optimal AmpErase®UNG enzyme activity (50°C; 2 min) and a step to activate the AmpliTaq Gold® DNA polymerase (95°C;10 min). This was followed by 40 cycles of a melting step (95°C; 15 sec) and an annealing/extension step (60°C; 1 min).

Component	Volume (μl)
Nuclease-free water (promega)	11.25 - x
2X TaqMan® Universal PCR master mix	12.5
20X Target Primers and Probe	1.25
cDNA sample	x
Total Volume	**25**

Table 2.8: Reaction mixture for Taqman PCR reactions using PDAR and AoD products.

in each sample was determined. The difference between the Ct value of the endogenous control gene (18sRNA) and the Ct value obtained for each cytokine was calculated for each sample:

Eqn 2.1: **Ct cytokine - Ct 18sRNA = ΔCt**

This allowed normalisation of the data and a direct comparison between cytokine expression in different samples. In order to compare differences in cytokine expression between different time-points (Tx), the ΔCt obtained for each sample and cytokine was then compared with expression levels at day 0 (control sample T0):

Eqn 2.2: **ΔCt (sample Tx) - ΔCt (control sample T0) = ΔΔCt**

The standard deviation of the ΔCt value is numerically equal to the ΔΔCt standard deviation as subtraction of arbitrary values was undertaken in determination of ΔΔCt.

To determine the relative quantification of each cytokine at each time-point the following value was derived: $2^{-\Delta\Delta Ct}$

2.2.9 Constant expression of endogenous control (18sRNA) following S. typhimurium LPS challenge

To ensure that the endogenous control, 18sRNA was not upregulated during infection *Salmonella Typhimurium* lipopolysaccharide (LPS), diluted in sterile PBS to 1.7 mg/ml, was administered to 3 female BALB/c mice intraperitoneally (100 µl). Mice were culled by cervical dislocation 1 hour following challenge, spleens were dissected and placed into Trizol (800 µl). Spleens were also removed from 3 untreated control BALB/c mice.

Spleens were mashed in Trizol™ using micropestles and samples were stored at –80°C overnight. RNA was extracted from the samples, DNA contamination was removed and samples were reverse-transcribed using the Omniscript™ method and were stored at –20°C overnight. Prior to PCR, the concentration of cDNA in each sample was determined by spectrophotometry. Sample cDNA (10 μl) was added to distilled water (490 μl) in a 1 ml cuvette (dilution factor = 1:50) and the absorbance of samples at 260 nm and 280 nm was measured. The amount of DNA in each sample was calculated as follows (Cavaluzzi & Borer, 2004):

Eqn 2.3: **A260 of 1.0 = 50μg/ml DNA**

Therefore: A260 x 50 (dilution factor) x 50μg/ml = [cDNA]

The sample volume was limited by the amount of cDNA sample retrieved following reverse transcription. The maximum sample volume for the PCR reaction was 2.5 μl, therefore, the sample with the lowest concentration had a set volume of 2.5 μl. The amount of cDNA in 2.5 μl of this sample was calculated to be 81.25 ng (Eqn 2.3). The volume of the remaining samples required to provide 81.25 ng of cDNA was calculated and added to the reaction mixture. The volume of nuclease-free water in the reaction mix was adjusted accordingly for each sample to ensure a constant final reaction volume of 25 μl. The PCR reaction mix (25 μl) for 18sRNA, TNFα and IL-1β was prepared as above (Table 2.8). All samples were run in triplicate and nuclease-free water controls were prepared for each reaction to ensure no contaminating DNA was present.

2.2.10 *Antibody depletion experiments*

2.2.10.1 *Neutrophil depletion*

2.2.10.1.1 *Depleting properties of RB6-8C5 antibodies*

In order to determine the specificity and efficacy of RB6-8C5 antibodies, BALB/c mice (n=6) were dosed with 500 µg RB6-8C5 or isotype control (Mac5) antibodies (Table 2.1). Spleens were excised 24 h later, processed and spleen cell suspensions were stained for flow cytometric analysis using the stain combinations in Table 2.9.

2.2.10.1.2 *Role of neutrophils during B. mallei infection*

Female BALB/c mice (n=6) were injected intraperitoneally with 200 µl (2.5 mg/ml) RB6-8C5 antibodies or control antibodies of the same isotype (Mac5; IgG2b) to give a final dose of 500 µg 24 h prior to infection. Mice were then dosed with antibody every 3 days. Animals were culled when a predetermined endpoint was reached.

2.2.10.2 *TNFα neutralisation*

2.2.10.2.1 *Depleting properties of MP6-XT22 antibodies*

The specificity of the TNFα antibody was investigated *in vitro* under sterile conditions. J774A.1 macrophages were added to 24 well plates in DMEM media, containing 10% FCS and L-glutamine, at a cell concentration of $2x10^6$/ml and were incubated overnight (37°C; 5% CO_2). Recombinant TNFα was diluted in media (0-50ng/ml) and was then added to cells in the presence or absence of 500µg/ml TNFα antibodies or Mac5 isotype control matched antibodies. Cells were reincubated for 24 h (37°C; 5% CO_2) and then harvested, washed and resuspended in staining buffer (100 µl) containing CD54-PE antibody. Cells were incubated for 30 min, resuspended in 1% paraformaldehyde and

Fluorochrome		
FITC	**PE**	**PE-Cy5**
CD11c (AHIgG1)	Gr-1 (rIgG2bκ)	B220 (rIgG2aκ)
Gr-1 (rIgG2bκ)	CD11b (rIgG2b)	F4/80 (rIgG2b)
DX5 (rIgMk)	Gr-1	CD3 (AHIgG1κ)

Table 2.9: Stains used for flow cytometric analysis of spleen samples. Gr-1^+ populations were identified by specific antibodies conjugated to fluorochrome labels: FITC: Fluorescein Isothiocyanate; PE: Phycoerythrin; PE-Cy5: phycoerythrin-cychrome-5; CD: complimentarity determining. The isotypes of the antibodies are bracketed () next to the appropriate marker. AH: Armenian hamster; R: rat; IgG: immunoglobulin G.

macrophage activation was measured by flow cytometry.

2.2.10.2.2 Role of TNFα during B. mallei infection

The effect of TNFα neutralisation during *B. mallei* infection was assessed. TNFα antibodies (clone: MP6-XT22) (500 μg) were administered intraperitoneally (200 μl at 2.5mg/ml) to BALB/c mice (n=6/group). Mice were given initial doses 24 h prior to infection with *B. mallei*, at day 1 p.i. and day 4 p.i. (Table 2.10). Mice were then re-dosed twice weekly (Scanga *et al*, 1999). An infected control group (n=6) was administered with the same dose of isotype control antibody (Mac49) at the same time as TNFα antibody dosing. Mice were culled 7 days post-infection, unless a humane endpoint was reached at an earlier time-point and spleens were removed for bacteriological analysis. In a further experiment, BALB/c mice (n=6) were infected with *B. mallei* and dosed intraperitoneally with TNFα antibodies (500 μg) at day 42 p.i. and re-dosed twice weekly. An infected control group (n=6) were dosed with control antibodies (Mac49) at the same timepoints. Mice were culled 14 days following the initial antibody dose unless a humane endpoint had already been reached.

Group	**Antibody dosing regime (Days post-infection)**
A (24 hours Pre-infection)	Day –1, +4
B (24 hours post-infection)	Day +1, +5
C (4 days post-infection)	Day + 4
D (Isotype control: Mac49)	Day –1, +1, +4, +5

Table 2.10: Description of TNFα neutralisation groups and the antibody dosing regimen for each group post-infection. Groups A-C were dosed with 500 μg MP6-XT22 TNFα antibody and Group D was dosed with 500 μg Mac49 control antibody at the timepoints stated post-infection

2.2.10.3 T cell depletion

2.2.10.3.1 Depleting properties of T cell antibodies

In order to determine the individual and combined effects of the antibodies YTS191 and YTS169 on specific T cell subsets, BALB/c mice (n=3/group) were injected intraperitoneally with an initial loading dose of antibody (500 μg). An isotype control group (Mac5) was dosed simultaneously with 500 μg antibody. Mice were injected with a maintenance dose of 250 μg after 48 h and were culled by cervical dislocation the following day. Spleens were removed from all animals and flow cytometry was performed on spleen suspensions. Cytotoxic T cells were identified by staining with CD8-FITC (RIgG2ak) and CD3-PE (HIgG1) and T helper cells were identified by staining with CD3-FITC (HIgG1k) and CD4-PE (IgG2ak).

2.2.10.3.2 Role of T cells during B. mallei infection

T helper cells ($CD4^+$ T cells) and/or cytotoxic T cells ($CD8^+$ T cells) were depleted prior to infection with *B. mallei*. An initial loading dose of 500 μg CD4 (YTS 191) and/or CD8 (YTS 169) antibodies or control antibodies (Mac5) was administered intraperitoneally to BALB/c mice (n=6/group) 3 days prior to infection. A second dose (250 μg) of appropriate antibody was administered 24 h prior to infection and then every 5 days until the end of the experiment (Table 2.11). This dosing regimen was based on previous experimental data (Scanga *et al*, 2000)). Mice were culled 28 days p.i. unless preceded by a predetermined endpoint.

T-cell Depletion group (Antibody Clone)	**Antibody Dosing Regime (Days post-infection)**
Anti-CD8 (YTS 169)	
Anti-CD4 (YTS 191)	Day –3 (500 μg), –1 (250 μg), +5 (250 μg), +10 (250 μg), +15 (250 μg), +20 (250 μg), +25 (250 μg)
Anti-CD4 and anti-CD8 (YTS 169 + YTS 191)	
Isotype control (Mac 5)	

Table 2.11: Description of T cell depletion groups. The antibody dosing regimen for each group post-infection was the same.

2.2.11 *In vitro stimulation of spleen cells*

2.2.11.1 *Preparation and stimulation of spleen cell suspensions*

Spleens were aseptically removed from mice and placed into media (RPMI 1640 supplemented with 10% FCS, penicillin, streptomycin and 10 mM L-glutamine). Spleens were mashed into media to create single cell suspensions and centrifuged (300 g, 5 min). Erythrocytes were lysed by adding red blood cell lysis buffer (8 ml) to cell pellets and incubating at room temperature for 10 min. Cell suspensions were washed in media and counted by trypan blue exclusion staining. Cell suspensions were adjusted to $5x10^6$ cells/ml by resuspending in warmed RPMI 1640 media. Cells suspensions were then plated at 100 µl/well in 96-well flat-bottom culture plates in triplicate wells for each mouse. Cells were incubated with 100 µl/well of $1x10^6$ cfu/ml (final concentration) heat-killed *B. mallei*, IL-6 (10-200ng/ml) or medium control (5% CO_2; 37°C). A combination of IL-12 (5ng/ml) and IL-18 (5ng/ml) was added to wells in triplicate for each mouse as a positive control for IFN-γ production.

2.2.11.2 *Intracellular IFN-γ staining for flow cytometry*

Supernatants were harvested from each well after 24 h and were frozen (-20°C) prior to cytometric bead array analysis to detect secreted cytokines. For intracellular staining, Golgi plug™ (1 µl of 1:10 dilution) was added to cultures 3.5 h prior to the end of stimulation and cells were harvested from wells. Cells from triplicate wells for each mouse were pooled for intracellular staining. Cells were washed in staining buffer and extracellular markers were stained using DX5-FITC (NK cells) and CD3-PC5 (T cells) and incubated in the dark (30 min, RT). Samples were then washed using staining buffer and were resuspended in 100µl Leucoperm™ Reagent A (15 min, RT). Cells were washed in

staining buffer, resuspended in 100 μl Leucoperm™ Reagent B containing IFN-γ-PE antibody or isotype control antibody (rat IgG1-PE) and incubated in the dark (30 min, RT). Cells were then washed in staining buffer and resuspended in 2% paraformaldehyde solution prior to flow cytometric analysis.

2.2.12 Statistical Analysis

2.2.12.1 Analysis of temperature data

Statistical analysis of temperature data was performed by a two-way Analysis of variation (ANOVA) test to determine whether infected and uninfected mice had significantly different temperatures over the time course of infection. The ANOVA was performed on the average of the uninfected and infected mice over time and F-values were calculated. Trendlines and correlation coefficients were determined using Microsoft Excel.

2.2.12.2 Analysis of in vivo infection data

Data obtained during analysis of real-time RT-PCR, serum cytokines and flow cytometry during infection in Immune Analysis 2 was determined to be non-parametric and, therefore, data is represented as median ± 99% confidence intervals. A Kruskal-Wallis Test was performed to determine the overall significance of the data and the significance of individual data points was determined using a Mann-Whitney U Test. The statistical significance of data obtained at each time-point during the infection was compared with uninfected control animals unless otherwise stated. A predetermined p-value of less than 0.01 was deemed to indicate statistical significance ($\alpha=0.01$). This lower significance level was chosen to reduce the chance that differences observed were due to chance because of the multiple comparisons made between the medians using the Mann-Whitney-U test; the Mann-Whitney U post-test does not automatically correct for multiple comparisons (Chapters 4-6).

2.2.12.3 *Analysis of in vitro stimulation data*

Data obtained in *in vitro* stimulation assays was parametric and was analysed using a one-way ANOVA test followed by a Tukey's t-test. A p-value of less than 0.05 was deemed to indicate statistical significance (α=0.05). This higher significance level was chosen as the Tukey's post-test automatically corrects for multiple comparisons between the means and, therefore, the significance level did not need to compensate for the fact that multiple comparisons were made on the data (Chapter 7).

Chapter 3

Characterisation of an intraperitoneal model of *B. mallei* infection in BALB/c mice.

3.1 Introduction

In depth characterisation of host immune responses to pathogens is important to determine potential protective and detrimental aspects of the immune response. Although some work has been performed to characterise the pathology of disease caused by *B. mallei*, little work has been performed to investigate host immune responses to *B. mallei* infection. Intraperitoneal models of infection in both outbred (Porton) and inbred (BALB/c and C57BL/6) mouse strains (Fritz *et al*, 2000; Amemiya *et al*, 2002) have been used to describe the pathology of *B. mallei* infection where a non-acute, systemic infection follows intraperitoneal infection. Bacteria entering the peritoneal (abdominal) cavity cause infection and histopathological changes in several organs including the spleen, lymph nodes, bone marrow and liver (Fritz *et al*, 2000). The spleen has an important function in clearing the blood of circulating pathogens but may also be colonised by pathogens including *B. mallei* which targets the spleen during infection in humans (Georgiades & Fishman, 2001).

In this chapter, an intraperitoneal model of *B. mallei* infection in BALB/c mice is described. The aims of this chapter were to determine:

- The time course of bacterial colonisation of the spleen
- Systemic changes occurring during infection by monitoring body temperature and signs of infection.

This model was consequently used to investigate host immune responses to *B. mallei* infection described in chapters 4-6.

3.2 Methods

In the following studies characterising temperature, signs of disease and splenic bacterial colonisation, female BALB/c mice were challenged i.p. with 10^6 cfu *B. mallei* (ATCC 23344). Temperature and signs of disease were measured throughout the time course of infection until mice reached a predetermied endpoint and were culled. Bacterial colonisation in the spleen was investigated during infection at several time-points in 2 time course experiments: Immune Analysis (1) and Immune Analysis (2) (see Chapter 2; Table 2.2). Immune Analysis (1) encompassed a 36-day time course of infection. Immune Analysis (2) repeated investigation of responses 0-7 days post-infection (p.i.), including an additional time-point at 5 h p.i. and higher animal numbers (n=8) in order to counter variation in bacterial counts observed during Immune Analysis (1). In addition to bacterial colonisation of the spleen, cellular responses, splenic cytokine expression and systemic serum cytokine production were investigated during both immune analysis experiments, the results of which are described in chapters 4-6.

3.3 Results

3.3.1 Signs of disease and temperature changes

Signs of disease and body temperatures were monitored for 36 days following i.p. infection with 10^6 cfu *B. mallei* in infected and uninfected control mice. During the 36 days of monitoring, none of the animals reached a humane endpoint for culling and no animal died as a result of the infection. Mice remained in the isolator for up to 70 days post-infection (Figure 3.1). After 66 days, ruffling and hunching were observed - one animal was found dead and another was culled 2 days later following the development of abdominal swelling. Once terminal signs of disease were observed in the remaining animals, mice were culled and the study ended.

Infected mice were housed in two cages of 5 animals (cage A and cage B) and ruffling of fur was used as a consistent sign of illness in the mice. A comparison of average temperatures from infected mice from both cages A and B (n=10) and uninfected mice (n=5) over time revealed that infected mice had significantly different temperatures in comparison with uninfected mice ($p<0.05$) in both the morning and afternoon. Temperature in infected mice from both cages was significantly higher than controls at day 14 p.i. ($p<0.05$) (Figures 3.2 & 3.3). A division in signs and temperatures was observed between cages of infected mice (A and B) (Figures 3.2 & 3.3). Diurnal variation in symptoms and temperature were also observed. Slight ruffling was observed in the morning on days 5-9 in both control and infected animals in cages A and B. From days 12-14, slight ruffling was observed in the morning in 1/5 infected animals in cage B and 2/5 animals in cage A (Figure 3.2AM). Notable ruffling of fur was observed in all cage A mice in the morning and afternoon from days 22 to 36 (Figure 3.2AM & PM). This was

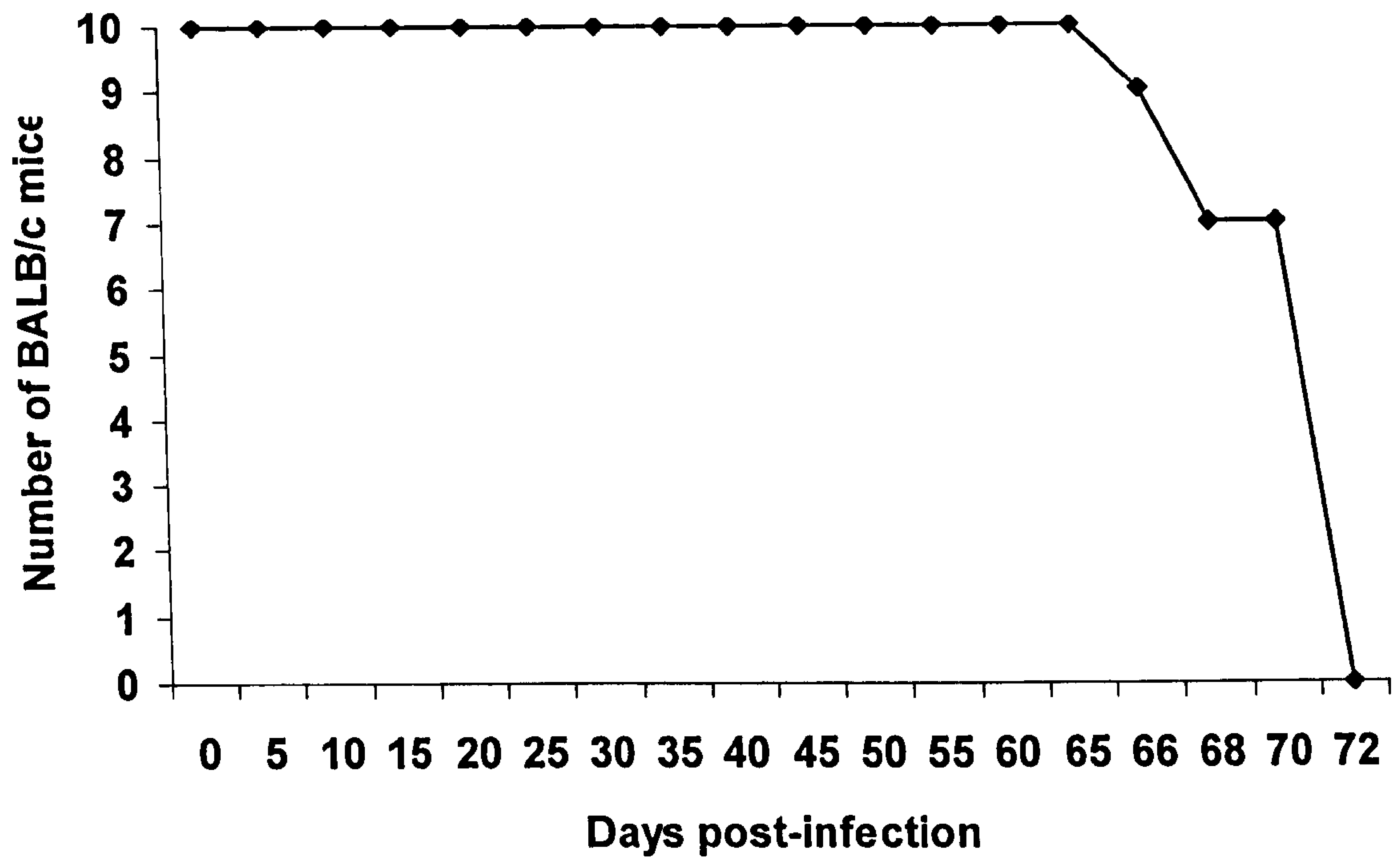


Figure 3.1: Number of BALB/c mice (n=10) surviving following intraperitoneal infection with 1 x 10^6 cfu *B. mallei*. At 66 days post-infection, one animal was found dead. Another mouse was culled 2 days later following the development of abdominal swelling. Terminal signs of disease were observed in the remaining mice 71-72 days p.i. which were then culled and the study ended.

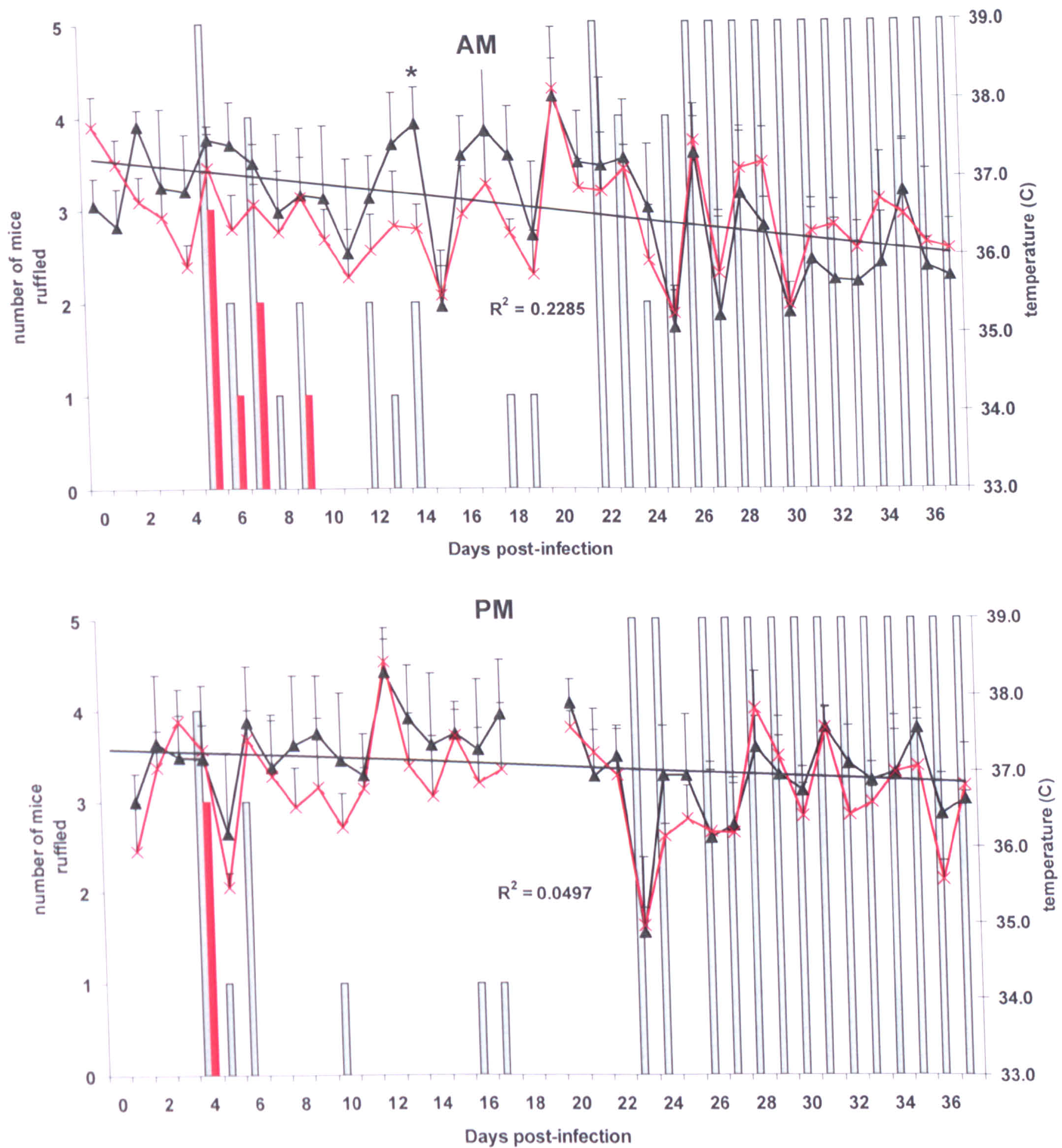


Figure 3.2: Temperature (°C) and symptoms measured in animals from cage A (n=5) in the morning (AM) and the afternoon (PM). Grey bars indicate the number of infected animals that showed signs of ruffling and black bars indicated the number of control animals (n=5) showing signs of ruffling. Black lines indicate average temperatures for infected animals and grey lines control temperatures. Error bars represent S.D. Trendlines and the correlation coefficient (R^2) are shown for infected animals only. Asterisk indicates a significant difference ($p<0.05$) between infected and control mice.

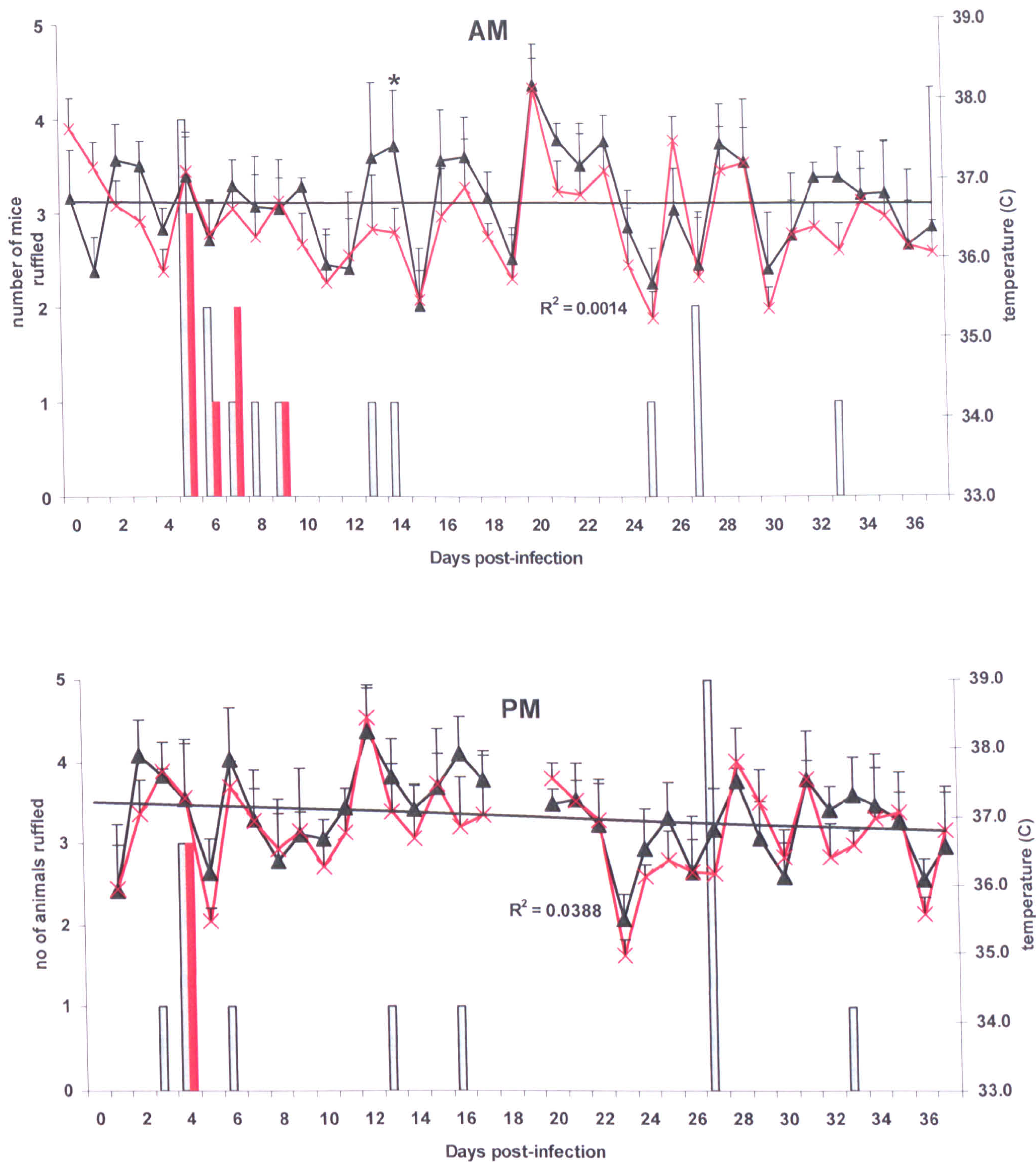


Figure 3.3: Temperature (°C) and symptoms measured in animals from cage B (n=5) in the morning (AM) and the afternoon (PM). Grey bars indicate the number of infected animals that showed signs of ruffling and black bars indicated the number of control animals (n=5) showing signs of ruffling. Black lines indicate average temperatures for infected animals and grey lines control temperatures. Error bars represent S.D.. Trendlines and the correlation coefficient (R^2) are shown for infected animals only. Asterisk indicates significant difference ($p<0.05$) between infected and control mice.

accompanied by a decreasing trend in morning temperature (R^2=0.23) which was not observed in control animals, cage B animals or infected animals during the afternoon (Figure 3.2AM). Ruffling was observed only sporadically in cage B mice from days 14-36 p.i. (Figure 3.3).

3.3.2 Bacteriological analysis and abscess formation

Bacteriological analysis was performed on spleens removed from BALB/c mice infected with 1 x 10^6 cfu *B. mallei*, identified as small, smooth, distinct pinkish-white colonies on Congo Red plates. Splenic colonisation was investigated on days 1 – 7 p.i. during both experimental time-course infections (Immune Analysis (1) and (2)) (Figure 3.4A & B). Similar counts were observed at days 1 and 7 p.i. in both studies although less variation was observed at days 3 and 5 p.i. in Immune Analysis (2).

Uniform bacterial colonisation of the spleen occurred 5 h p.i. during Immune Analysis (2) (Figure 3.4A). At 24 h p.i., *B. mallei* colonies were isolated from all mice. More than 1000 cfu were isolated from 5/8 mice although <10 cfu were isolated from one individual. Spleens from all mice were colonised 3 days p.i., although bacterial burdens were variable (10-10^3 cfu). At day 5 p.i., 5/8 mice were colonised with counts ranging from 135 – 3690 cfu. Splenomegaly was observed in some animals at day 7 p.i. and 7/8 mice were colonised with bacterial burdens similar to day 1. At day 14 p.i., splenomegaly and/or abscess formation was observed in all animals except one. Abscesses occurred in 2/10 mice at day 14 and were characterised by yellow pus surrounded by a hard, white casing. Multiple small abscesses were observed in the spleens of some animals. Abscess formation correlated with high bacterial counts (>10^6 bacteria/spleen) (Figure 3.5). Splenomegaly was not always accompanied by high bacterial counts. At day 21, 5/10 animals possessed abscesses accompanied by high levels of bacteria (>10^7 bacteria/spleen). At days 28 and

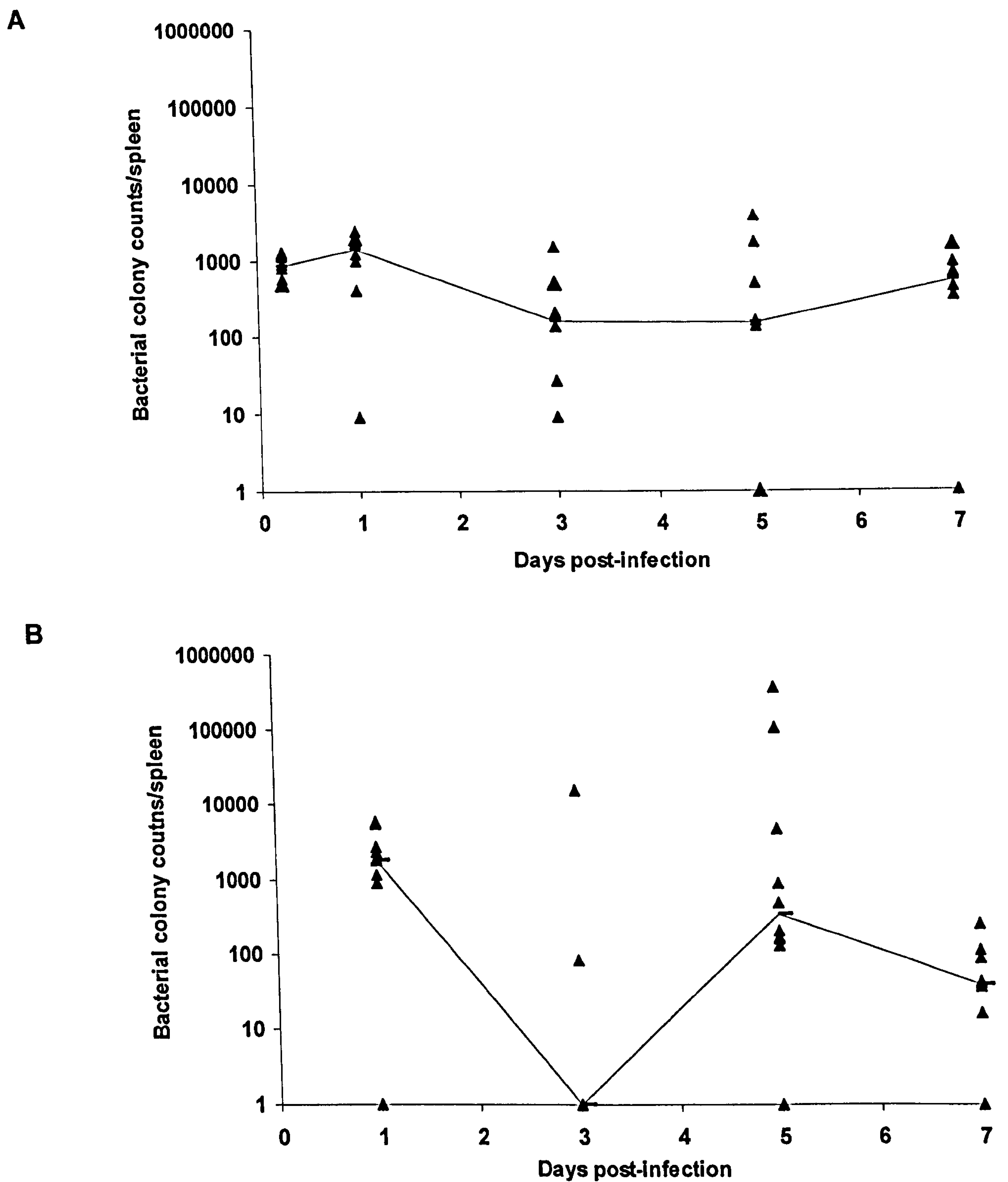


Figure 3.4: The number of colonies detected in the spleens of BALB/c mice infected with 1 x 10^6 cfu *B. mallei* over the first 7 days of infection during A) Immune Analysis (2) (n=8/timepoint) and B) Immune Analysis (1) (n=10/timepoint). Triangles represent individual animals. The line represents the median number of colony counts detected at each time-point post-infection.

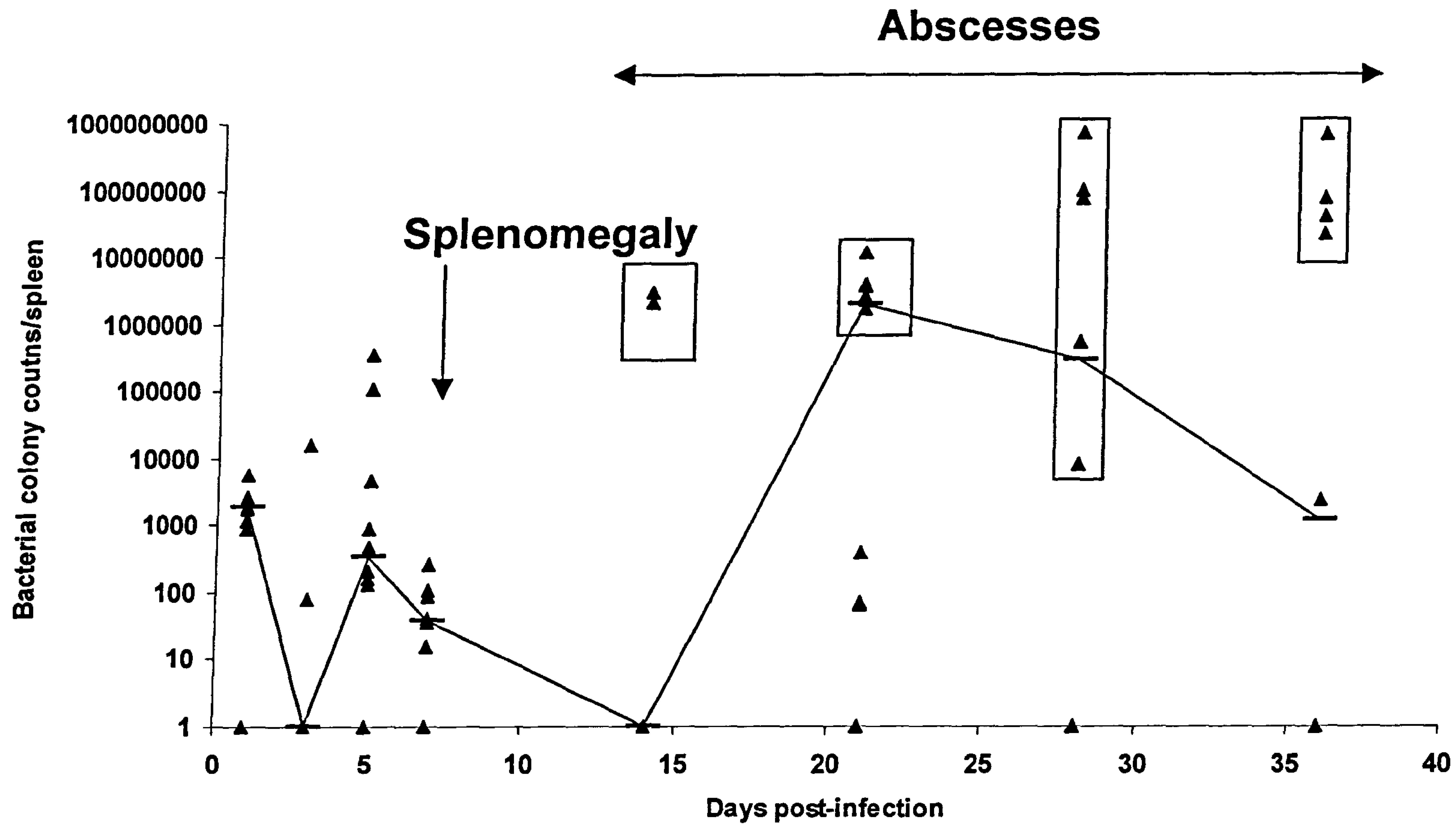


Figure 3.5: The number of colonies detected in the spleens of BALB/c mice infected with 1 x 10^6 cfu *B. mallei* over 36 days of infection. Triangles represent individual animals (n=10/timepoint). The line represents the median number of colony counts detected at each time-point post-infection. Splenomegaly began to develop 7 days post-infection and abscesses were observed from day 14 onwards. Boxes represent animals with abscessed spleens.

36, abscesses were observed in 6/10 and 4/10 mice respectively, and were larger than those found at previous time-points. No *B. mallei* colonies were detected in the remaining mice from days 14-36 suggesting an apparent clearance or latency of infection in these animals.

3.4 Discussion

Intraperitoneal infection of BALB/c mice with *B. mallei* caused the development of a chronic disease to which animals succumbed several weeks after the initial observation of abscesses. The course of *B. mallei* infection could be separated into 2 phases. During the initial phase of infection (Days 0-7) bacterial numbers appeared to be controlled by the host immune system. The number of bacterial colonies did not increase but remained between 10^2 and 10^4 cfu during this period of infection. During the second phase of infection (Days 14-70), bacterial numbers rapidly expanded in the spleen, abscess formation occurred and overt signs of disease developed with death ensuing within 70 days post-infection. This suggests that *B. mallei* was able to overcome host immune responses to allow uncontrolled bacterial replication leading to a systemic, fatal disease.

A heterogeneous infection, characterised by variable bacterial numbers, signs of disease and temperature changes was observed. This variability became more apparent after the first week of infection when animals either developed abscesses or appeared to clear the infection. Heterogeneity in BALB/c mice was also observed in another model of *B. mallei* infection (Fritz *et al*, 2000) and exists in cases of human glanders where a variety of symptoms are observed between infected individuals (Bernstein & Carling, 1909). The spleens of individual mice were colonised uniformly at 5h p.i.. This suggests that variation in bacterial colonisation of the spleen from day 1 following infection was not due to a variation in challenge dose and that mice were infected with the same initial loading dose. The cause of variable bacterial colonisation during the remainder of the infectious time

course in an inbred mouse strain is unknown. However, bacteria can undergo transformation during infection leading to the development of a chronic, latent strain. Loss of the capsular structure of *B. mallei* has been observed in experimental murine glanders (Fritz *et al*, 2000). This may have implications in the development of latent infection as the capsule is an important virulence factor (DeShazer *et al*, 2001; Popov *et al*, 2002). Latent *B. mallei* infection is present in natural populations (Bernstein & Carling, 1909) and the absence of splenic colonies in some individuals during the second phase of infection suggests that a latent infection may exist in these animals.

Multiple body-wide abscess formation is a common feature of human glanders (Srinivasan *et al*, 2001). Splenic abscess formation was observed in this model of *B. mallei* infection. Capsular polysaccharide structures appear to be important in the formation of abscesses during infection with *B. fragilis*. Interaction of the polysaccharide capsule of this bacterium with T cells is responsible for abscess formation during infection (Tzianabos *et al*, 1994). It would be interesting to determine whether the *B. mallei* capsular polysaccharide is important for abscess formation *in vivo*.

In recorded human cases of glanders, symptoms accompanied by fever and pyrexia normally occur in the afternoon (Howe *et al*, 1947). Temperature differences between control and infected animals were more marked in the morning in mice with overt signs of disease in comparison with afternoon temperatures in the same mice. Diurnal variations in mouse species means that they are normally more active at night and less active during daytime – the opposite to human activity, suggesting that these temperature changes may be analogous to human glanders. Temperature spiking also occurs throughout the day in human glanders with temporary, short-lived periods of pyrexia (Howe & Miller, 1947). The occurrence of temperature spiking during *B. mallei* infection in the mouse is not known, however, more regular monitoring (e.g. hourly) of core body temperature using an

implanted temperature probe may identify any temperature spikes occurring in the mouse model of *B. mallei* infection. This would prevent exclusion of temperature elevations occurring in between temperature monitoring in the morning and afternoon.

Periodic symptoms were observed in mice during the first three weeks of infection, however, this developed into continuous ruffling in half the mice from day 21 p.i.. During this period temperatures dropped below those of control values only in animals that were ruffled. Hypothermia, is a marker in acutely ill BALB/c mice during lipopolysaccharide-induced shock (Vlach *et al*, 2000) and the trend towards hypothermia in overtly ill mice in this study suggests that a drop in temperature may be indicative of the severity of illness. However, in order to substantiate this, a more sensitive technique (e.g. implanted temperature probe) could be used to measure core body temperature rather than peripheral temperature in a manner which is not subject to user variation.

Ruffling occurred occasionally in some control mice from days 5-9 p.i.. This may have been due to differential assessment of signs of disease by different investigators in the early part of the study, however, the reason for this should be more fully investigated in future studies. The distinct difference between the two separate cages of infected mice (cages A&B) in terms of signs of disease could also not be explained: both cages were challenged with the same dose of *B. mallei* from the same culture; signs of disease were recorded by the same operators at each time-point; all mice (from both cages) developed terminal signs of disease within days of each other. Variation was apparent in this model of *B. mallei* infection in terms of splenic bacterial colonisation, however, the clear cage-specific signs of disease would not be expected and should be investigated in further studies.

A heterogeneous model of chronic, i.p. *B. mallei* infection has been described in this chapter, characterised by abscess formation. Host immune responses occurring during infection in this model, and the immunological consequences of heterogeneity of disease, are described in Chapters 4-6.

Chapter 4

Characterisation of neutrophil and macrophage responses during *B. mallei* infection in BALB/c mice.

4.1 Introduction

Neutrophils and macrophages are immune cells important in innate defence against infection. They are involved in pathogen recognition and killing by a number of mechanisms including release of reactive oxygen and nitrogen species. They are also involved in the production of cytokines (eg. IL-12 and IL-18) involved in IFN-γ production and development of type 1 immunity. Neutrophils are essential for protection against infection with a number of intracellular pathogens including *L. monocytogenes* (Conlan & North, 1994), *S. typhimurium* and *Y. enterocolitica* (Conlan, 1997) and their protective role during *M. tuberculosis* infection has also been established (Pedrosa *et al*, 2000). Macrophages can be infected by a number of intracellular pathogens including *B. pseudomallei* (Harley *et al*, 1998) and *F. tularensis* (Clemens *et al*, 2005). Although macrophages instigate a number of bacterial killing mechanisms following infection, including the production of nitric oxide and pro-inflammatory cytokines, many intracellular pathogens are able to subvert these processes to enable survival and propagation within the macrophage (Jones *et al*, 1996).

Histopathological analysis has identified that neutrophils and macrophages infiltrate several organs during *B. mallei* infection (Fritz *et al*, 2000). However, the activation status and role of these cells in the progression of disease has not been characterised. Therefore, the aims of this chapter were to determine:

- The effect of *B. mallei* on host neutrophil and macrophage responses by measuring the infiltration and activation profiles of these cells during infection.
- The effectiveness of host neutrophil responses in protecting against *B. mallei* using depleting antibodies.

4.2 Methods

Neutrophil and macrophage responses were measured by flow cytometry following staining with lineage markers and markers of activation. Data described from the first 7 days of *B. mallei* infection were predominantly derived during Immune Analysis (2). However, where possible, this data was compared with data derived from Immune Analysis (1). Data relating to days 14-36 of infection were observed during Immune Analysis (1) (Table 2.2).

$Gr\text{-}1^+$ cell populations were depleted 24 h prior to infection following intraperitoneal injection with RB6-8C5 depleting antibodies or Mac5 isotype-matched control antibodies and mice were re-dosed every 3 days p.i.. BALB/c mice were infected with 10^6 cfu *B. mallei*. The specificity of $Gr\text{-}1^+$ antibodies was assessed following depletion with RB6-8C5 or Mac5 antibody in naïve BALB/c mice. $Gr\text{-}1^+$ populations were identified in the spleen by flow cytometry.

4.3 Results

4.3.1 Neutrophil responses during *B. mallei* infection

4.3.1.1 Splenic Neutrophil infiltration

During the first 7 days of infection in Immune Analysis (2) and Immune Analysis (1) (Table 2.2), the number of neutrophils was investigated in the spleens of infected BALB/c mice. During Immune Analysis (2), neutrophils were identified by their forward scatter:side scatter profiles (size and granularity) (Figure 4.1) and by examining the expression of high intensity Gr-1 on these cells (Figure 4.2A). In this experiment, the percentage of neutrophils within the spleen increased at 5 h p.i. ($p<0.01$) and remained elevated 24 h p.i. ($p<0.01$) returning to control levels 3 days p.i. (Figure 4.3). The percentage of neutrophils became elevated above control values again 7 days p.i. ($p<0.01$) (Figure 4.3). The number of neutrophils in the spleen at day 5 p.i. was related to bacterial colonisation with comparatively low neutrophil numbers present in spleens colonised by <10 cfu.

During Immune Analysis (1), the neutrophil marker (Clone: 7/4) was used and was found to have very low expression in BALB/c mice. Therefore, neutrophils were identified solely on their forward scatter:side scatter (FS:SS) profiles. In order to provide a relevant comparison, neutrophil data obtained during Immune Analysis (2) was further analysed and neutrophils were identified solely on their FS:SS characteristics. A similar proportion of neutrophils per sample were observed in uninfected BALB/c mice in both Immune Analysis (1) and (2) with 2-4% of cells having neutrophil size and granularity (Figure 4.4). At days 1 and 3 p.i., 6-8% of cells had neutrophil FS:SS properties in both experiments. At

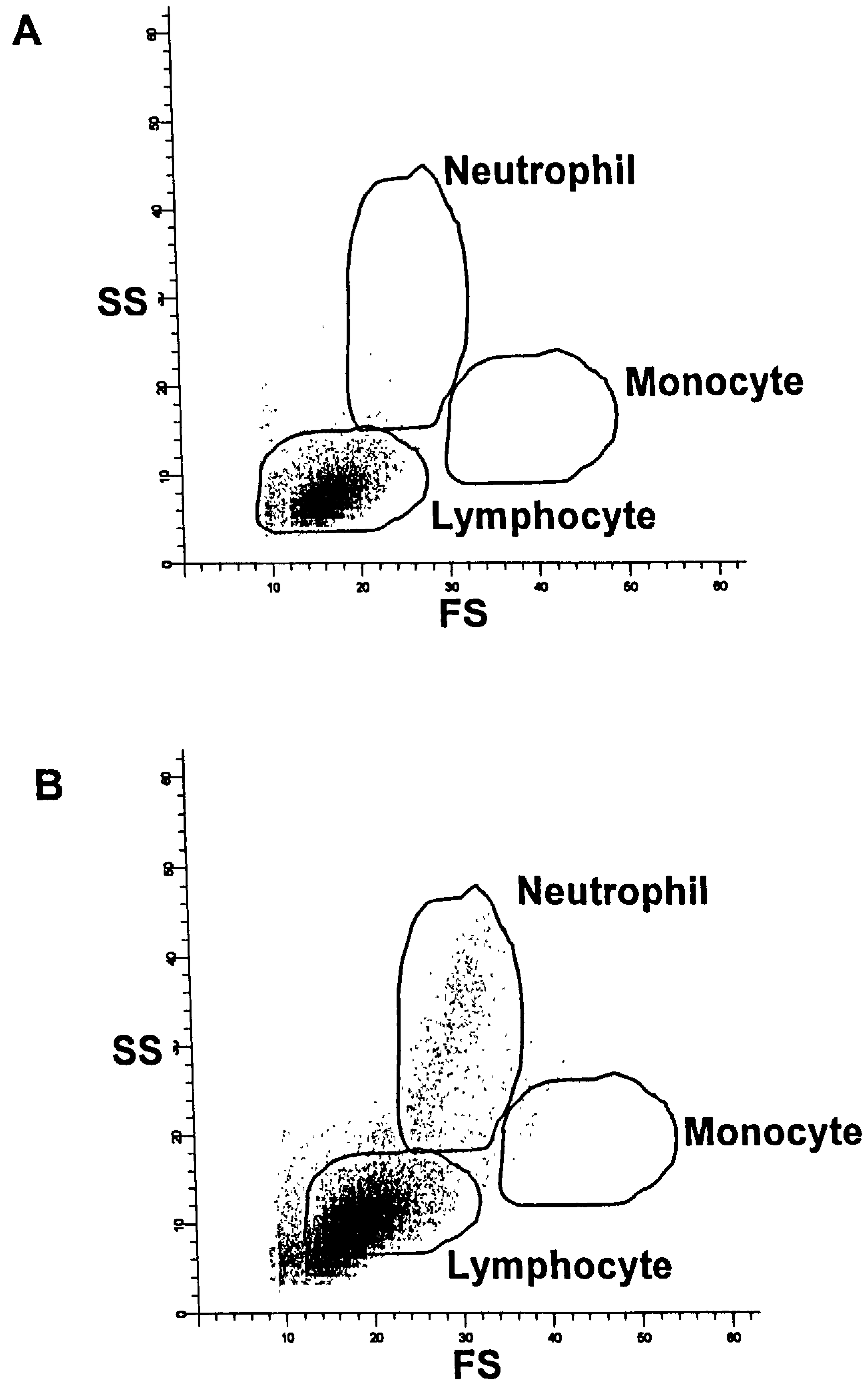


Figure 4.1: Representative splenic cell populations in a naïve and an infected BALB/c mouse. Forward scatter (FS):side scatter profiles (SS) of lymphocytes, macrophages/monocytes and neutrophils identified in A) a naïve and B) a *B. mallei* infected BALB/c mouse spleen. FS represents cell size and SS represents cellular granularity.

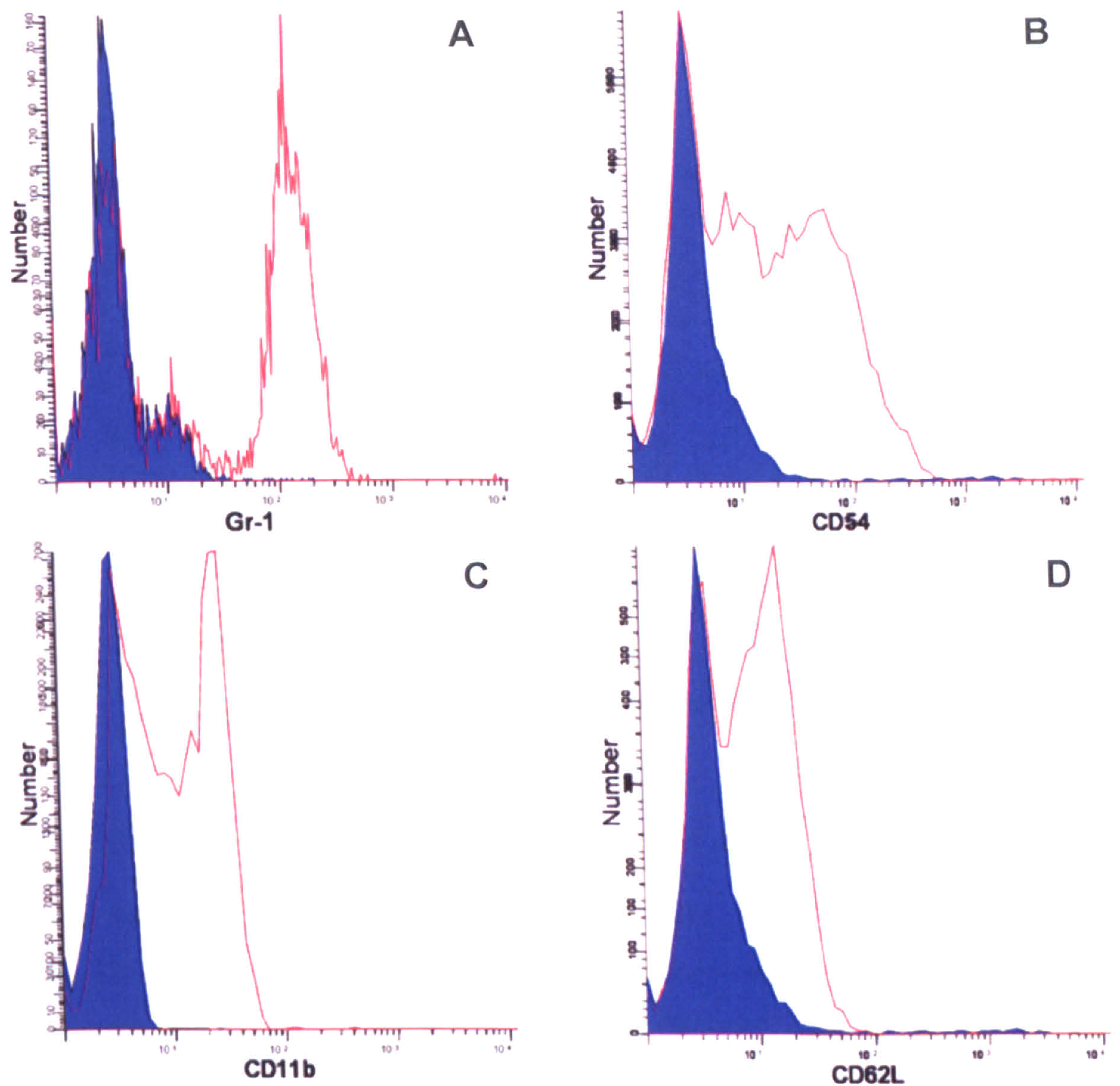


Figure 4.2: Representative flow cytometry histograms to indicate positive staining profiles of neutrophils during *B. mallei* infection. Splenic cells obtained from BALB/c mice following infection with *B. mallei* during Immune Analysis (2) were identified by staining with A) Gr-1 and co-stained with antibodies specific for B) CD54; C) CD11b and D) CD62L. Blue plots indicate negative staining determined using appropriate isotype controls and red plots indicate positive fluorescence staining.

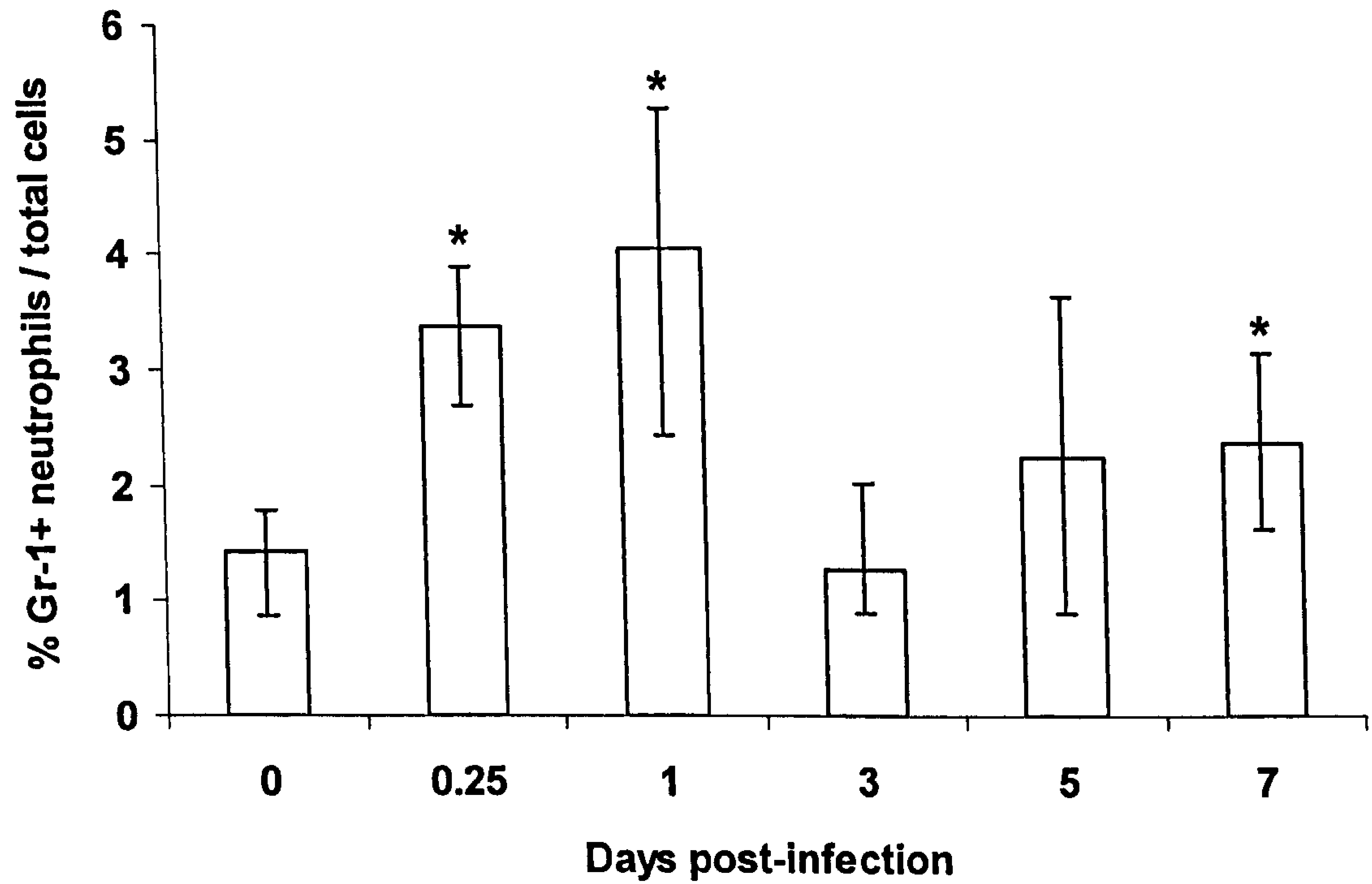


Figure 4.3: Splenic Gr-1^+ neutrophil populations during the first 7 days post-infection with *B. mallei*. The median percentage of Gr-1^{Hi} neutrophils detected as a proportion of total cells collected per sample (n=8/timepoint) during Immune Analysis (2). Error bars represent 99% confidence intervals. Asterisk(s) indicate statistical significance ($p<0.01$) in comparison with day 0 (uninfected).

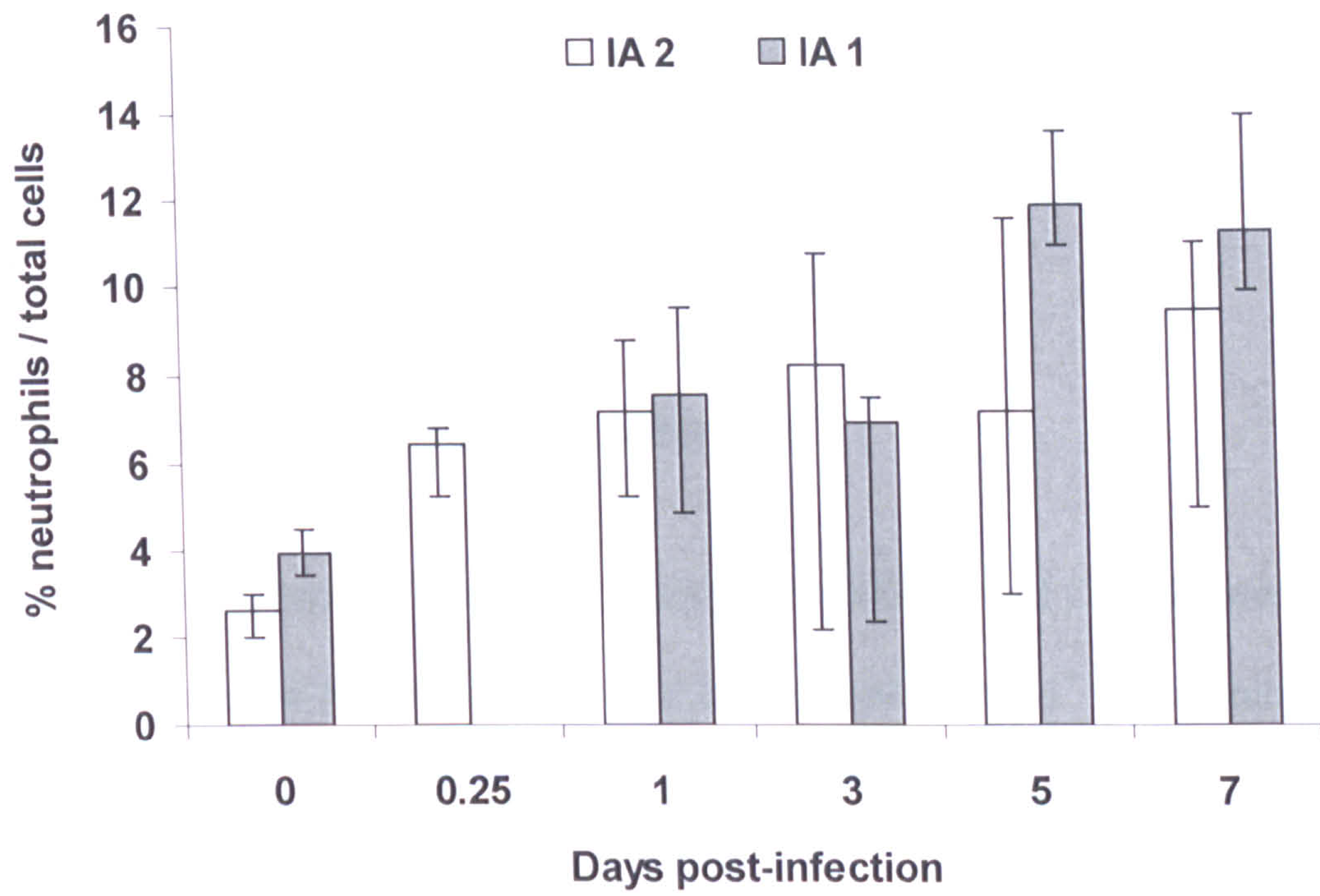


Figure 4.4: Splenic neutrophil populations during the first 7 days post-infection with *B. mallei* during Immune Analysis (1) and Immune Analysis (2). The median percentage of neutrophils (identified by FS:SS profiles alone) as a proportion of total cells collected per sample during Immune Analysis 1 (IA 1) (n=5/timepoint) or Immune Analysis 2 (IA 2) (n=8/timepoint). Error bars represent 99% confidence intervals.

day 5, the proportion of neutrophils increased in Immune Analysis (1) although in Immune Analysis (2) neutrophil percentages remained at similar levels to days 1 and 3. At day 7 p.i., neutrophil percentages remained elevated above control levels and were 10-12% of total cells per sample in both experiments (Figure 4.4).

4.3.1.2 Splenic Neutrophil activation

Cell surface markers involved in inflammation (CD54, CD11b and CD62L) were identified using plots displayed in Figure 4.2 and were investigated on Gr-1$^+$ neutrophils at 5 h and days 1, 3, 5 and 7 p.i. during Immune Analysis 2. A significant increase in CD11b expression ($p<0.01$) on Gr-1$^+$ neutrophils (Figure 4.5A) was accompanied by a significant decrease in CD62L expression ($p<0.01$) 5 h p.i. (Figure 4.5B). The increase in CD11b and decrease in CD62L expression was observed on approximately the same percentage (30%) of neutrophils. Expression of both markers returned to levels observed in uninfected mice 24 h p.i.. CD11b expression remained constant until an increase in expression was observed again at day 7 p.i. ($p<0.01$). A large decrease in CD62L expression (50% lower than control values) occurred at day 3 p.i. ($p<0.01$). Expression of CD62L recovered to control levels at days 5 and 7 p.i.. The percentage of neutrophils expressing both markers (CD62L$^+$CD11b$^+$) remained within control values until day 3 p.i. when the percentage of CD62L$^+$CD11b$^+$ neutrophils decreased significantly below control levels ($p<0.01$) (Figure 4.5C). An increase in neutrophils expressing both CD62L and CD11b occurred at day 5 and was higher than control values 7 days p.i. ($p<0.01$).

The activation marker CD54 (ICAM-1) was also investigated on neutrophils during *B. mallei* infection. Less than 2% of Gr-1$^+$ neutrophils expressed CD54 in uninfected mice (Figure 4.6). Within 5 h p.i., expression of CD54 on Gr-1$^+$ neutrophils increased to 24 ±

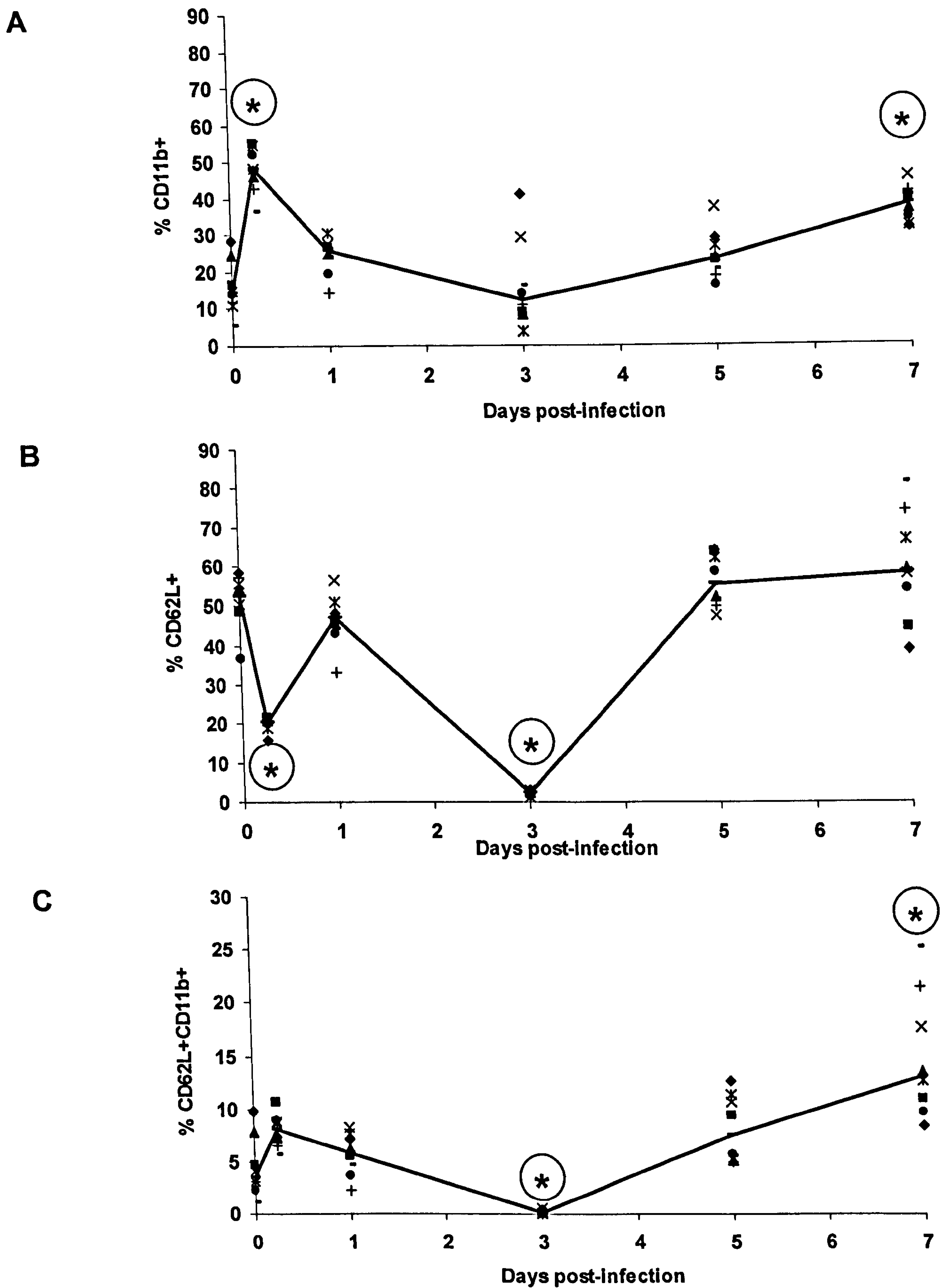


Figure 4.5: Activation status of splenic Gr-1^+ neutrophils during the first 7 days of *B. mallei* infection in Immune Analysis (2). Percentage of neutrophils expressing A) CD11b; B) CD62L; C) both CD62L and CD11b. Line indicates median. Individual points at each time-point represent individual BALB/c mice (n=8/timepoint). Boxes indicate animals with <10cfu /spleen. Circled asterisks indicate that activation is significantly elevated in comparison with day 0 (uninfected) ($p<0.01$).

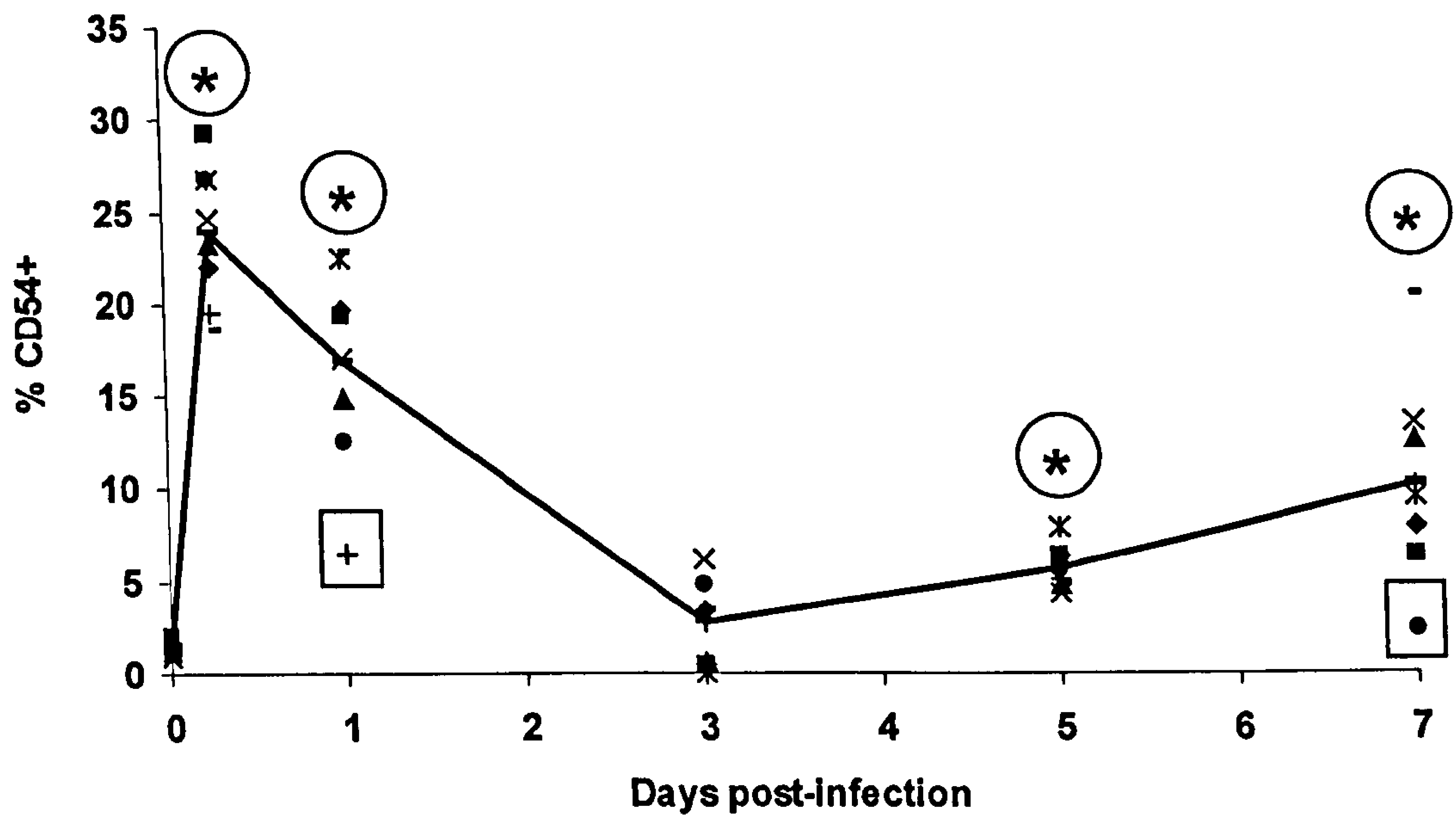


Figure 4.6: Activation status of splenic Gr-1+ neutrophils during the first 7 days of *B. mallei* infection in Immune Analysis (2). Percentage of neutrophils expressing CD54 during the first 7 days post-infection. Line indicates median. Individual points at each time-point represent individual BALB/c mice (n=8/timepoint). Boxes indicate mice with <10 cfu /spleen. Circled asterisks indicate that activation is statistically elevated ($p<0.01$) in comparison with day 0 (uninfected).

4% ($p<0.01$) and remained elevated 24 h p.i. (17 ± 6%) ($p<0.01$). CD54 expression returned to control levels 3 days p.i. but increased significantly above controls at days 5 and 7 p.i. ($p<0.01$) (Figure 4.6). CD54 expression at days 1 and 7 p.i. was related to bacterial colonisation of the spleen (Figure 4.6). Neutrophil responses characterised during Immune Analysis 1, revealed that on days 21-28 p.i., CD54 expression on neutrophils was elevated only in mice with splenic abscesses and high bacterial loads (Figure 4.7A & B).

4.3.1.3 Characterisation of splenic Gr-1+ populations

The neutrophil population (Gr-1^{Hi}) during *B. mallei* infection comprised the majority of splenic Gr-1^+ cells. Further characterisation of Gr-1^{Hi} cells with neutrophil forward scatter:side scatter properties demonstrated that they also co-expressed CD11b but did not express the macrophage marker F4/80 (Figure 4.8A). Other splenic cell populations also expressed Gr-1. These included a small Gr-1^+F4/80^+ population (Figure 4.8B), a Gr-1^+ $CD3^+$ T cell population and a rare population of Gr-1^+CD11c^+B220^+ cells which expressed markers characteristic of plasmacytoid dendritic cells. No Gr-1^+ NK cells were identified.

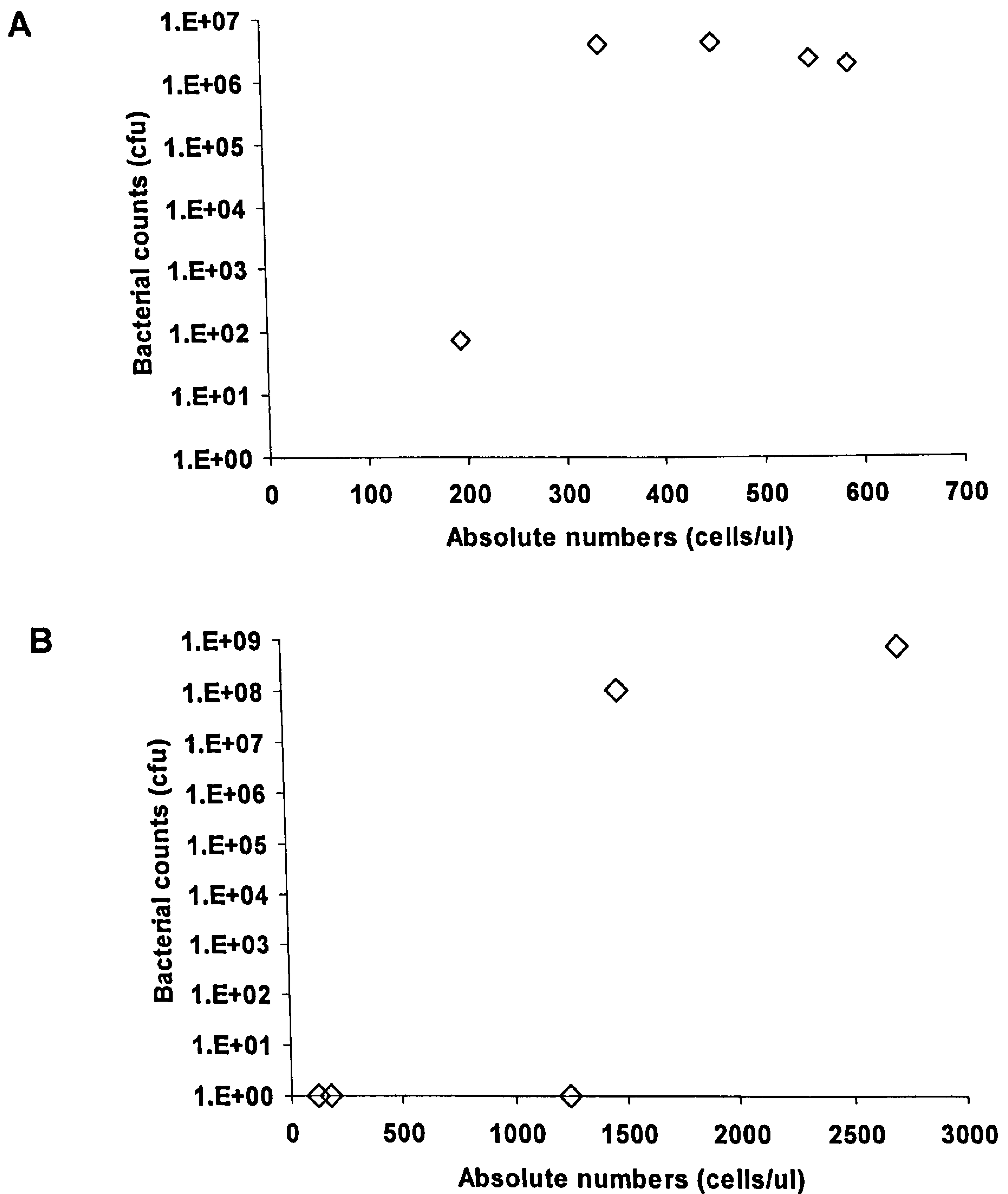
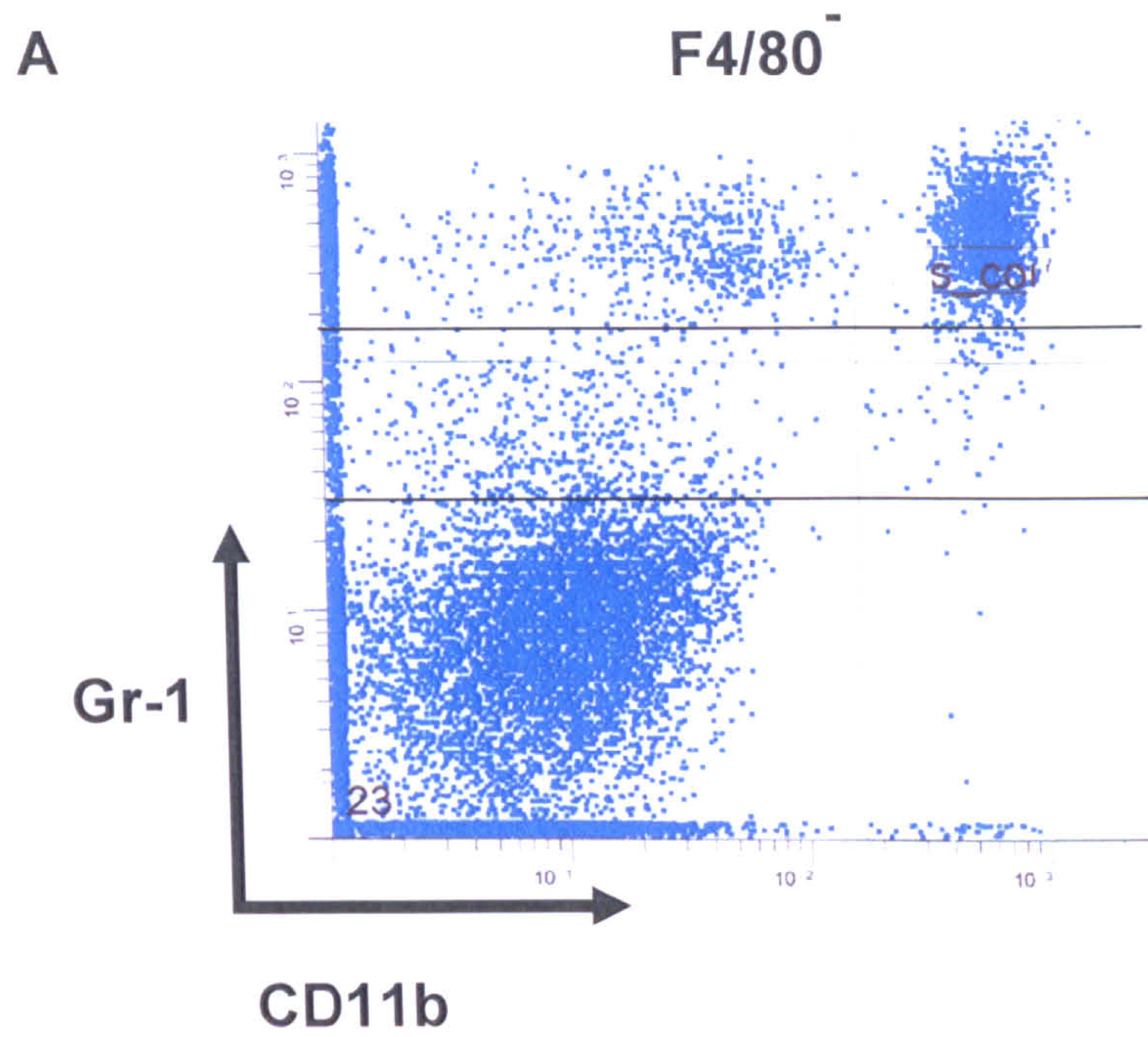


Figure 4.7: CD54$^+$ neutrophils are associated with abscessed spleens. The absolute number of CD54$^+$ neutrophils (7/4) and bacterial colonies identified in the spleens of individual BALB/c mice (n=5/timepoint) infected with *B. mallei* at A) 21 days post-infection and B) 28 days post-infection during Immune Analysis (1). Diamonds represent individual BALB/c mice.



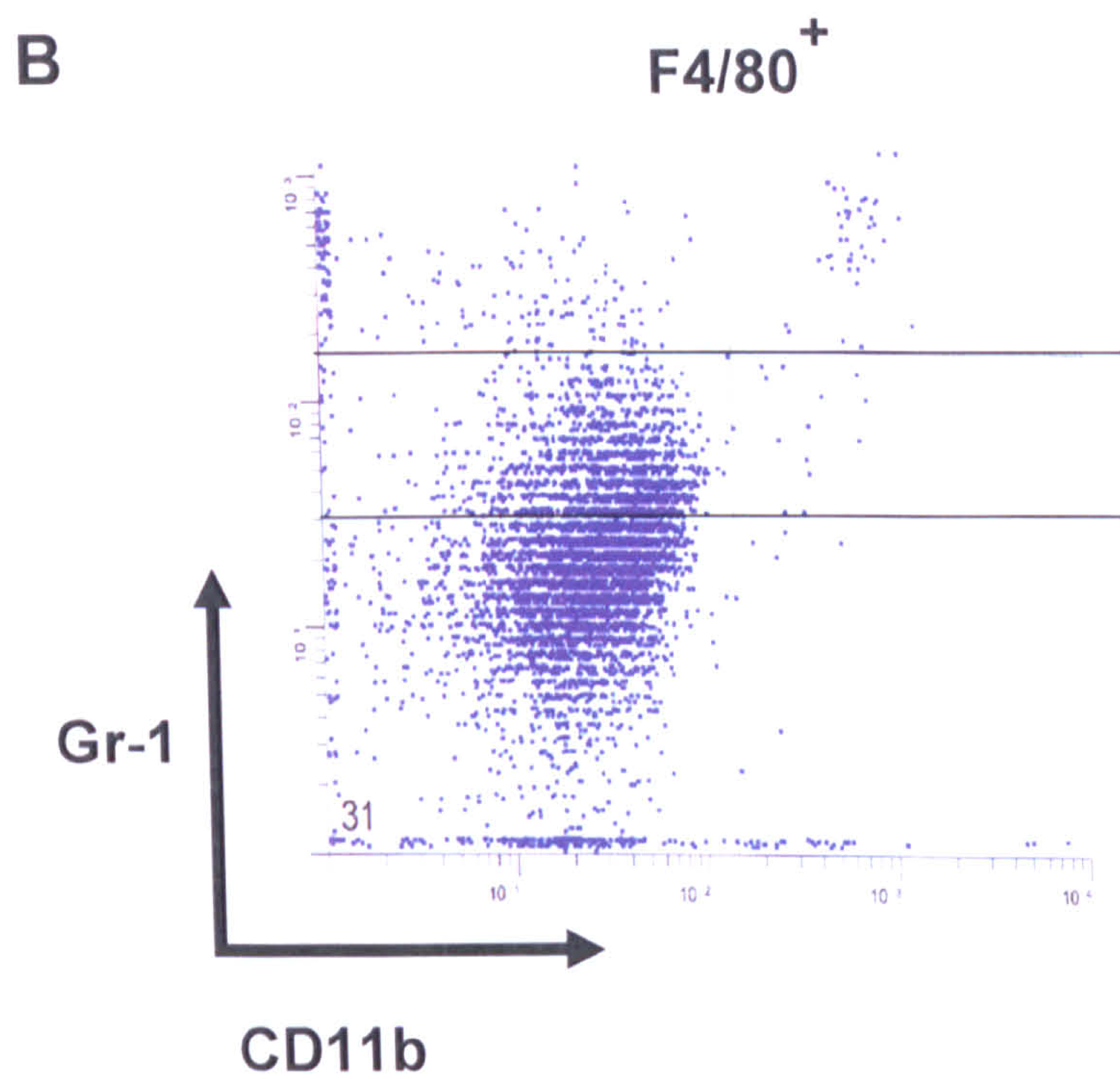


Figure 4.8: Gr-1$^+$ neutrophil and macrophage populations identified in the spleens of naïve BALB/c mice. Gr-1 and CD11b expression on A) F4/80$^-$ (neutrophils); B) F4/80$^+$ (macrophages) identified in splenic cell suspensions extracted from naïve BALB/c mice. This data is representative of splenic cell populations from 6 BALB/c mice.

4.3.1.3 Depletion of Gr-1$^+$ populations during B. mallei infection

The role of neutrophils and other Gr-1$^+$ cells during *B. mallei* infection was investigated using depleting antibodies. The majority of Gr-1$^+$ cell populations identified in the spleen were depleted by Gr-1 (RB6-8C5) antibodies but not Mac5 isotype-matched control antibodies ($p<0.05$) (Figure 4.9). Following *B. mallei* infection of BALB/c mice administered with Gr-1 antibodies 24 h prior to infection, all animals died within 5 days p.i. (Figure 4.10). Infected animals administered with isotype control (Mac5) antibodies did not die as a result of the infection.

4.3.2 Macrophage responses

4.3.2.1 Splenic Macrophage infiltration

Splenic macrophage numbers and their activation status were investigated during the first 7 days of infection (Immune Analysis 2). Macrophages were identified by their size and granularity (Figure 4.1) and their expression of F4/80, a macrophage lineage marker. The number of macrophages in the spleen increased significantly within 5 h p.i. continuing to increase by 24 h p.i. ($p<0.01$) (Figure 4.11). The number and percentage of macrophages remained significantly elevated from days 1-5 p.i. ($p<0.01$) (Figure 4.11) and macrophage numbers increased further 7 days p.i. in comparison with control mice ($p<0.01$) (Figure 4.11).

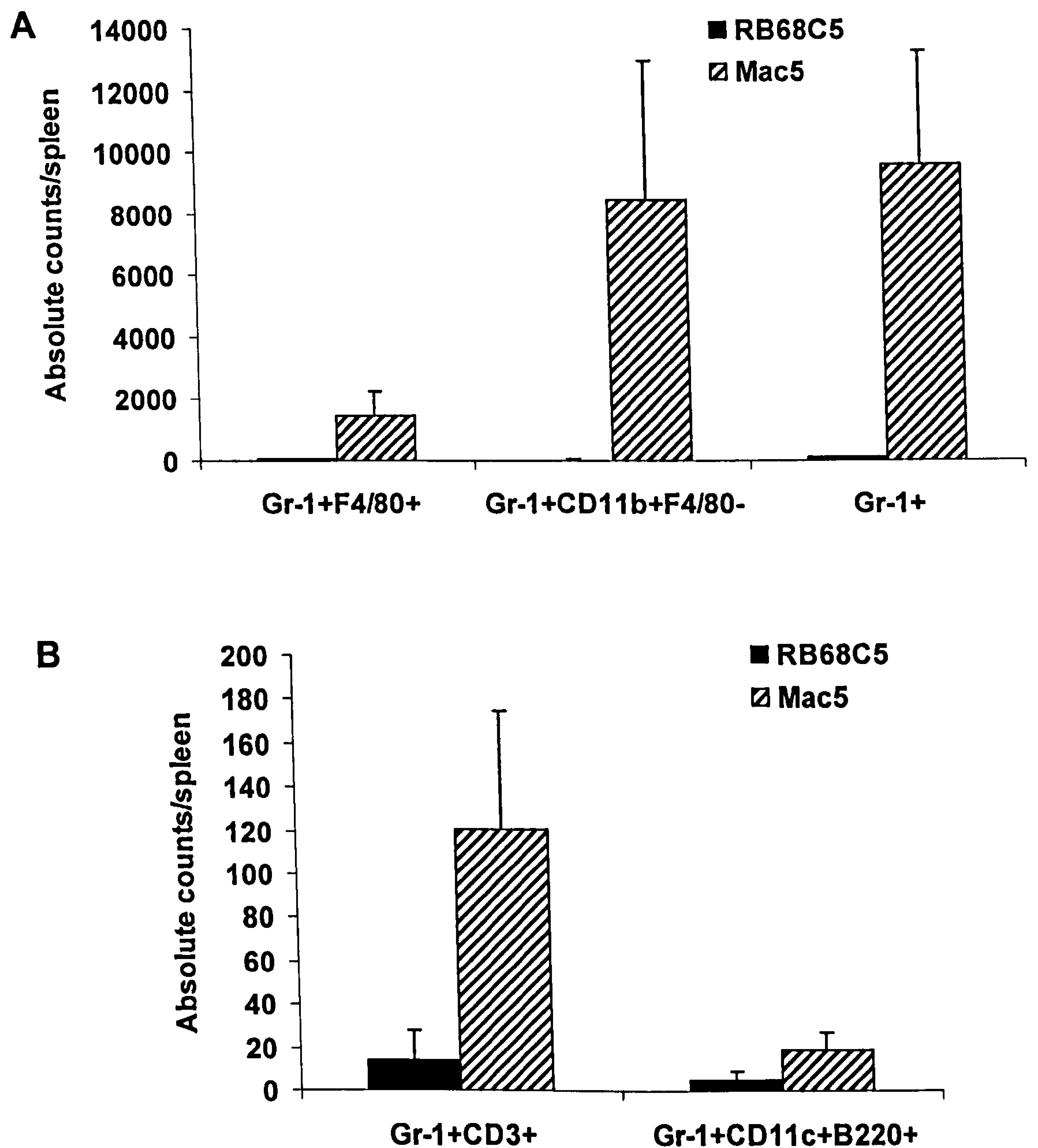


Figure 4.9: Splenic Gr-1$^+$ cell populations depleted by RB6-8C5 antibodies. Mean numbers of Gr-1$^+$ populations in the spleens of BALB/c mice (n=6/group) treated with either 500 µg RB6-8C5 depleting antibodies or isotype-matched Mac5 antibodies. A) Gr-1$^+$ CD11b$^+$F4/80$^-$ neutrophils and Gr-1$^+$ F4/80$^+$ macrophages; B) Gr-1$^+$CD3$^+$ T cell and Gr-1$^+$ CD11c$^+$B220$^+$ plasmacytoid dendritic cell populations. Error bars indicate standard deviation.

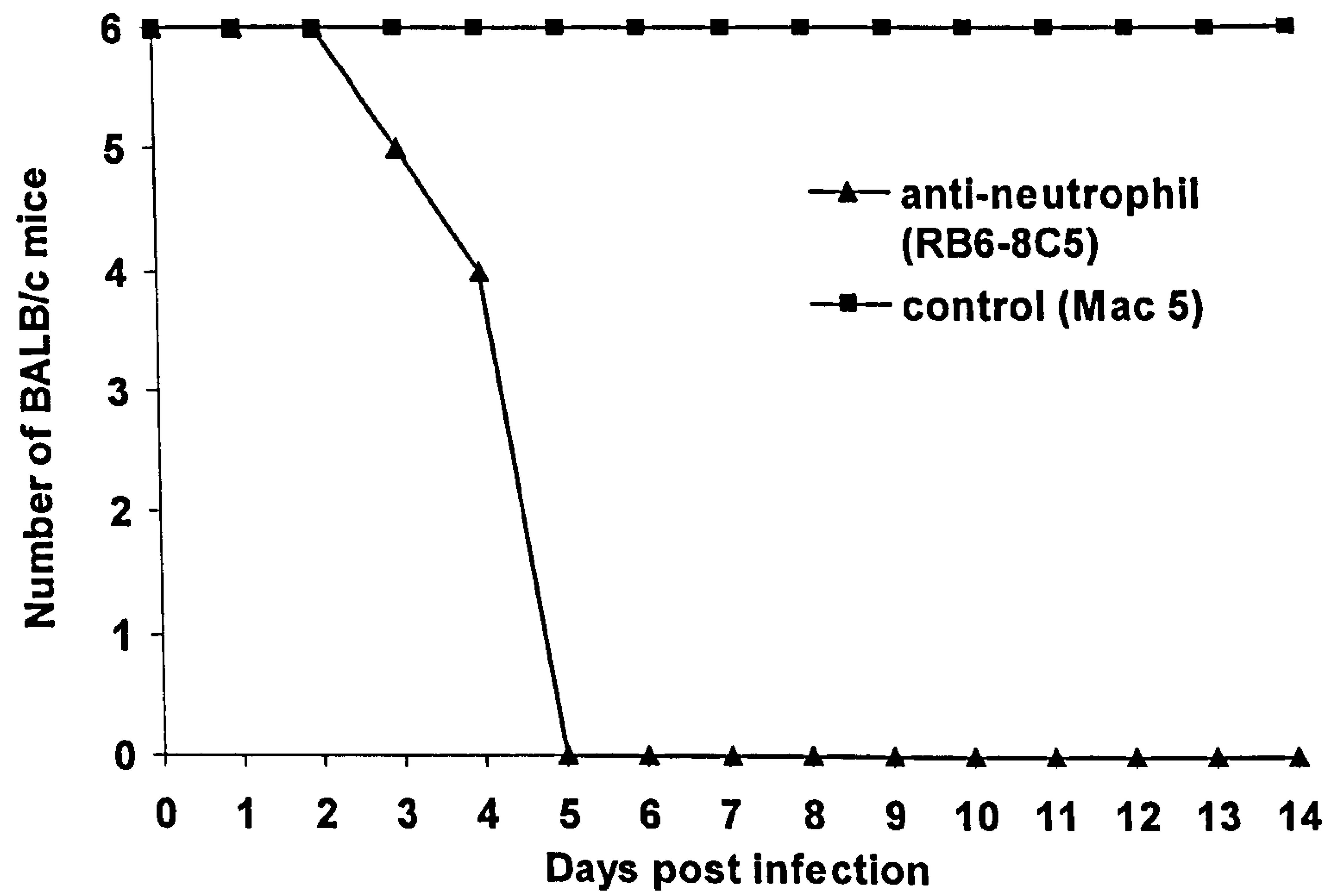


Figure 4.10: Survival of BALB/c mice following depletion of Gr-1^{+} cells with 500 μg of Gr-1 (RB6-8C5) antibody during *B. mallei* infection. Groups of BALB/c mice (n=6) were dosed with Gr-1 or isotype-control matched (Mac5) antibodies 24 hours prior to infection with *B. mallei.*

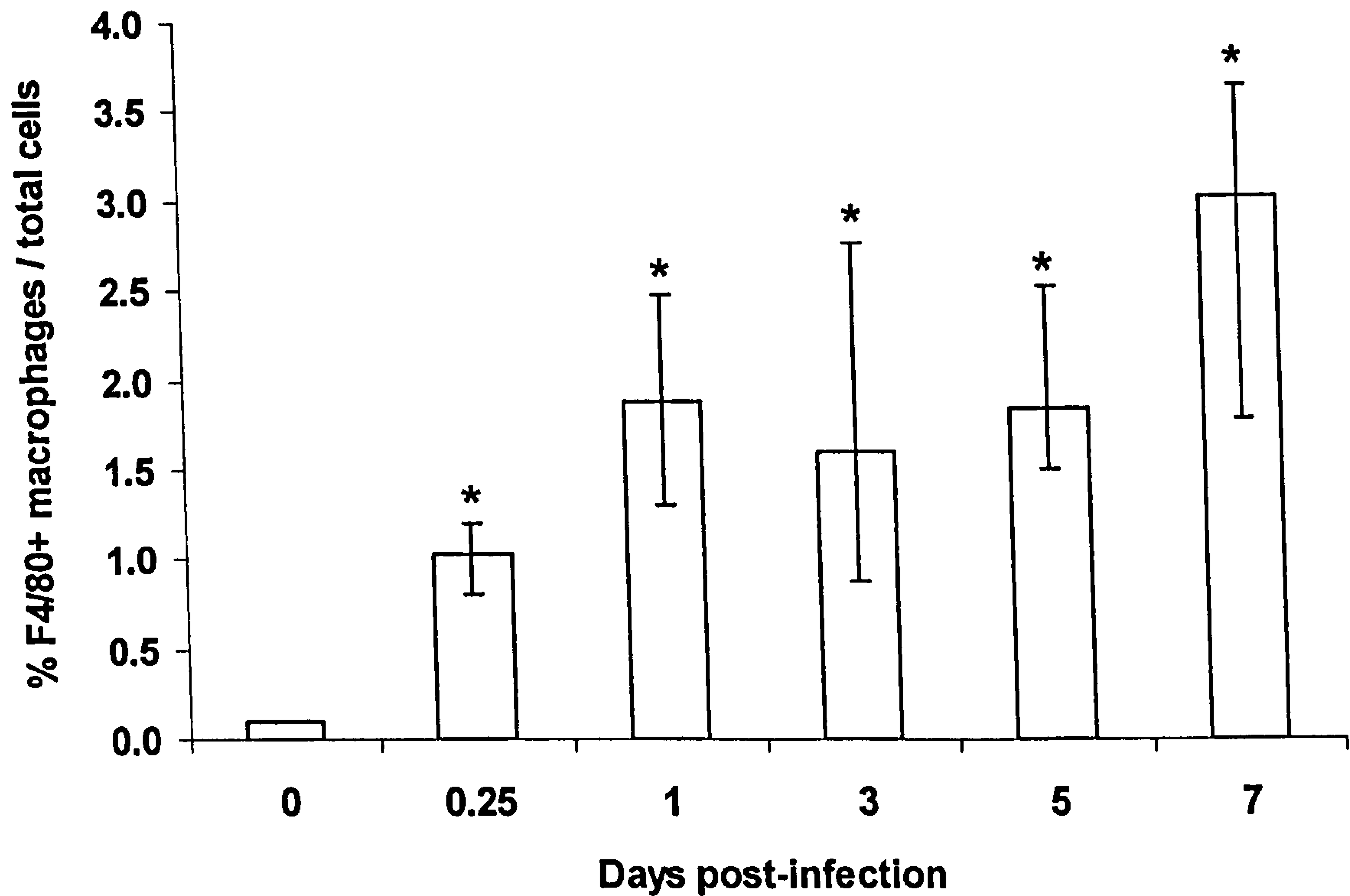


Figure 4.11: Splenic F4/80^{+} macrophage populations during the first 7 days of *B. mallei* infection in Immune Analysis (2). The median percentage of F4/80^{+} macrophages detected in spleens extracted from BALB/c mice (n=8/timepoint) over the time course of infection as a proportion of total cells collected per sample. Error bars indicate 99% confidence intervals. Asterisk(s) indicate statistical significance ($p<0.01$) in comparison with day 0 (uninfected).

4.3.2.2 *Splenic Macrophage activation*

Expression of the activation markers CD54, MHCII and CD11b on macrophages were investigated during *B. mallei* infection. Between 50 and 60% of resting macrophages in uninfected mice expressed CD54 at intermediate intensity whereas less than 10% expressed CD54 at high intensity. Increases in high intensity expression of CD54 ($CD54^{hi}$) were investigated to measure changes in the activation status of the macrophage population i.e. increases in the percentage of macrophages with high numbers of CD54 molecules per cell (Figure 4.12A). Significant increases in the percentage of $CD54^{hi}$ macrophages occurred 5h p.i. ($p<0.01$) with further increases 24 h p.i ($p<0.01$) (Figure 4.12A). A decrease in the percentage of $CD54^{hi}$ macrophages occurred at day 3 p.i. to uninfected levels and then increased in number again at days 5 and 7 p.i. ($p<0.01$). The expression of MHCII was also investigated on macrophages by measuring expression of IA/IE, a haplotype of MHCII expressed in BALB/c mice. In resting macrophages, approximately 90% expressed IA/IE at intermediate levels although less than 20% expressed IA/IE at high levels. The number of macrophages expressing IA/IE at high levels (IA/IE^{hi}) was investigated during infection (Figure 4.12B). The percentage of IA/IE^{hi} macrophages increased above control values 24 h p.i. ($p<0.01$). The percentage of IA/IE^{hi} macrophages decreased at day 3 p.i. but was elevated 5 days p.i. ($p<0.01$) and remained elevated 7 days p.i. in 7/8 mice although this was not significant. The expression of CD11b on macrophages did not increase above control levels until 5 days p.i. ($p<0.01$) (Figure 4.13C).

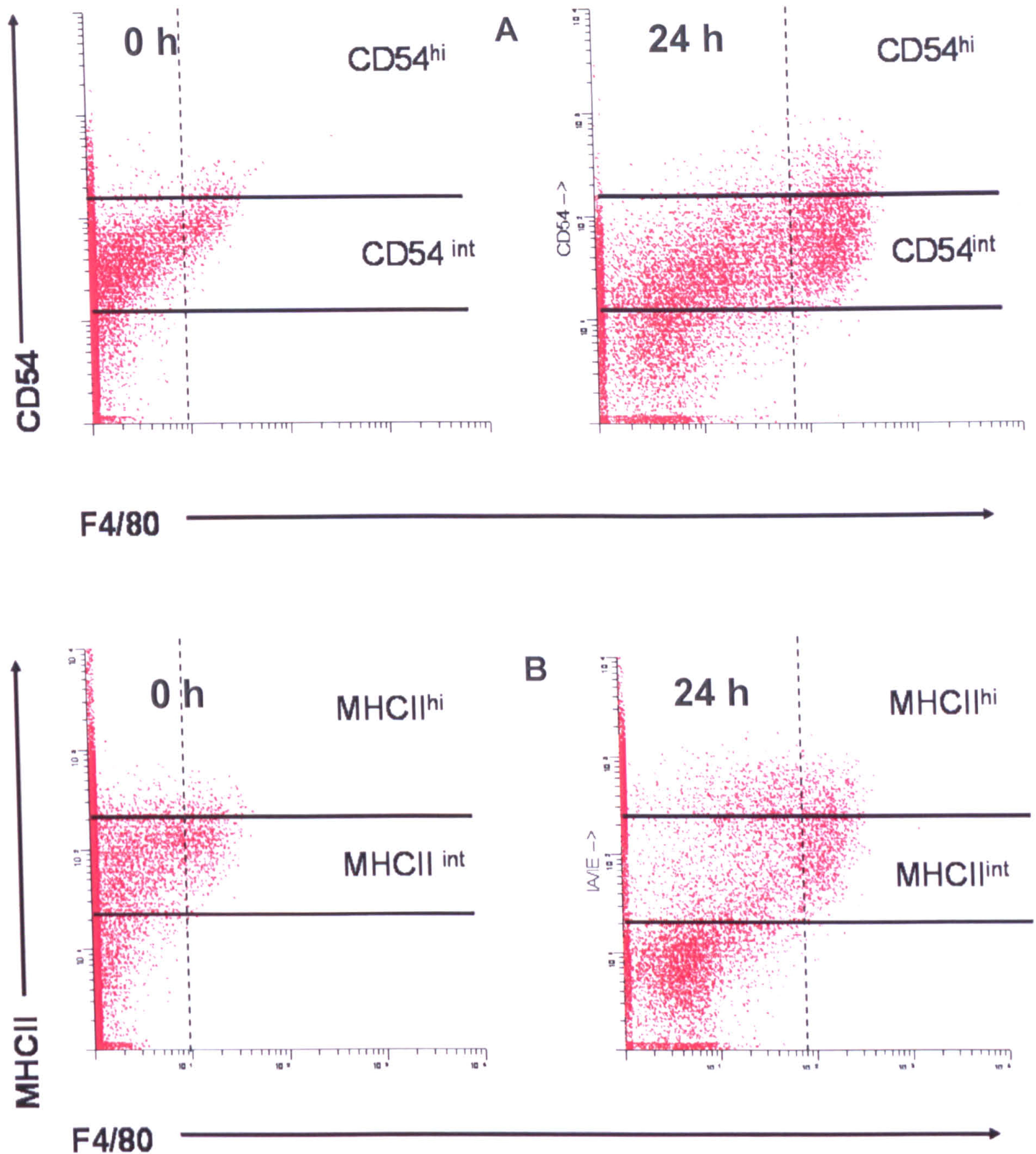


Figure 4.12: F4/80 macrophage populations expressing different levels of CD54 and MHCII. Flow cytometry plots showing high (hi) and intermediate (int) levels of expression of A) CD54 and B) MHCII in splenic F4/80^{+} macrophages from uninfected BALB/c mice (0 h) and mice infected with *B. mallei* 24 h p.i. (24 h) during Immune Analysis 2. This data is representative of individual BALB/c mice (n=8/timepoint). Dotted line represents the fluorescence intensity of the F4/80 isotype control. Data to the right of this line indicates F4/80^{+} cells.

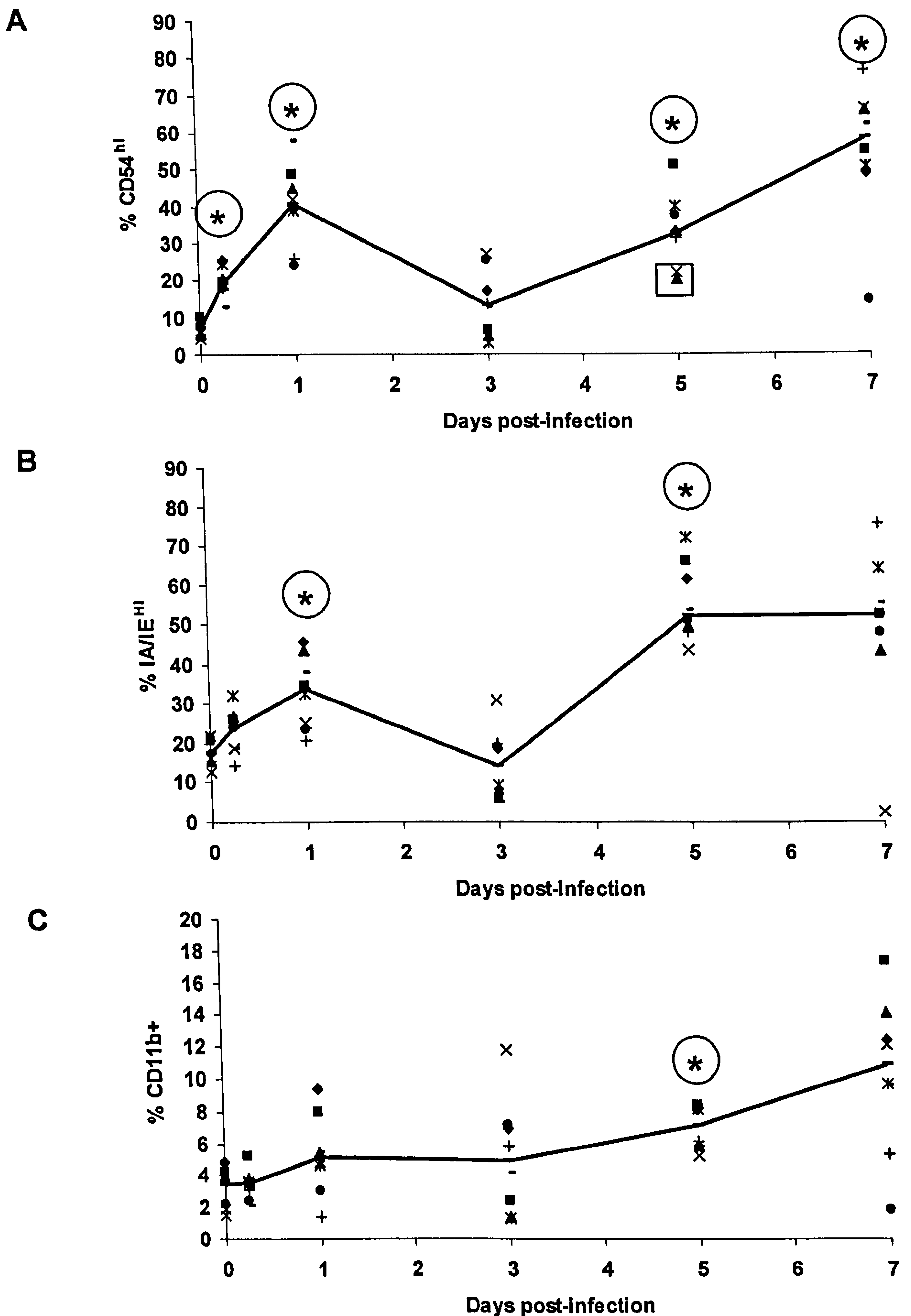


Figure 4.13: Activation status of splenic F4/80^{+} macrophages during the first 7 days of *B. mallei* infection in Immune Analysis (2). Percentage of macrophages expressing A) high intensity CD54; B) high intensity IA/IE (MHC II); C) CD11b. Line indicates median. Individual points at each time-point represent individual BALB/c mice (n=8/timepoint). Boxes indicate animals with <10cfu /spleen. Circled asterisks indicate that activation is statistically elevated (p<0.01) in comparison with day 0 (uninfected).

4.4 Discussion

Characterisation of neutrophil and macrophage responses in the spleen was performed in order to investigate the host's innate cellular immunological response to *B. mallei* infection. The spleen is composed of a number of resident cell populations including a small percentage of neutrophils and macrophages. Within 24 hours following infection, the number of Gr-1$^+$ neutrophils and macrophages in the spleen had doubled. Influx of neutrophils and macrophages into the spleen has previously been reported during *B. mallei* infection following histological examination (Fritz *et al*, 2000) and also occurs during infection with other intracellular bacteria including *S. typhimurium* (Kirby *et al*, 2002) and *L. monocytogenes* (Conlan, 1997). Infiltration of these phagocytic cells into the spleen suggests that they are involved in the host immune response to *B. mallei* at this site of infection.

Gr-1 is commonly used as a marker for identification of neutrophils (Tateda *et al*, 2001), however, another antibody (7/4) may also be used for identifying neutrophils. This antibody was found to have very low expression in BALB/c mice in Immune Analysis (1), therefore, Gr-1 was used in Immune Analysis (2) to refine the analysis of the neutrophil population. Examination of the size and granularity of neutrophils alone lead to a higher percentage of neutrophils being identified in comparison with those identified using Gr-1. A number of other cells including some monocytes, eosinophils and blasting T cells share the same FS:SS characteristics as neutrophils which may have lead to a higher percentage of neutrophils being identified using their size and granularity alone. Comparison of the neutrophil population between Immune Analysis 1 and 2 was performed using FS:SS characteristics alone. A comparable proportion of neutrophils were identified in the two experiments showing reproducibility of the analysis at each timepoint except day 5 p.i. when a higher proportion of neutrophils were identified in Immune Analysis 1. This

discrepancy could be addressed in future studies, although the use of Gr-1 should be used to identify the neutrophil population.

Analysis of the macrophage/monocyte population could not be compared between Immune Analysis (1) and (2). In Immune Analysis (1), CD14 (Lipopolysaccharide receptor) was used, however, this was found to be weakly expressed on macrophages and did not provide a coherent picture of the macrophage population. Use of the antibody for F4/80 allowed a better defined macrophage population to be discerned in Immune Analysis (2). A comparison of the macrophage/monocyte population between the 2 experiments, based on FS:SS profile alone, was not possible as the size and granularity of this cell type is more diffuse. F4/80 should be used in future studies to identify the macrophage population and provide further evidence for reproducibility of the macrophage data discerned in Immune Analysis (2).

Neutrophils infiltrate tissues from the blood using cell-cell interactions between adhesion molecules on the neutrophil and the endothelial lining of blood vessels. CD62L (L-selectin) is involved in adhesion of neutrophils to epithelial surfaces and rolling across the surface of blood vessels. CD62L expression on neutrophils decreased dramatically 5h p.i.. Loss or 'shedding' of CD62L during infection occurs following neutrophil migration into the tissue possibly to capture migrated cells (Kishimoto *et al*, 1989) and to limit leukocyte aggregation in the tissues (Walcheck *et al*, 1996). The loss of CD62L expression coincides with neutrophil influx into the spleen 5 h p.i.. By 24 h p.i., CD62L expression returned to control levels. Neutrophil numbers did not change significantly between 5 and 24 h p.i. suggesting that the neutrophils entering the spleen 5 h p.i. remained *in situ.* Concurrent increases in CD11b expression were also observed 5 h p.i.. CD11b is also involved in neutrophil migration by interacting with CD54 on endothelial cells allowing migration from the blood vessels into the tissues (Agace *et al*, 1995). Concurrent increases in CD11b

expression with decreased CD62L expression have also been observed during infection (Wang *et al*, 1998) possibly highlighting the differing roles of these receptors in neutrophil adhesion and migration during *B. mallei* infection. CD11b is also the receptor for the C3 component of the complement cascade aiding neutrophil (Egan & Gordon, 1996) and macrophage (Clemens *et al*, 2005) phagocytosis by complement mediated fixation of bacteria. Upregulation of CD11b may also aid uptake of *B. mallei* into these cells early in infection. A loss of CD62L expressing neutrophils was again observed 3 days p.i. although the percentage of neutrophils and activated CD11b (and CD54) expression returned to control levels. The reason for the loss of CD62L$^+$ neutrophils is unknown, although it may be a mechanism for retaining neutrophils with the spleen to aid the fight against infection.

Upregulation of CD54 (ICAM-1) on both macrophages and neutrophils 5 h p.i. demonstrated that these cells had become activated as a result of the infection. CD54 is an adhesion molecule involved in inflammatory responses. Upregulation of CD54 on neutrophils (Wang *et al*, 1998) and macrophages (Ghosh & Saxena, 2004) occurs during inflammation and infection. CD54 may be involved in aggregation of neutrophils during the inflammatory response (Wang *et al*, 1997) although its role on macrophages within hours of infection is uncertain. The recognition of bacterial components by pattern recognition receptors on macrophages and neutrophils allows them to respond to infection immediately following contact with the pathogen. The receptors involved in recognition of *B. mallei* are unknown, however, the early (5 h p.i.) activation of macrophages and neutrophils suggests that these cells interact directly with the bacillus. CD54 expression was also upregulated on neutrophils during the later stages of infection and was associated with abscesses. This suggests that an ongoing inflammatory response is occurring in abscessed tissue although it appears to be ineffective in controlling bacterial growth.

In addition to their role in innate responses, macrophages are also involved in the development of adaptive immune responses by presenting processed antigen (in association with MHCII on the macrophage surface) to $CD4^+$ T cells (Neild *et al*, 2005). IFN-γ is a potent macrophage activator that specifically initiates gene expression of MHCII through signalling via the IFN-γ receptor on the macrophage surface (Schroder *et al*, 2004). Increased macrophage MHCII expression, observed 24 h p.i., may signify the involvement of early IFN-γ responses during *B. mallei* infection. Increased expression of macrophage MHCII was also observed 5 and 7 days p.i.. Increased expression of MHCII suggests that *B. mallei* infected macrophages are capable of presenting antigen to T cells and stimulating cell-mediated immunity. CD54 expression was also upregulated at days 5 and 7 p.i. suggesting a potential role for CD54 in antigen presentation during *B. mallei* infection. Macrophage CD54 acts as a costimulatory molecule during MHC I presentation of antigen (Lebedeva *et al*, 2005) to $CD8^+$ T cells. These cells are cytotoxic for host cells infected with intracellular pathogens and may therefore be involved in protective responses during *B. mallei* infection.

Macrophage activation and influx to the spleen during infection suggests that macrophages may play a role in *B. mallei*-mediated immunity. Studies involving inducible nitric oxide synthase (iNOS) knockout mice revealed that nitric oxide was not important for survival or control of bacterial replication during *B. mallei* infection (Lukaszewski, 2004). Nitric oxide is one of a number of mechanisms of macrophage-mediated pathogen killing. *B. pseudomallei*, a close relative of *B. mallei*, is able to subvert macrophage-mediated killing by nitric oxide to survive and multiply inside macrophages (Utaisincharoen *et al*, 2001). Macrophage (and neutrophil) responses were downregulated 3 days p.i. suggesting that *B. mallei* may be modulating the host immune response and, therefore, the relationship between *B. mallei* and macrophages during infection should be investigated further.

The antibody RB6-8C5 (anti-Gr-1) has been used during infection studies to demonstrate the role of neutrophils in protection against infection with intracellular pathogens including *L. pneumophila* (Tateda *et al*, 2001), *F. tularensis* (Sjostedt *et al*, 1996) and *S. typhimurium* (Vassiloyanakopoulos *et al*, 1998). Therefore, these antibodies were used in this study to determine the role of neutrophils during *B. mallei* infection. An essential role for Gr-1^+ cells was demonstrated during innate responses to *B. mallei* infection following treatment with RB6-8C5 antibodies. The predominant population depleted by RB6-8C5 antibody were neutrophils and, therefore, their contribution to Gr-1-mediated immunity to *B. mallei* is likely to be important. However, examination of spleen cells from naïve mice found that a number of Gr-1^+ cell types were depleted. These included Gr-1^+ T cell, macrophage and plasmacytoid dendritic cell (pDC) populations. Gr-1 is expressed on a subset of memory $CD8^+$ T cells which can be depleted by RB6-8C5 treatment (Matsuzaki *et al*, 2003). Memory $CD8^+$ T cells can respond to cytokines released very early in infection to produce IFN-γ and other cytokines to aid the innate immune response in response to *B. pseudomallei* (Lertmemongkolchai *et al*, 2001). Recruitment of Gr-1^+ macrophages into the tissues during *T. gondii* infection has also been found to be important in protective immunity by secreting type 1 cytokines (Robben *et al*, 2005) Murine pDCs express Gr-1 and are rapidly able to recognise and respond to bacterial components (Nakano *et al*, 2001), initiating innate immune responses by activating NK cells and macrophages. Consequently depletion of all these Gr-1^+ populations by RB6-8C5 may affect the outcome of *B. mallei* infection and their role requires further examination.

In this chapter, activation and infiltration of neutrophils and macrophages was identified in the spleen, demonstrating that these cells are responding to *B. mallei* infection. Gr-1^+ cells were found to be essential for protection in innate responses during *B. mallei* infection, although the contribution of neutrophils to this effect was likely to be important, it could not be characterised.

Chapter 5

Characterisation of lymphocyte responses during *B. mallei* infection in BALB/c mice.

5.1 Introduction

T and B cells are involved in both innate and adaptive responses to infection and have a range of functions in immunity. T cells differentiate in response to cytokines secreted during innate immune responses to polarise the immune response towards type 1 (IL-12, IFN-γ) or type 2 (IL-4) immunity depending on the infective pathogen. $CD8^+$ T cells and $CD4^+$ Th1 cells are particularly important in immunity to intracellular pathogens through cytotoxic killing and secretion of IFN-γ, respectively. Some T cell subsets are able to secrete cytokines non-specifically in response to bacterial components and cytokines, thus contributing to innate immune responses (Kambayashi *et al*, 2003). The interaction of T cells with B cells and other antigen presenting cells, including macrophages and dendritic cells, allows the development of effector and antigen-specific T cell populations (Constant *et al*, 1995). Antigen-specific responses are important for killing pathogens not cleared by the initial, innate responses and in the development of memory responses to protect against further challenge with the pathogen.

B cells are involved in the processing and presentation of antigen to T cells in the formation of specific, adaptive immune responses. B cells secrete opsonising antibody in response to type 1 cytokines early in infection to enhance the phagocytosis of pathogens by neutrophils and macrophages (Kozel *et al*, 2004). The clonal expansion of plasma cell populations which secrete specific antibody to neutralise pathogens during extracellular stages of infection is supported by the presentation of processed antigen by B cells to T cells. The aims of this chapter were to determine:

- The effect of *B. mallei* on host T and B cell responses by measuring the infiltration and activation profiles of these cells during infection.
- The effectiveness of host T cell responses in protecting against *B. mallei* using depleting antibodies.

5.2 Methods

T and B cell responses were measured by flow cytometry following staining with lineage markers and markers of activation. Data described from the first 7 days of *B. mallei* infection was derived predominantly during Immune Analysis (2) and where possible, this data was compared with data derived from Immune Analysis (1). Data relating to 14-36 days of infection was determined during Immune Analysis (1) (Table 2.2).

$CD4^+$ and $CD8^+$ T cell populations were depleted 72 h and 24 h prior to infection following i.p. injection with YTS169 and/or YTS191 depleting antibodies or Mac5 isotype-matched control antibodies. Mice were then re-dosed with antibodies every 5 days following infection. BALB/c mice were then infected with 10^6 cfu *B. mallei*. The specificity of CD4 and CD8 antibodies was assessed following depletion with YTS169 and/or YTS191 antibodies or Mac5 control antibody in naïve BALB/c mice. T cell populations were identified in the spleen by flow cytometry.

5.3 Results

5.3.1 Lymphocyte responses

The responses of lymphocytes (T cells and B cells) were investigated. Distinct lymphocyte populations share similar size and granularity profiles (Figure 4.1) and were identified as individual populations by their exclusive expression of CD3 (T cells) (Figure 5.1A) and CD19 (B cells) (Figure 5.1B).

5.3.1.2 T cell and B cell infiltration

In Immune Analysis (2), $CD3^+$ T cells comprised 30% of the lymphocyte population in uninfected mice and this percentage of T cells remained at control levels 5 h, 24 h and 72 h p.i. (Figure 5.2). At days 5 and 7 p.i, the percentage of T cells was significantly decreased below control levels ($p<0.01$) (Figure 5.2). A similar kinetic profile of the T cell population as a percentage of total splenic cell composition during *B. mallei* infection was observed during both Immune Analysis (1) and Immune Analysis (2) (Figure 5.3): In uninfected mice $CD3^+$ T cells comprised 30% of the total splenic cell composition, however, by day 7 p.i., less than 20% of total splenic cells were $CD3^+$ (Figure 5.3).

In Immune Analysis (2), B cells comprised 50% of splenic cell composition in uninfected mice and almost 50% of the total lymphocyte population (Figure 5.2). The B cell population reduced by almost 50% within 5 h p.i. compared with uninfected mice ($p<0.01$) although it returned to control levels 24 h p.i. and remained at control levels 72 h p.i. (Figure 5.2). A further comparable reduction in the percentage of B cells occurred 5 days p.i. and the B cell population remained diminished 7 days p.i. ($p<0.01$) although the B cell population appeared to be recovering at this timepoint (Figure 5.2).

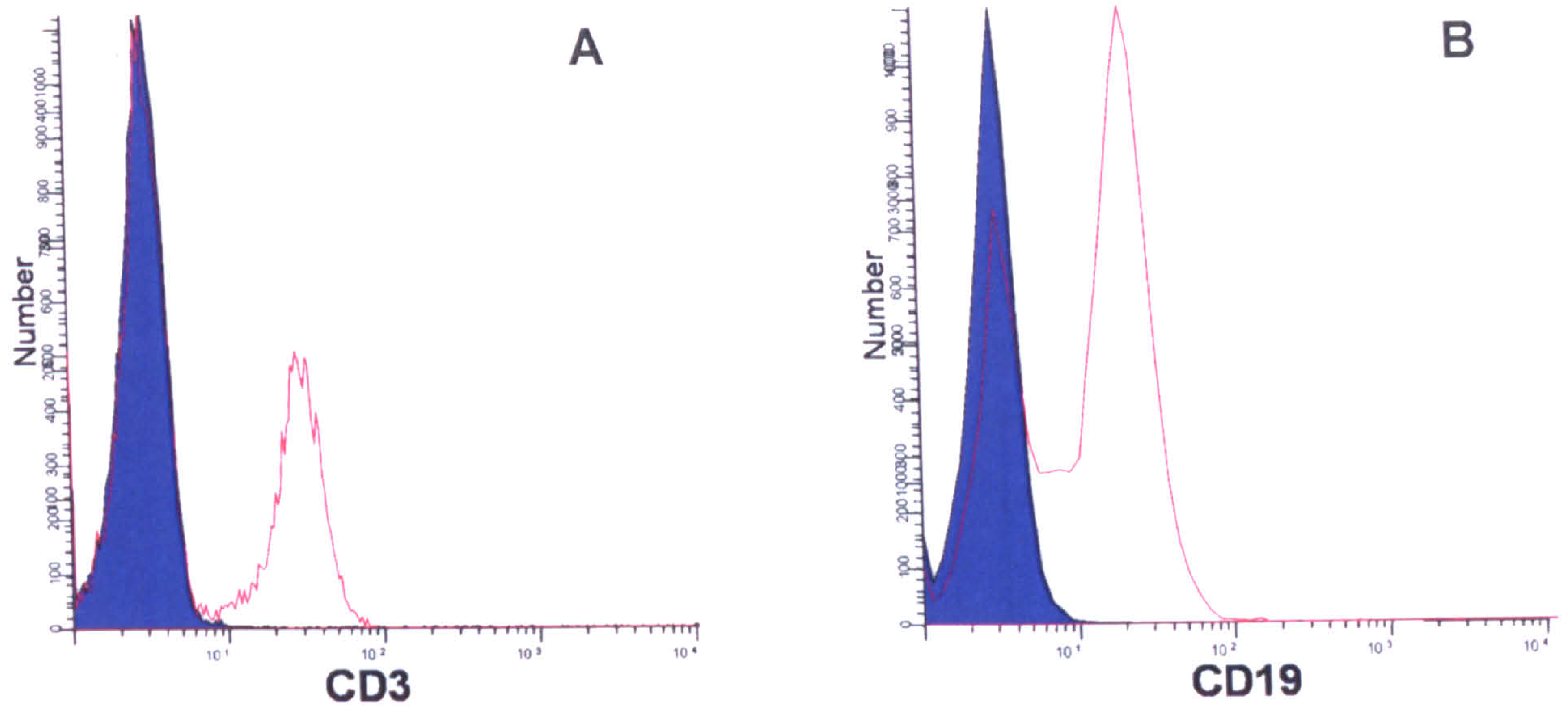


Figure 5.1: Representative flow cytometry histograms to indicate positive staining profiles of CD3$^+$ T and CD19$^+$ B cells during *B. mallei* infection. Splenic lymphocytes obtained from BALB/c mice following infection with *B. mallei* during Immune Analysis 2 were stained with antibodies specific for A) CD3; B) CD19; and appropriate isotype-matched control antibodies. Blue plots indicate negative staining determined using appropriate isotype controls and red plots indicate positive fluorescence staining.

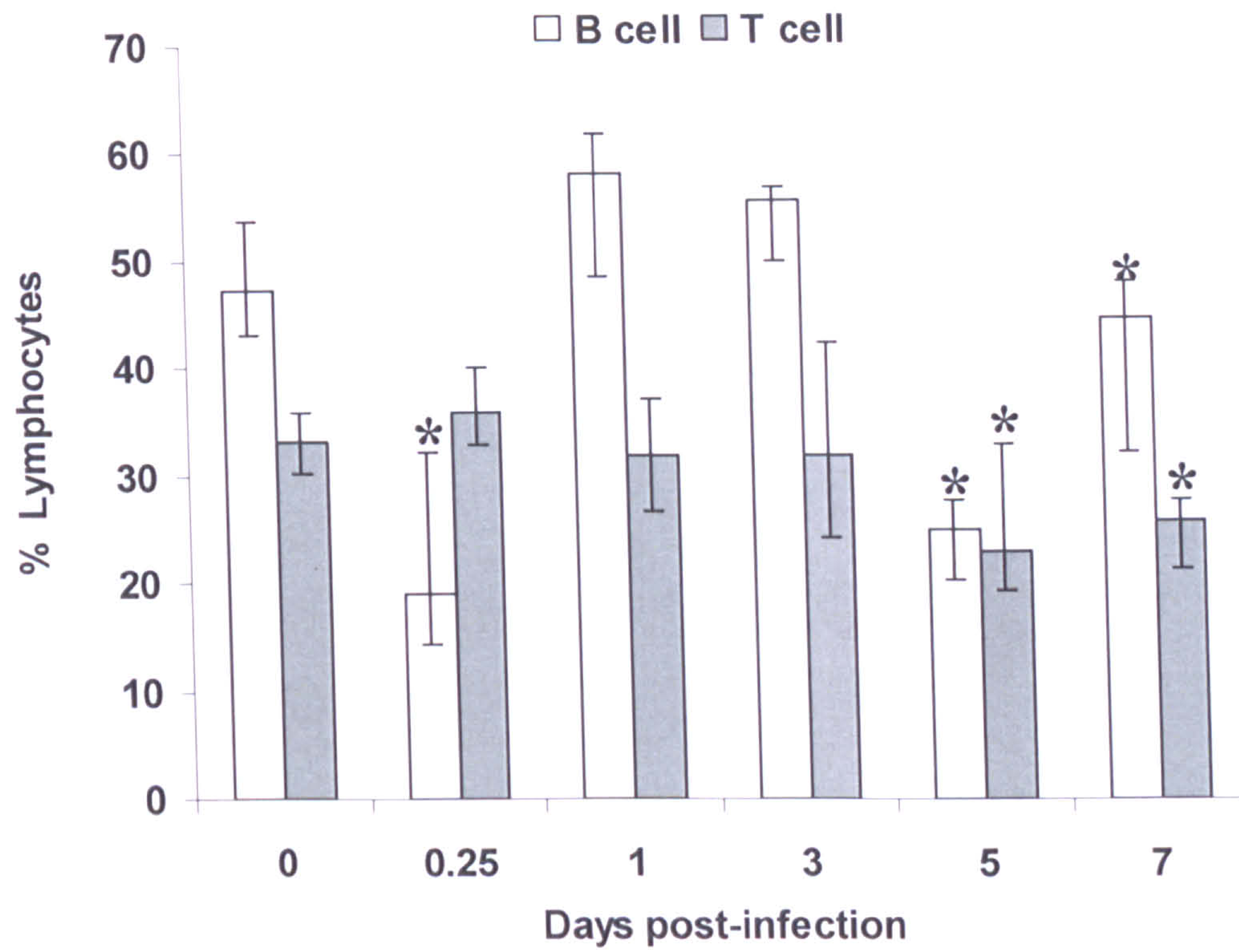


Figure 5.2: Splenic CD3$^+$ T cell and CD19$^+$ B cell populations during *B. mallei* infection (Immune Analysis (2)). The median percentage of splenic T cells and B cells within the lymphocyte population collected per sample. Spleens were extracted from BALB/c mice (n=8/timepoint) over the time course of infection. Error bars indicate 99% confidence intervals. Asterisk(s) indicate statistical significance ($p<0.01$) in comparison with day 0 (uninfected).

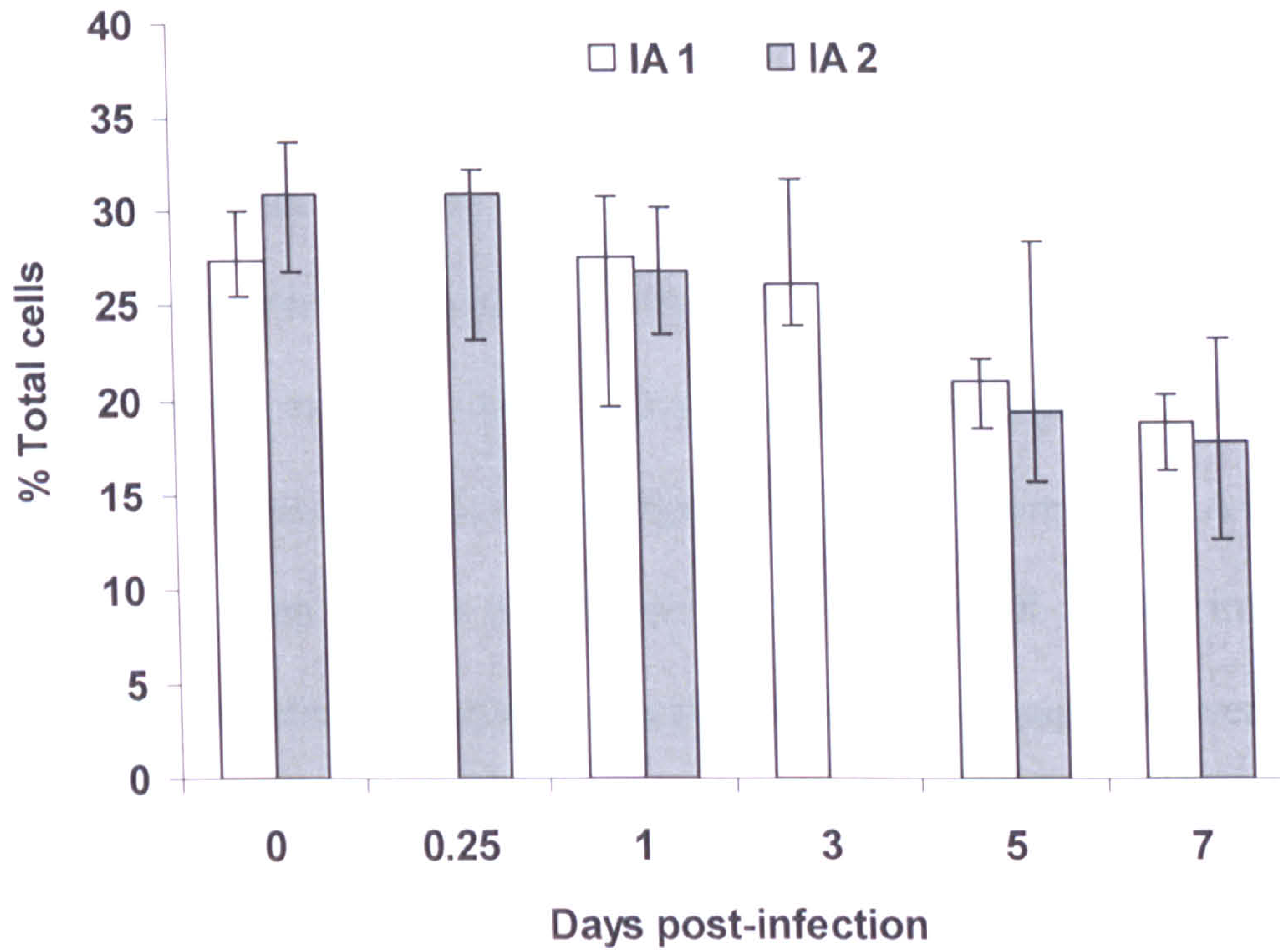


Figure 5.3: A comparison of the splenic $CD3^{+}$ T cell population in BALB/c mice during *B. mallei* infection in Immune Analysis (1) and Immune Analysis (2). The median percentage of splenic T cells detected as a proportion of total cells collected per sample. White bars indicate data collected from Immune Analysis (1) (IA 1) (n=5/timepoint) and grey bars indicate data collected from Immune Analysis (2) (IA 2) (n=8/timepoint). Error bars indicate 99% confidence intervals. Asterisk(s) indicate statistical significance ($p<0.01$) in comparison with day 0 (uninfected).

5.3.1.3 T cell activation

T cell activation was measured by cell surface expression of CD25, CD54 and CD44 on $CD3^+$ T cells (Figure 5.4). In Immune Analysis (2), the percentage of $CD25^+$ T cells decreased below uninfected control levels 5 h p.i. ($p<0.01$) (Figure 5.5A). The $CD25^+$ population returned to control levels 24 h p.i. and became significantly elevated 3 days p.i. ($p<0.01$) returning to control levels at days 5 and 7 p.i. (Figure 5.5A). A similar profile of CD25 expression on T cells during the first 7 days of *B. mallei* infection was also determined in data derived during Immune Analysis (1) although the overall percentage of T cells expressing CD25 was lower and CD25 expression was not higher than control levels 3 days p.i. (Figure 5.5B).

In Immune Analysis (2), CD54 expression on T cells was elevated above control levels 24 h p.i. ($p<0.01$) but was not different from control values at any other timepoint (Figure 5.6A). In Immune Analysis (1), CD54 expression was also elevated 24 h p.i. and was maximally elevated above control values 5 days p.i. ($p<0.01$) (Figure 5.6B). CD54 expression on $CD3^+$ T cells was associated with splenic abscesses from days 21-36 during Immune Analysis (1) and abscessed mice expressed higher levels of CD54 on T cells than non-abscessed mice (Figure 5.7).

The activation status of individual T cell subsets in Immune Analysis (2) was measured by high intensity expression of CD44 ($CD44^{Hi}$) on $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells. The proportion of $CD4^+$ T cells expressing $CD44^{Hi}$ increased above control levels 24 h p.i. and remained elevated at days 3 and 7 p.i. ($p<0.01$) (Figure 5.8A). Expression of $CD44^{Hi}$ on CD8 T cells was significantly lower 5 h p.i. ($p<0.01$) but returned to control levels 24 h p.i.. $CD44^{Hi}$ expression on CD8 T cells decreased again 7 days p.i. ($p<0.01$) (Figure 5.8B).

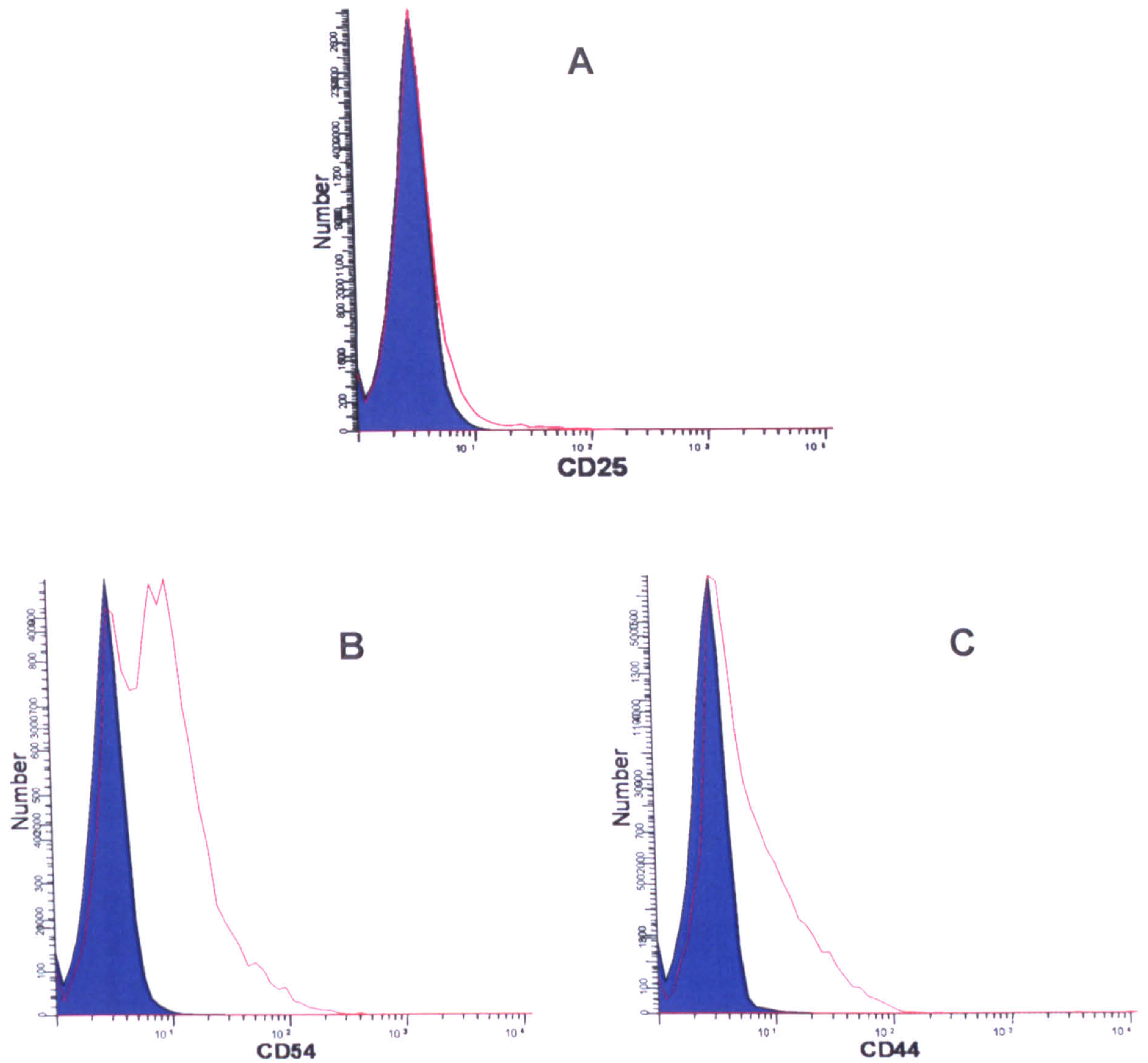


Figure 5.4: Representative flow cytometry histograms indicating positive staining profiles of T cell activation markers during *B. mallei* infection. Splenic CD3^{+} T cells obtained from BALB/c mice following infection with *B. mallei* during Immune Analysis (2) were co-stained with antibodies specific for A) CD25; B) CD54 and C) CD44. Blue plots indicate negative staining determined using appropriate isotype controls and red plots indicate positive fluorescence staining.

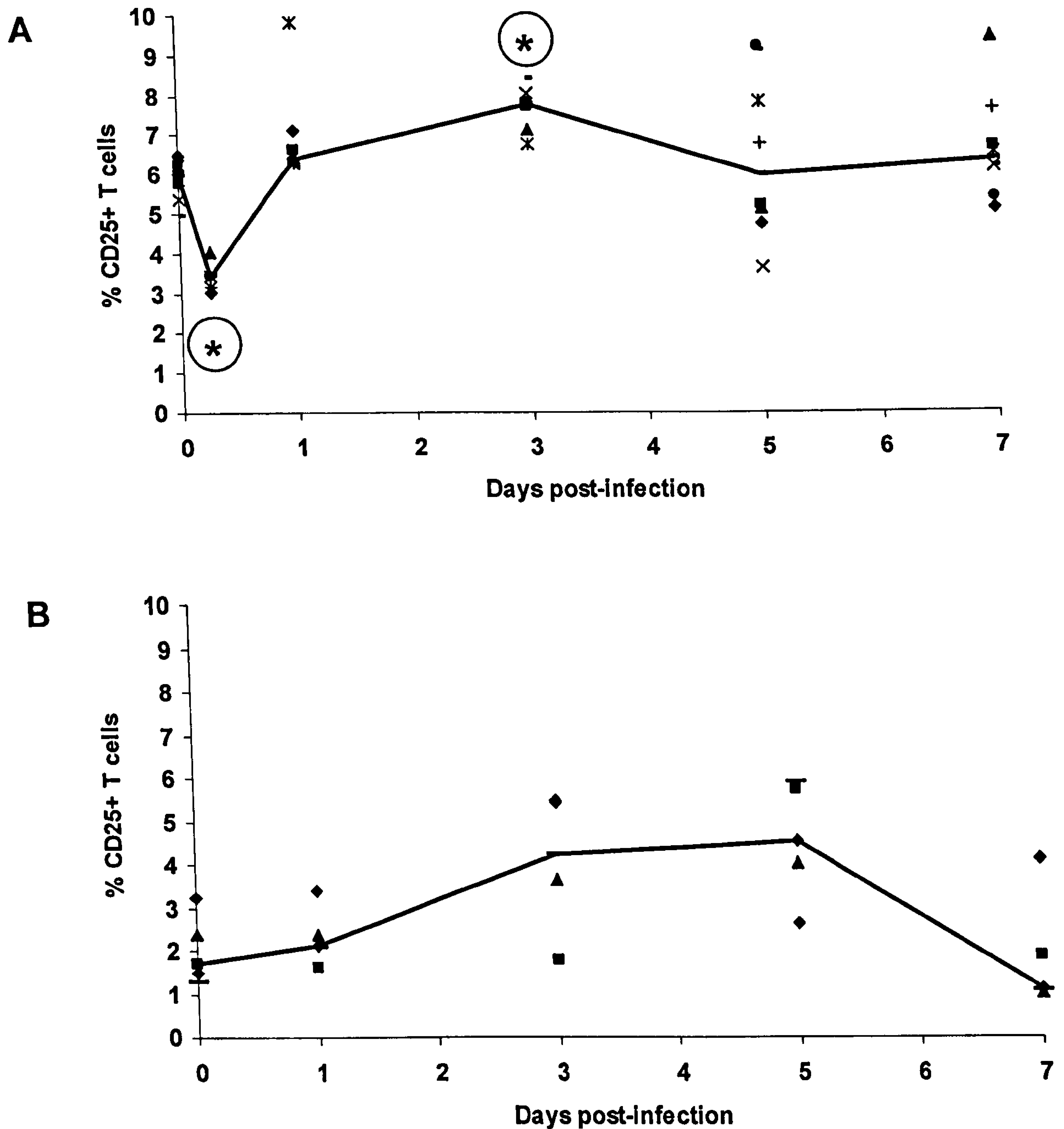


Figure 5.5: CD25 expression on splenic CD3^{+} T cells during the first 7 days of *B. mallei* infection in Immune Analysis (1) and Immune Analysis (2). Percentage of T cells expressing CD25 during A) Immune Analysis (2) (n=8/timepoint); B) Immune Analysis (1) (n=5/timepoint). Line indicates median and individual points at each time-point represent individual BALB/c mice. Circled asterisks indicate statistical significance ($p<0.01$) in comparison with day 0 (uninfected).

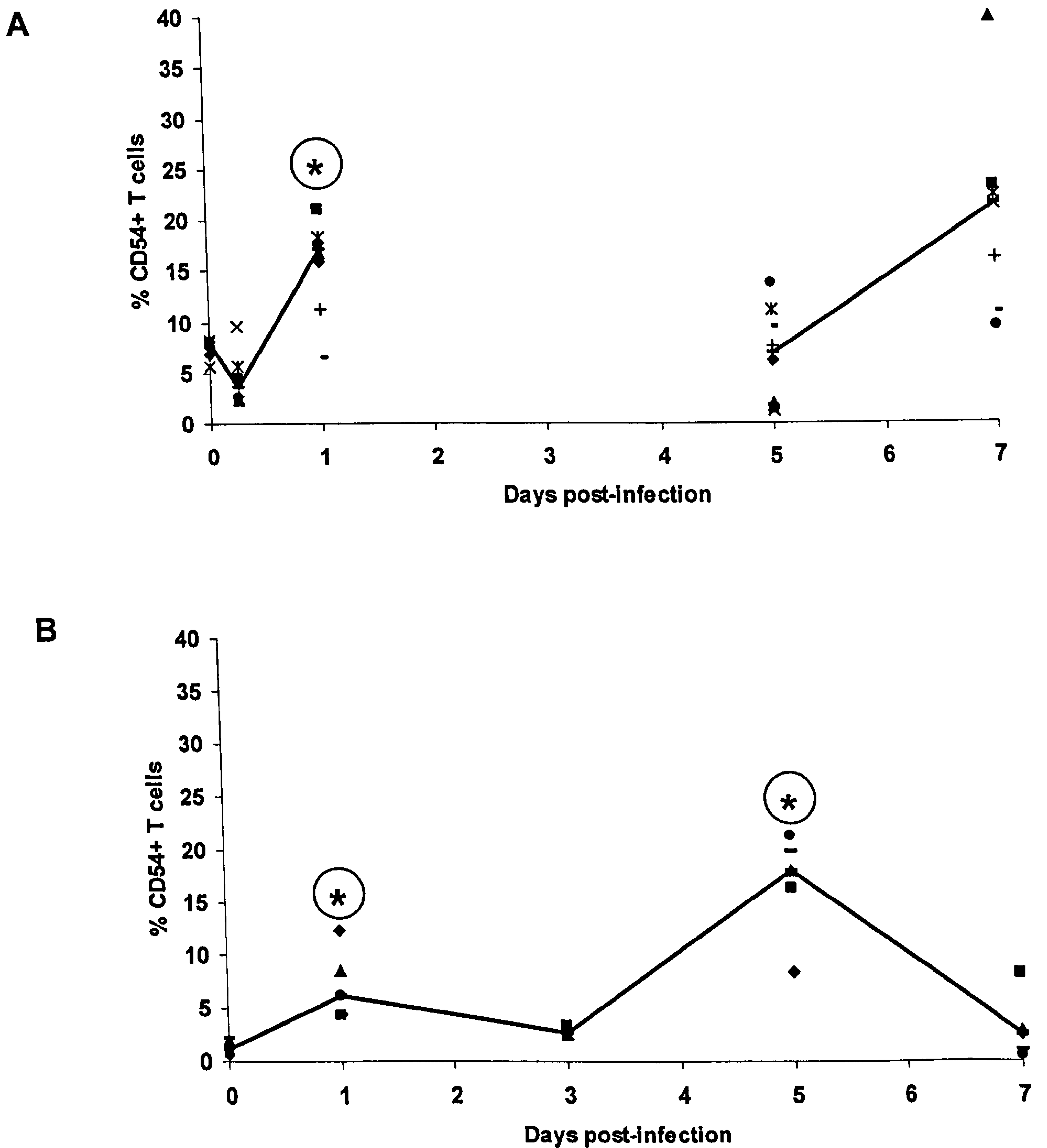


Figure 5.6: CD54 expression on splenic $CD3^+$ T cells during the first 7 days of *B. mallei* infection in Immune Analysis (1) and Immune Analysis (2). Percentage of T cells expressing CD54 during A) Immune Analysis (2) (n=8/timepoint); B) Immune Analysis (1) (n=5/timepoint). Line indicates median and individual points at each time-point represent individual BALB/c mice. Circled asterisks indicate statistical significance ($p<0.01$) in comparison with day 0 (uninfected).

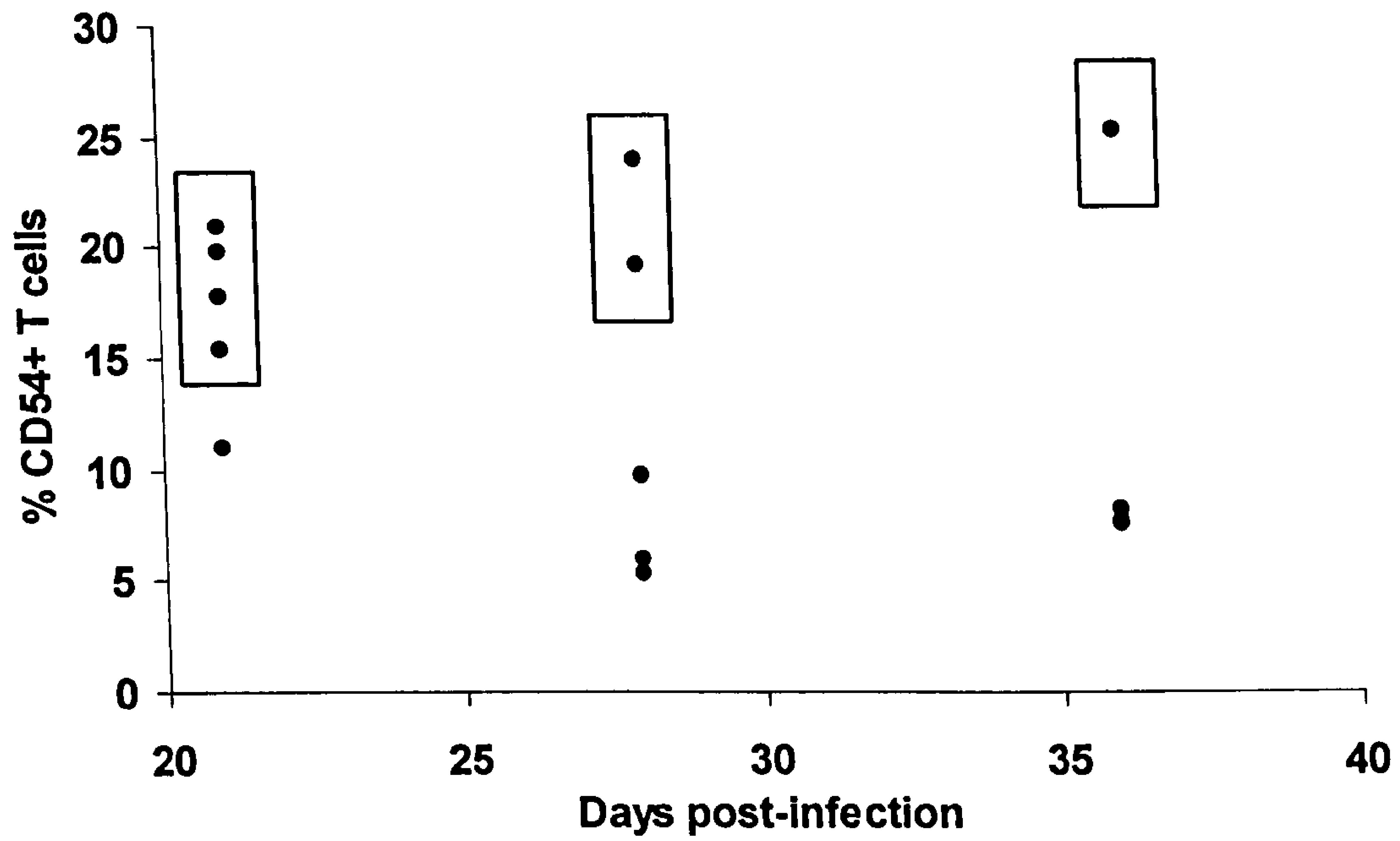


Figure 5.7: CD54 expression on splenic T cells from 21-36 days post-infection with *B. mallei* during Immune Analysis (1). The percentage of T cells expressing $CD54^{+}$ on individual BALB/c mice (n=5/timepoint). Boxes indicate mice with splenic abscesses.

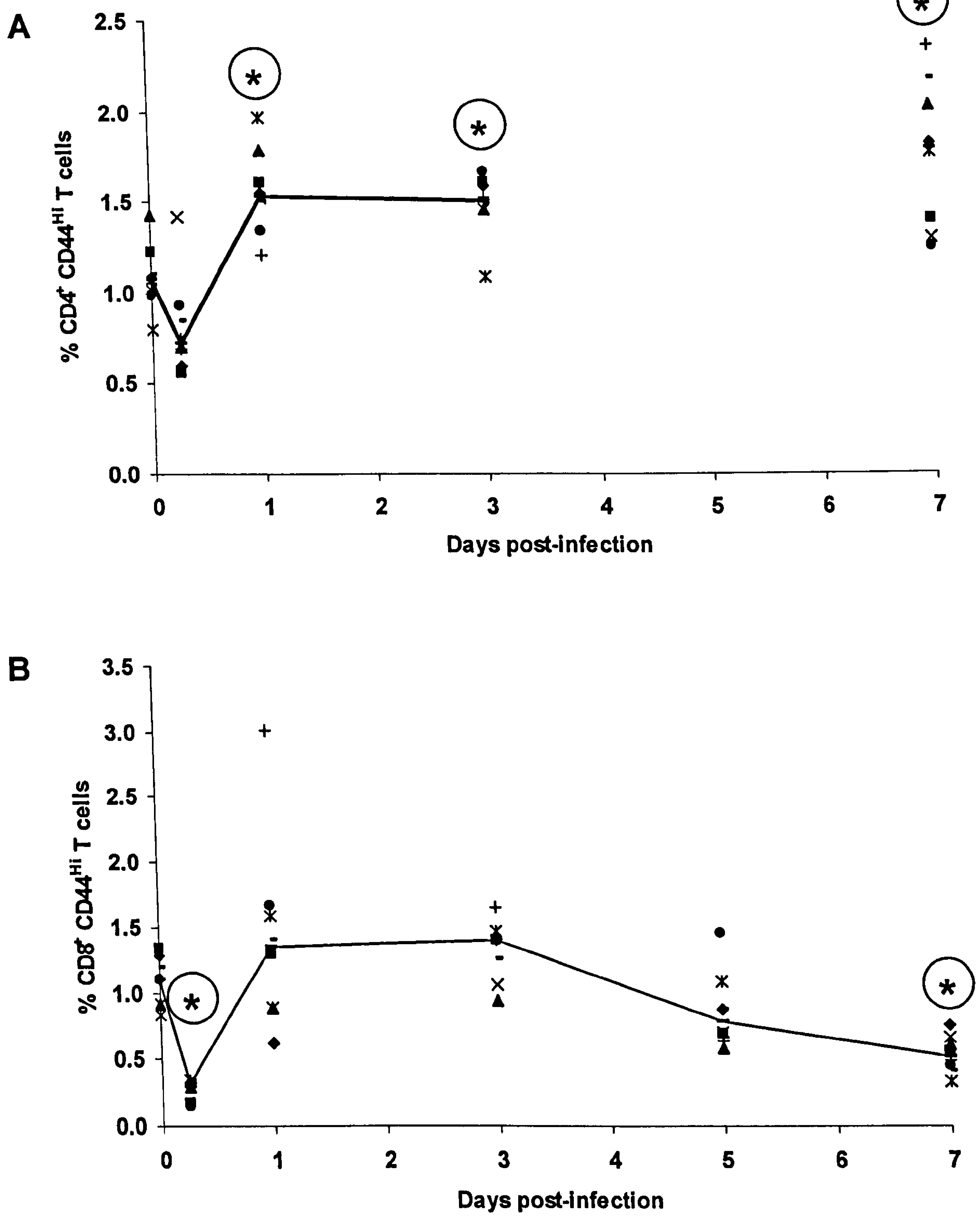


Figure 5.8: High intensity expression of CD44 on T helper and cytotoxic T cell subsets during the first 7 days of *B. mallei* infection during Immune Analysis 2. Percentage of A) $CD3^{+}CD4^{+}$ T cells; B) $CD3^{+}$ $CD8^{+}$ T cells expressing CD44 at high intensity ($CD44^{Hi}$). Line indicates median and individual points at each time-point represent individual BALB/c mice (n=8/timepoint). Circled asterisks indicate statistical significance ($p<0.01$) in comparison with day 0 (uninfected).

5.3.2 The role of T cells during *B. mallei* infection

The progression of *B. mallei* infection in the absence of $CD8^+$ and $CD4^+$ T cells was assessed using depleting antibodies. The specificity of anti-CD4 and anti-CD8 antibodies was assessed prior to infection of antibody-treated mice.

5.3.2.1 Specificity of anti-CD4 and CD8 antibodies

The efficacy of anti-T cell antibodies was assessed using flow cytometry to detect T cell subsets in the spleens of treated mice. No $CD4^+$ T cells were detected in the spleens of mice dosed with anti-CD4 (YTS191) antibodies (Figure 5.9A). The number of $CD8^+$ T cells present in the spleens of mice dosed with anti-CD4 (YTS191) antibodies was not significantly different from mice treated with control (Mac5) antibodies ($p<0.05$) (Figure 5.9B). This demonstrates that anti-CD4 (YTS191) antibodies specifically deplete $CD4^+$ T cells without affecting the $CD8^+$ T cell population. In the spleens of mice treated with anti-CD8 antibodies (YTS169), no $CD8^+$ T cells were detected (Figure 5.9B) and $CD4^+$ T cell numbers were not significantly different from mice treated with control antibodies ($p<0.05$) (Figure 5.9A) demonstrating the specificity of depletion with YTS169. When both YTS191 and YTS169 were administered simultaneously neither $CD4^+$ T cells nor $CD8^+$ T cells could be detected (Figure 5.9 & B). A population of $CD3^+CD4^-CD8^-$ T cells was detected following treatment with a combination of both antibodies (Figure 5.9C).

5.3.2.2 T cell depletion during *B. mallei* infection

The effects of T cell depletion on survival of BALB/c mice following infection with *B. mallei* were assessed. $CD4^+$ T helper cells or $CD8^+$ cytotoxic T cells were depleted from

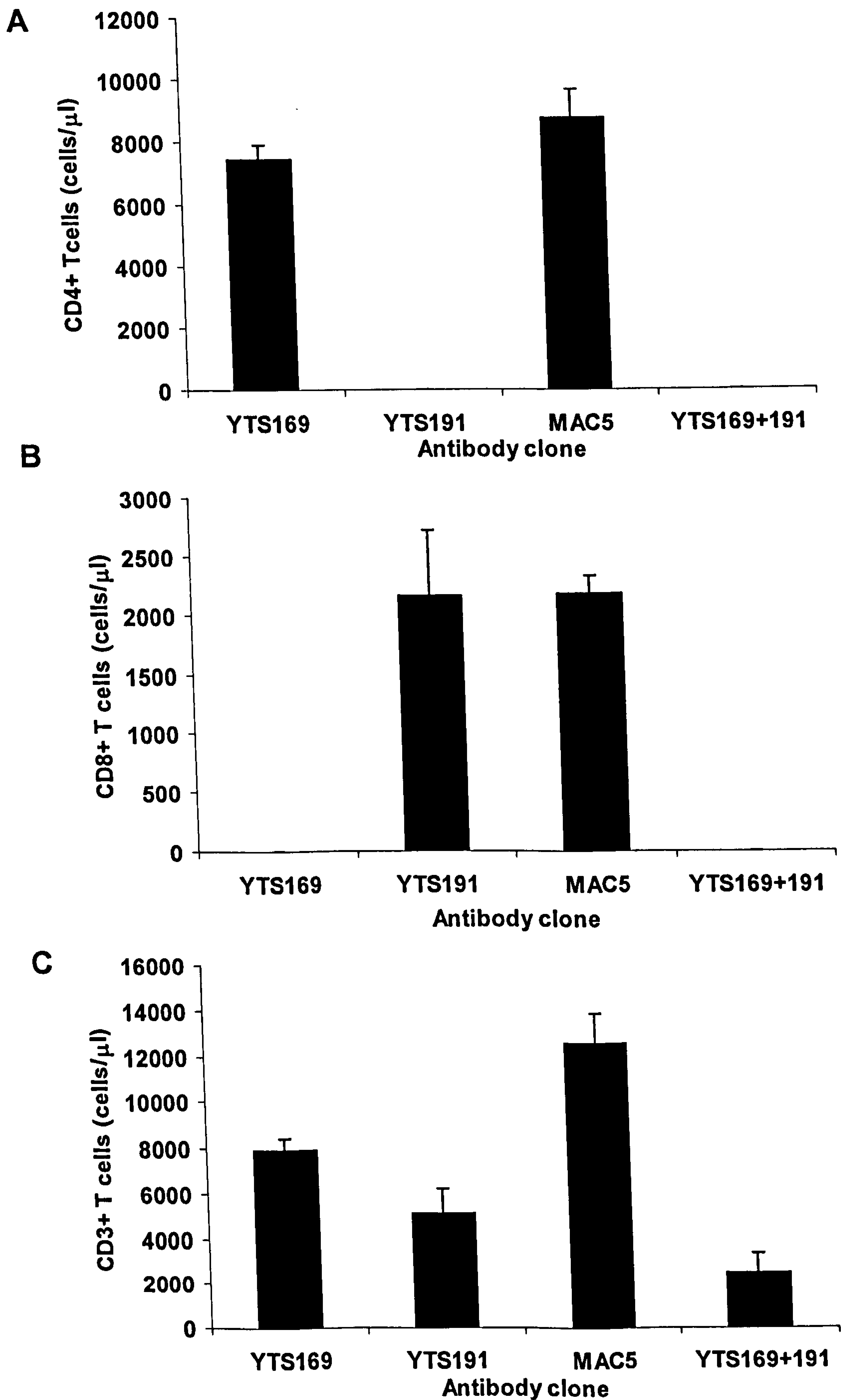


Figure 5.9: Splenic T cell populations in BALB/c mice identified by flow cytometry following administration of CD4 (YTS 191) and/or CD8 (YTS 169) or isotype control antibodies (Mac5) to BALB/c mice (n=3/group). A) Mean absolute number of $CD4^{+}$ T cells; B) Mean absolute number of $CD8^{+}$ T cells; C) Mean absolute number of $CD3^{+}$ T cells identified in the spleens of BALB/c mice. Error Bars represent standard deviation.

BALB/c mice prior to infection with *B. mallei* using the antibodies YTS 191 and YTS169 respectively. The effect of removing both T cell subsets was assessed by administering both YTS191 and YTS169 antibodies in combination. Depletion of the $CD4^+$ T cell population caused 3/6 animals to die 21 days p.i. (Figure 5.10). Following depletion of the $CD8^+$ T cell population, 2/6 animals died at day 14 p.i.. Mice (4/6) died 14 days p.i. following depletion of both T cell subsets simultaneously and a further animal died 23 days post-infection (Figure 5.10). Infected animals treated with isotype-matched control antibodies (Mac5) did not die as a result of the infection (Figure 5.10).

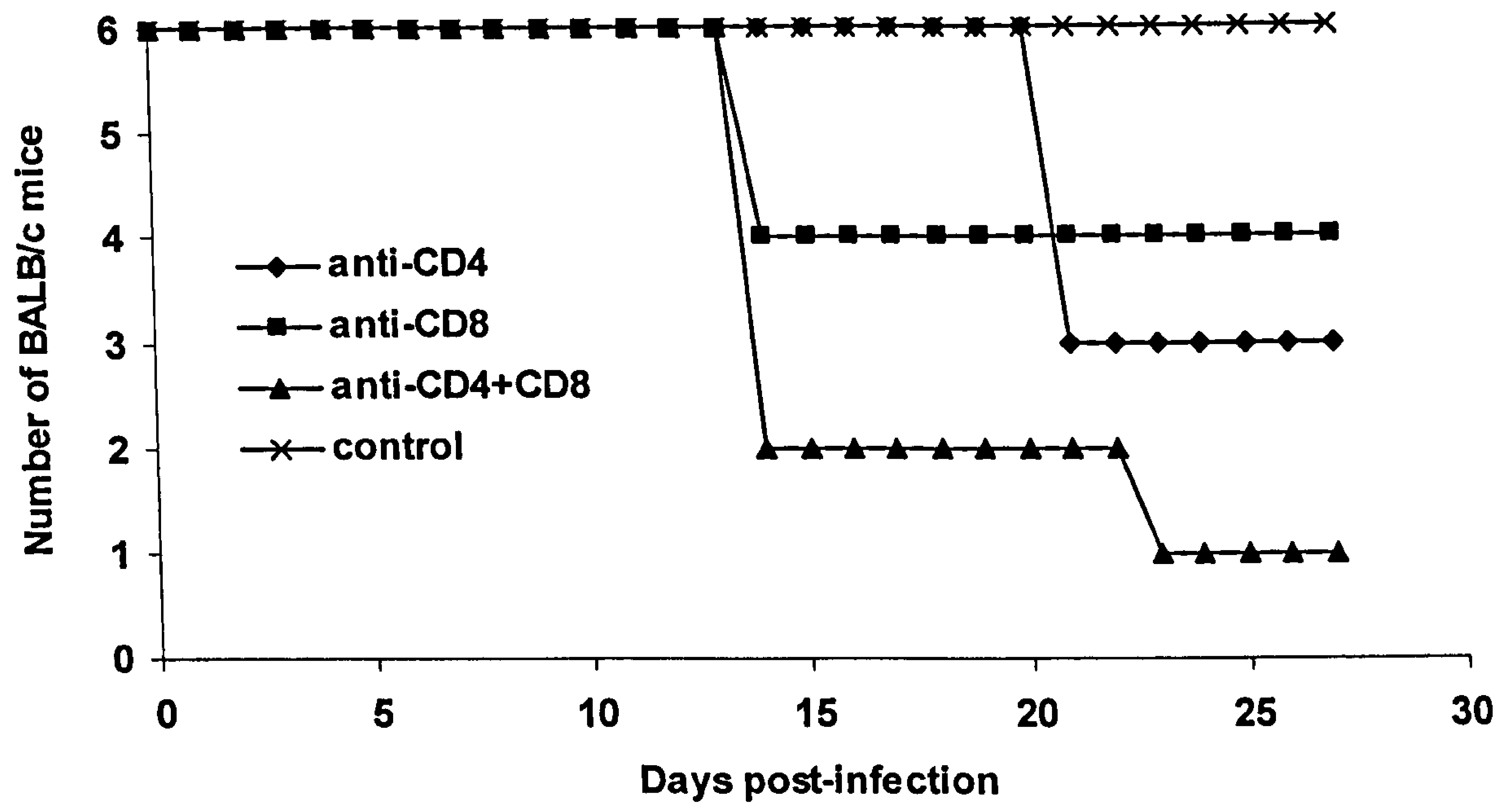


Figure 5.10: Survival of BALB/c mice following depletion of T cell populations during *B. mallei* infection. Groups of mice (n=6) were dosed with CD4 antibodies (YTS169), CD8 antibodies (YTS191), both CD4 and CD8 antibodies (YTS169 + YTS191) or isotype-matched control antibodies (Mac5) 24 hours prior to infection with 10^6 cfu *B. mallei*.

5.4 Discussion

The work presented in this chapter further aimed to characterise the cellular mechanisms involved in the host immune response to *B. mallei* by examining lymphocyte responses during infection. In uninfected, immunocompetent BALB/c mice, lymphocytes comprised the majority of spleen cells. A significant decrease in the B cell population occurred 5 h p.i., recovering to normal levels within 24 h. Splenic B cell depletion within hours of infection has been observed in response to the supertoxins of *S. aureus* caused by a combination of trafficking and apoptosis of specific B cell populations (Goodyear & Silverman, 2004). However, it is unknown whether a *B. mallei* 'superantigen' exists or is released during infection. The recovery and maintenance of the B cell population 24 h p.i. to 72 h p.i. suggests that B cells are migrating into the spleen from the blood or lymphatic circulation. However, further work is required to establish the reasons for these changes in the B cell population.

The functional contribution of B cells in protective immunity to *B. mallei* infection was not investigated, however, previous studies involving infection of B cell knockout mice showed that removal of B cells did not affect survival or bacterial proliferation during *B. mallei* infection (Lukaszewski, 2004). This suggests that antibody-mediated immunity is not essential for control of *B. mallei* infection *in vivo*. This was also observed in a non-human primate model of infection where humoral immunity was not found to be protective during re-infection with *B. mallei* in monkeys with high titres of antibody (Manzeniuk *et al*, 1997).

Decreases in the percentage of activated $CD25^+$ T cells and $CD8^+$ T cells with a $CD44^{Hi}$ memory phenotype occurred 5 h p.i. while T cell numbers remained constant. T cell populations continually move between the blood and tissues in surveillance for pathogens

(Butcher & Picker, 1996). Movement of cell populations between the blood and the spleen allows mixing of activated T cells, which have been presented with antigen, with naïve T cells. This suggests that an increase in naïve T cells in the spleen, causes dilution of these activated splenic T cell populations during *B. mallei* infection. However, the actual mechanisms involved in lymphocyte trafficking during *B. mallei* infection are unknown. An increase in the percentage of $CD54^+$ T cells and $CD4^+$ T cells with a $CD44^{Hi}$ memory phenotype was observed 24 h p.i., although the number of T cells in the spleen remained constant. This suggests that local activation of T cells is occurring within the spleen in response to *B. mallei*. CD54 is upregulated on T cells during infection with *M. tuberculosis* (Mitra *et al*, 2005) and expression of CD54 on T cells may be important in the differentiation of naïve $CD4^+$ T cells into Th1 cells in response to IL-27 (Owaki *et al*, 2005) suggesting the development of type 1 immunity during *B. mallei* infection. Memory $CD4^+$ T cells are able to respond to cytokines and proliferate independently of antigen (Eberl *et al*, 2000) suggesting that these cells may be responding during *B. mallei* infection. The discrepancy in CD54 expression between Immune Analysis (1) and (2) at day 5 p.i. should be investigated in further studies.

The number of T cells and B cells in the spleen remained at control levels 72 h p.i. but diminished 5-7 days following infection. Histopathological analysis of the spleen during *B. mallei* infection in BALB/c mice revealed lymphoid depletion of the white pulp between days 3-7 post-infection (Fritz *et al*, 2000). The white pulp is composed mainly of T cells and B cells and it is possible that destruction of the splenic architecture is responsible for the decrease in lymphocyte numbers observed during *B. mallei* infection. During infection with other pathogens, antigen-specific T cell responses develop around 5-7 days p.i.. Selective depletion of non-specific T cell populations or circulation of antigen-specific T cell populations may also lead to decreased T cell numbers observed in the spleen.

Neither $CD4^+$ T helper cells nor $CD8^+$ cytotoxic T cells were important for survival during the initial phases of *B. mallei* infection although they were both essential for survival 2-3 weeks following *B. mallei* infection. This suggests that T cells may play a role in controlling the infection by adaptive immune mechanisms (Haque *et al*, 2005). Adaptive immunity develops following antigen presentation to T cells in the presence of polarising cytokines, allowing the development of antigen-specific T cells to specifically target the infective pathogen. $CD8^+$ T cells are important in cytotoxic killing of infected host cells and, therefore, may be important in killing macrophages (or other cells) presenting *B. mallei* antigen. The importance of both $CD4^+$ and $CD8^+$ subsets in *B. mallei* mediated-immunity is highlighted by the decreased survival time in the absence of both subsets, suggesting that a mixed antigen-specific response is required for protection against *B. mallei* infection.

Following treatment with both CD4 and CD8 antibodies some $CD3^+$ ($CD4^-CD8^-$) T cells were not depleted. T cells may be important in innate responses to intracellular infection by producing IFN-γ in response to proinflammatory cytokines and bacterial components (Lertmemongkolchai *et al*, 2001; Kambayashi *et al*, 2003). Subsets of $CD3^+CD4^-CD8^-$ T cells exist which are involved in innate immune responses to infection with *F. tularensis* (Cowley *et al*, 2005). It is possible that this subset and other $CD4^-CD8^-$ subsets including $\gamma\delta$ T cells (Kawakami, 2004) may constitute part of the CD3 population remaining following depletion of CD4 and CD8 subsets and may provide protection against early *B. mallei* infection.

In immunocompetent mice, splenic abscesses are prevalent within 2 weeks following *B. mallei* infection. $CD4^+$ T cells are known to be important for abscess formation following infection with *B. fragilis* (Tzianabos *et al*, 2000; Chung *et al*, 2003). The role of T cells in

abscess formation during *B. mallei* infection is unknown; however, CD54 expression was elevated on T cells in abscessed mice suggesting an active role for T cells within abscessed tissue. In addition to this, mice begin to die 21 days p.i. following depletion of $CD4^+$ T cells when abscesses are known to have formed in the spleens of immunocompetent animals. Disruption in the formation or maintenance of abscesses could lead to systemic release of bacteria leading to sepsis and death.

Chapter 6

Characterisation of cytokine responses during *B. mallei* infection in BALB/c mice.

6.1 Introduction

Cells communicate and propagate immune responses by releasing a range of proteins with a diverse range of functions allowing appropriate immune responses to develop. TNFα, IL-6 and IL-1β are pro-inflammatory cytokines released during early infection and are involved in inducing innate, bactericidal mechanisms in neutrophils, macrophages and DCs and propagating inflammatory reactions. Chemokines (e.g. MCP-1) and colony stimulating factors (e.g.GM-CSF) are involved in the recruitment, development and maintenance of cellular inflammatory responses. IFN-γ is produced in the early stages of infection and is involved in development of type 1 immune responses in conjunction with a number of other cytokines including IL-27, IL-18 and IL-12. Type 1 responses are important for killing intracellular pathogens by activating the cytotoxic responses of macrophages and T cells and killing host cells infected with intracellular pathogens.

In order to complement investigation of the cellular mechanisms involved in the immune response to *B. mallei*, this chapter describes splenic and systemic cytokine responses occurring during *B. mallei* infection. The aims were to determine:

- The effect of *B. mallei* on type 1 and proinflammatory cytokine host responses by measuring splenic cytokine mRNA and serum cytokine protein during infection. Type 1 and proinflammatory cytokines were chosen to provide a comparison between *B. mallei* and the cytokines responses important during infection with other intracellular pathogens.
- The effectiveness of the type 1 cytokine IFN-γ and proinflammatory cytokine TNF-α in protection against *B. mallei* infection using knockout mice and depleting antibodies respectively.

6.2 Methods

Expression (mRNA) of a range of cytokines involved in pro-inflammatory responses and type 1 immunity were investigated in the spleen using RT-PCR. In addition to this, cytokine protein levels in the serum were investigated using a flow cytometric assay to determine systemic responses during *B. mallei* infection. Data retrieved during the first 7 days of infection in Immune Analysis (2) is described in detail (Chapter 2; Table 2.2). Trends in cytokine responses and their relationship to bacterial colonisation and abscess formation were observed in Immune Analysis (1).

In order to assess the role of IFN-γ in immunity to *B. mallei* infection, IFN-γ KO mice on both BALB/c and C57BL/6 backgrounds were infected with 10^6 cfu *B. mallei* (ATCC 23344). Mice were also infected with other doses stated in the results (section 6.3.5.1). The effects of TNFα were assessed in BALB/c mice dosed i.p. with 500 μg TNFα antibodies (MP6-XT22) or 500 μg control (Mac49) antibodies at several time-points during and prior to infection. Mice were again challenged i.p. with 10^6 cfu *B. mallei.*

6.3 Results

6.3.1 Validation of endogenous control (18sRNA)

Analysis of cytokine expression was investigated by real-time RT-PCR in the spleens of BALB/c mice. The use of 18sRNA as an endogenous control was validated in order to ensure that 18sRNA expression remained constant and was not upregulated in response to the inflammatory stimulus, lipopolysaccharide (LPS), a component of Gram-negative bacterial cell walls. Expression of 18sRNA was not upregulated one hour following administration of LPS, in comparison with control animals, although the expression of inflammatory cytokines (TNFα and IL-1β) was significantly elevated as expected ($p<0.05$) (Figure 6.1). Therefore, 18sRNA was determined to be a suitable endogenous control for cytokine expression during RT-PCR analysis.

6.3.2 Splenic cytokine mRNA responses

Expression of a range of cytokines TNFα, IL-1β, IL-12p35, IL-12p40, IL-6, IFN-γ, GM-CSF, IL-18, IL-27 and IL-10 was investigated during two time course experiments of *B. mallei* infection (Immune Analysis 1 & 2) by comparing cDNA produced from the spleens of infected and uninfected animals. Background levels of all cytokines investigated were detected in control animals. Comparable trends in cytokine expression were observed during both time course studies during the first 7 days of infection (Figure 6.2). The predominant cytokines in both studies, peaking at 24 h p.i., were IFN-γ, IL-6 and IL-27. Differential results existed for IL-10 which was elevated in the initial time course study within 24 h p.i. but not in the repeated study.

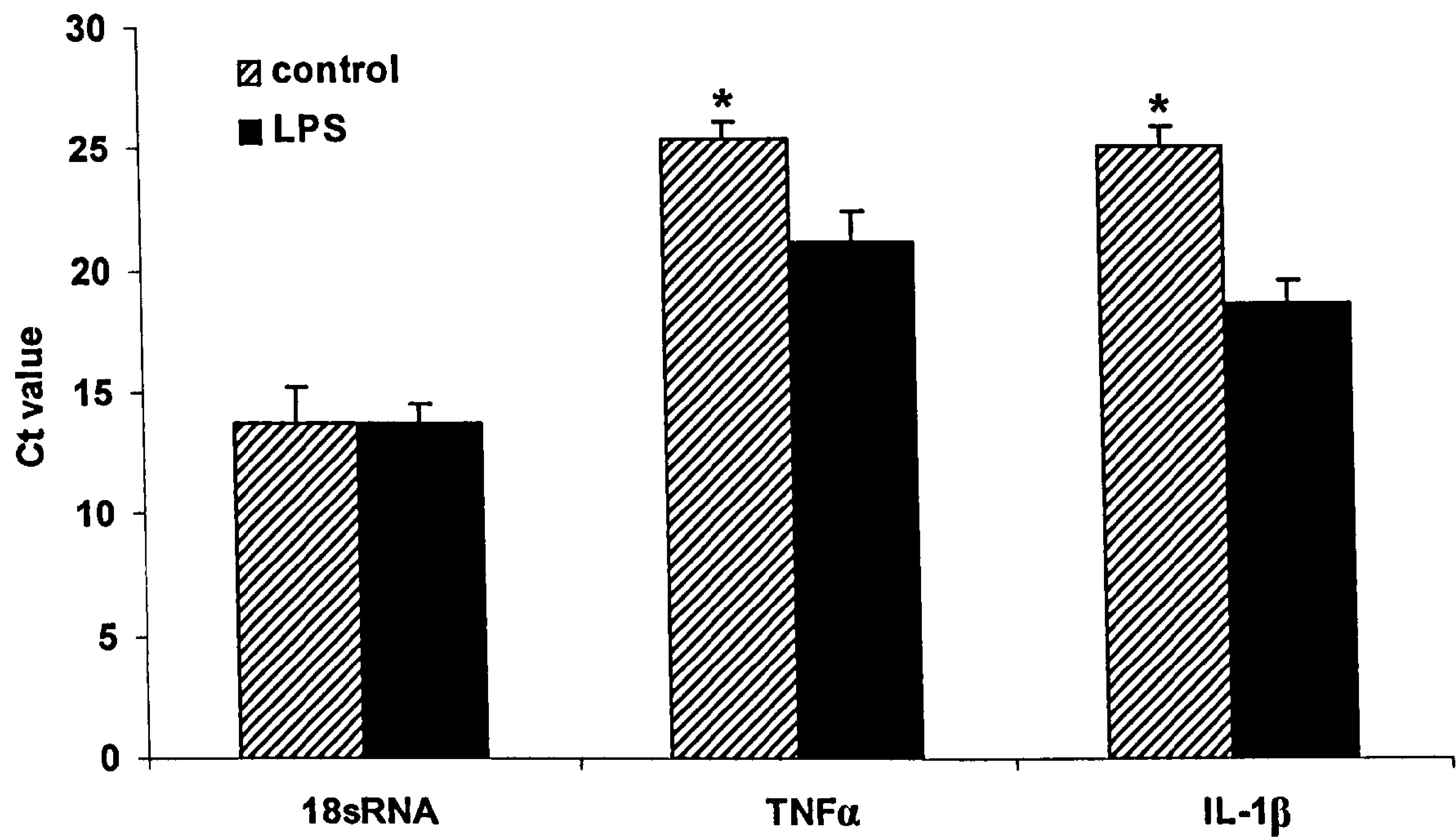


Figure 6.1: Mean threshold cycle (Ct) values for 18sRNA, TNFα and IL-1β in the spleens of BALB/c mice (n=3) following LPS challenge or saline-control mice. Error bars indicate standard deviation. Asterisks indicate statistical significance ($p<0.05$) between control and LPS treated mice.

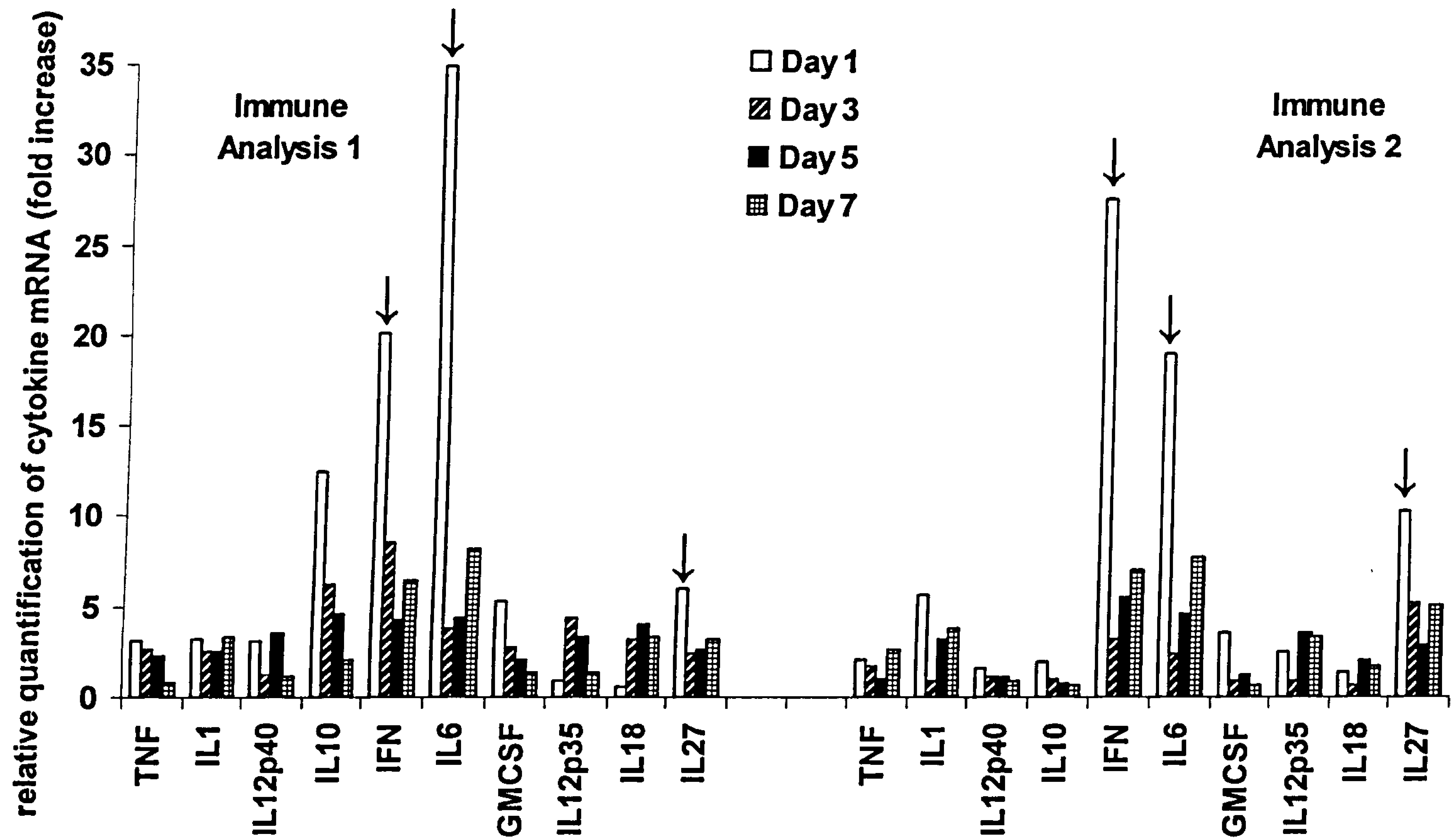


Figure 6.2: Comparison of the relative fold increase in splenic mRNA expression between infected and control BALB/c mice of cytokines investigated from 24 hours to 7 days post-infection with *B. mallei* in two separate infection time-course experiments: Immune Analysis 1 (n=5/timepoint) and Immune Analysis 2 (Day 0: n=7; Day1: n=6; Day 3: n=6; Day 5: n=5; Day 7: n=4). Arrows highlight predominant cytokine responses in both experiments.

6.3.3 Splenic cytokine expression during B. mallei infection

The data shown below was retrieved from Immune Analysis (2). Splenic cytokine expression was investigated in BALB/c mice (n=8/timepoint) during the first 7 days of infection. Data could not be retrieved from all samples taken due to RNA degradation or bacterial contamination, therefore, analysis of the data was performed initially using a Kruskal-Wallis test followed by a Mann-Whitney Test to account for variation in results obtained from differing numbers of animals at each time-point. RT-PCR was performed on a minimum of 4 samples per time-point.

Five hours post-infection with 10^6 cfu *B. mallei*, a significant increase in IL-18 and IL-1β expression was observed ($p<0.01$) in comparison with uninfected control levels (Figure 6.3). An increase in TNFα also occurred although this was not significant ($p=0.0124$) (Figure 6.4). Increases in cytokine expression were accompanied by a decrease in GM-CSF expression 5 h p.i. ($p<0.01$) (Figure 6.4). At 24 h p.i., IL-1β expression remained elevated ($p<0.01$) and IL-18 expression returned to control levels (Figure 6.3). Large, increases in IFN-γ (27-fold) and IL-27 (10-fold) expression ($p<0.01$) occurred 24 h p.i., accompanied by a 19-fold increase in IL-6 expression ($p=0.0124$) (Figure 6.5). A significant increase in GM-CSF at 24 h p.i. infection (compared with expression at 5 h p.i.) also occurred ($p<0.01$) (Figure 6.4). IL12p35 (but not IL12p40) and TNFα expression was significantly higher than control values over the 7 day time course of infection as determined by Kruskal-Wallis analysis ($p<0.01$). Significant differences at individual time-points could not be elucidated, possibly due to the variation in IL12p35 and TNFα expression in control mice (Figure 6.6).

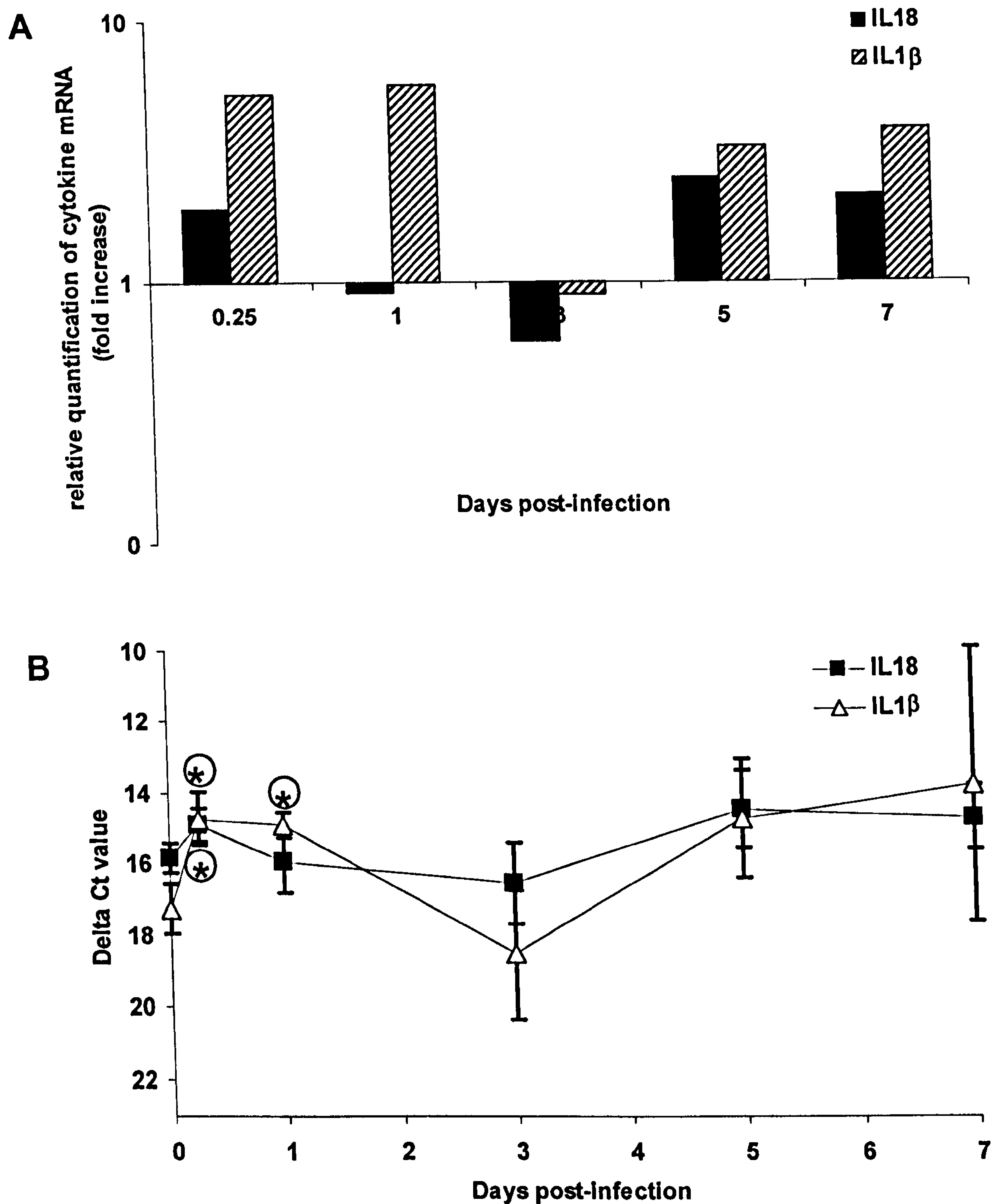


Figure 6.3: Splenic expression of IL-18 and IL-1β during *B. mallei* infection of BALB/c mice (Day 0: n=7; 5 h: n=6; Day1: n=6; Day 3: n=6; Day 5: n=5; Day 7: n=4). (A) Relative fold increase in expression of IL-18 and IL-1β compared with uninfected mice; (B) Median delta threshold cycle (Ct) values for IL-18 and IL-1β over the time course of the infection. Error bars indicate 99% confidence intervals. Asterisks (*) indicate statistical significance ($p<0.01$) in comparison with day 0 (uninfected).

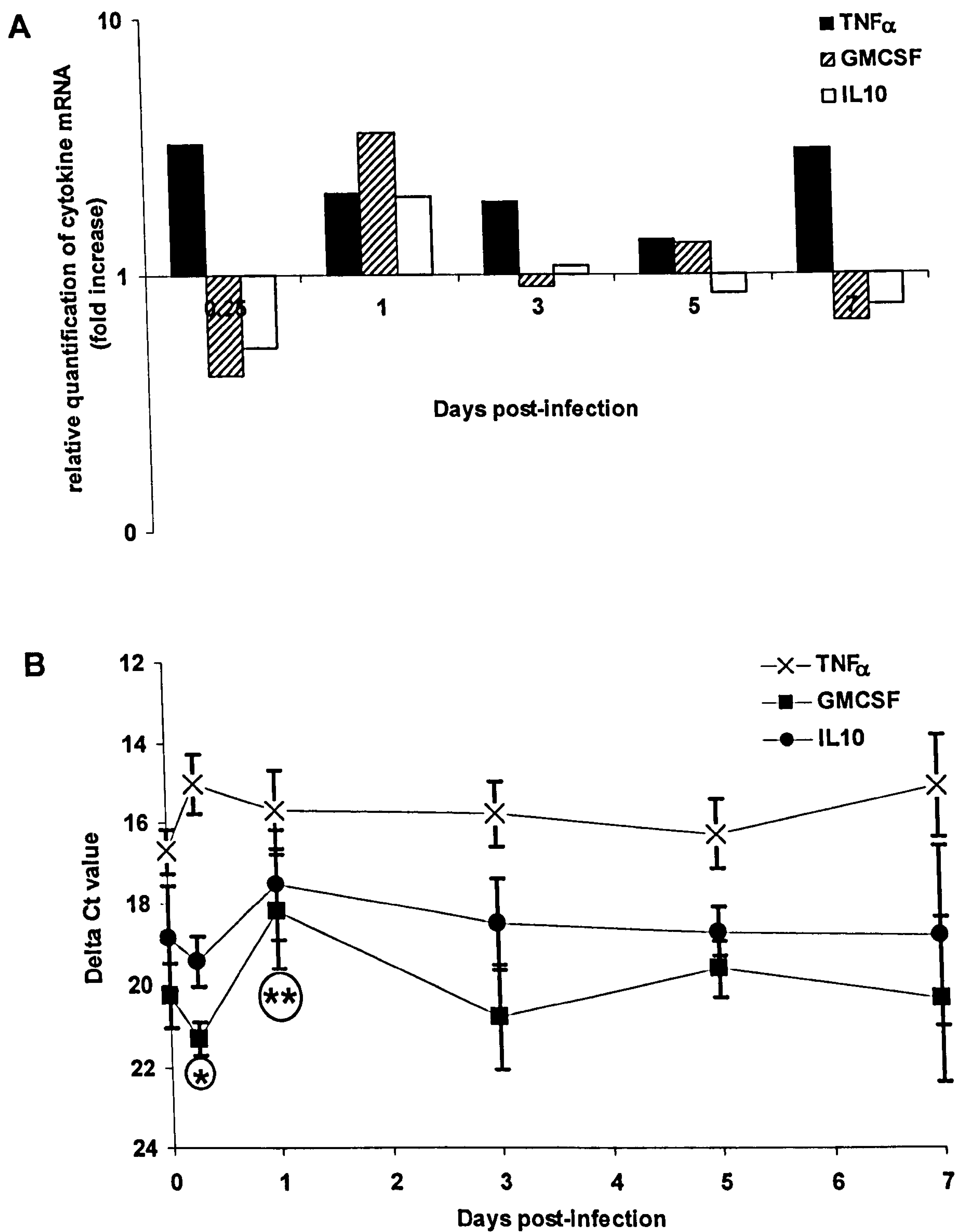


Figure 6.4: Splenic expression of TNFα, GM-CSF and IL-10 during *B. mallei* infection of BALB/c mice (Day 0: n=7; 5 h: n=6; Day1: n=6; Day 3: n=6; Day 5: n=5; Day 7: n=4). (A) Relative fold increase in expression of TNFα, GM-CSF and IL-10 compared with uninfected mice; (B) Median delta threshold cycle (Ct) values for TNFα, GM-CSF and IL-10 over the time course of the infection. Error bars indicate 99% confidence intervals. Circled asterisks (*) indicate statistical significance ($p<0.01$) in comparison with day 0 (uninfected);** indicates statistical significance in comparison with 5 h p.i..

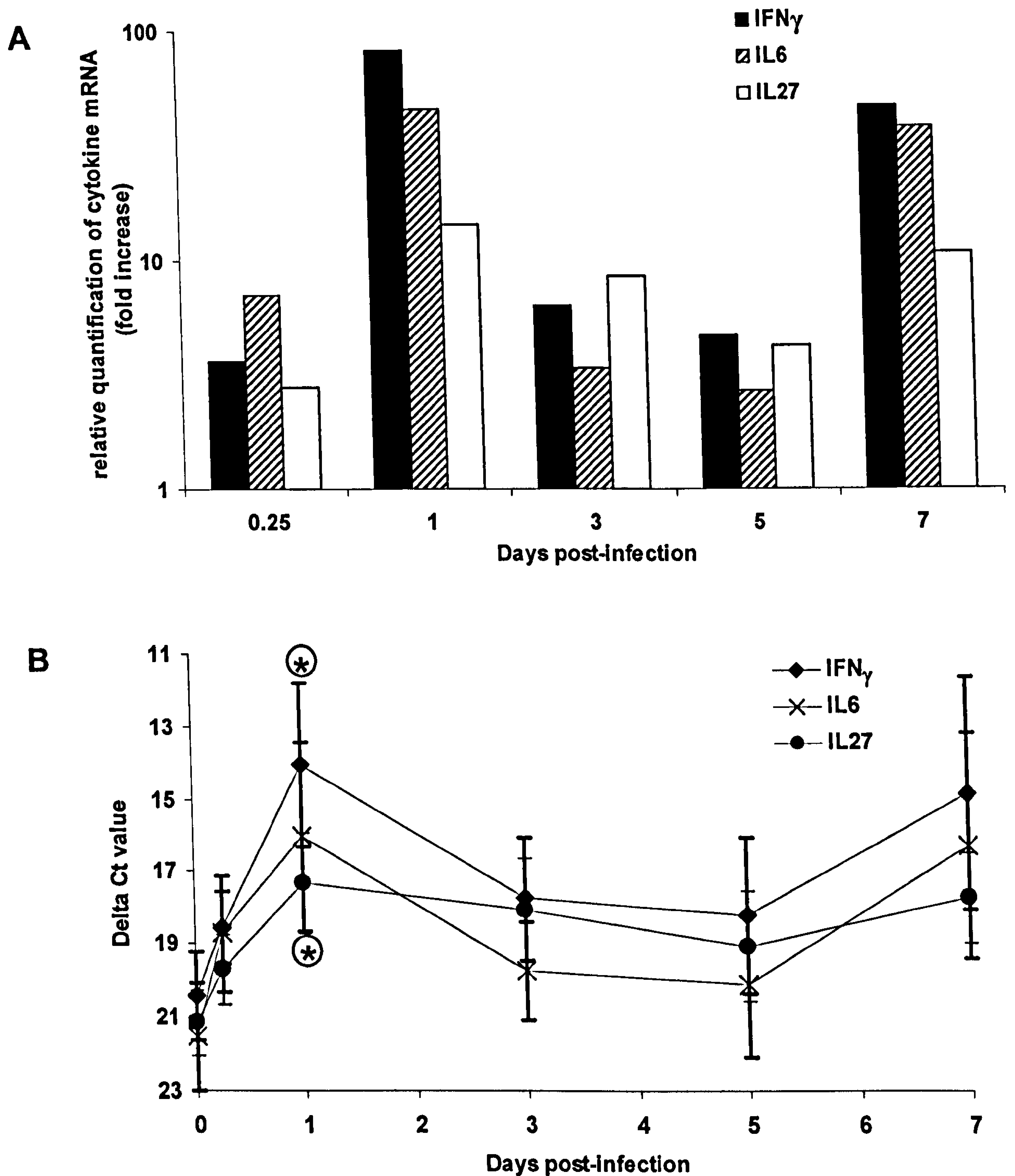


Figure 6.5: Splenic expression of IFN-γ, IL-6 and IL-27 during *B. mallei* infection of BALB/c mice (Day 0: n=7; 5 h: n=6; Day1: n=6; Day 3: n=6; Day 5: n=5; Day 7: n=4). (A) Relative fold increase in expression of IFN-γ, IL-6 and IL-27 compared with uninfected mice; (B) Median delta threshold cycle (Ct) values for IFN-γ, IL-6 and IL-27 over the time course of the infection. Error bars indicate 99% confidence intervals. Circled asterisks (*) indicate statistical significance ($p<0.01$) in comparison with day 0 (uninfected).

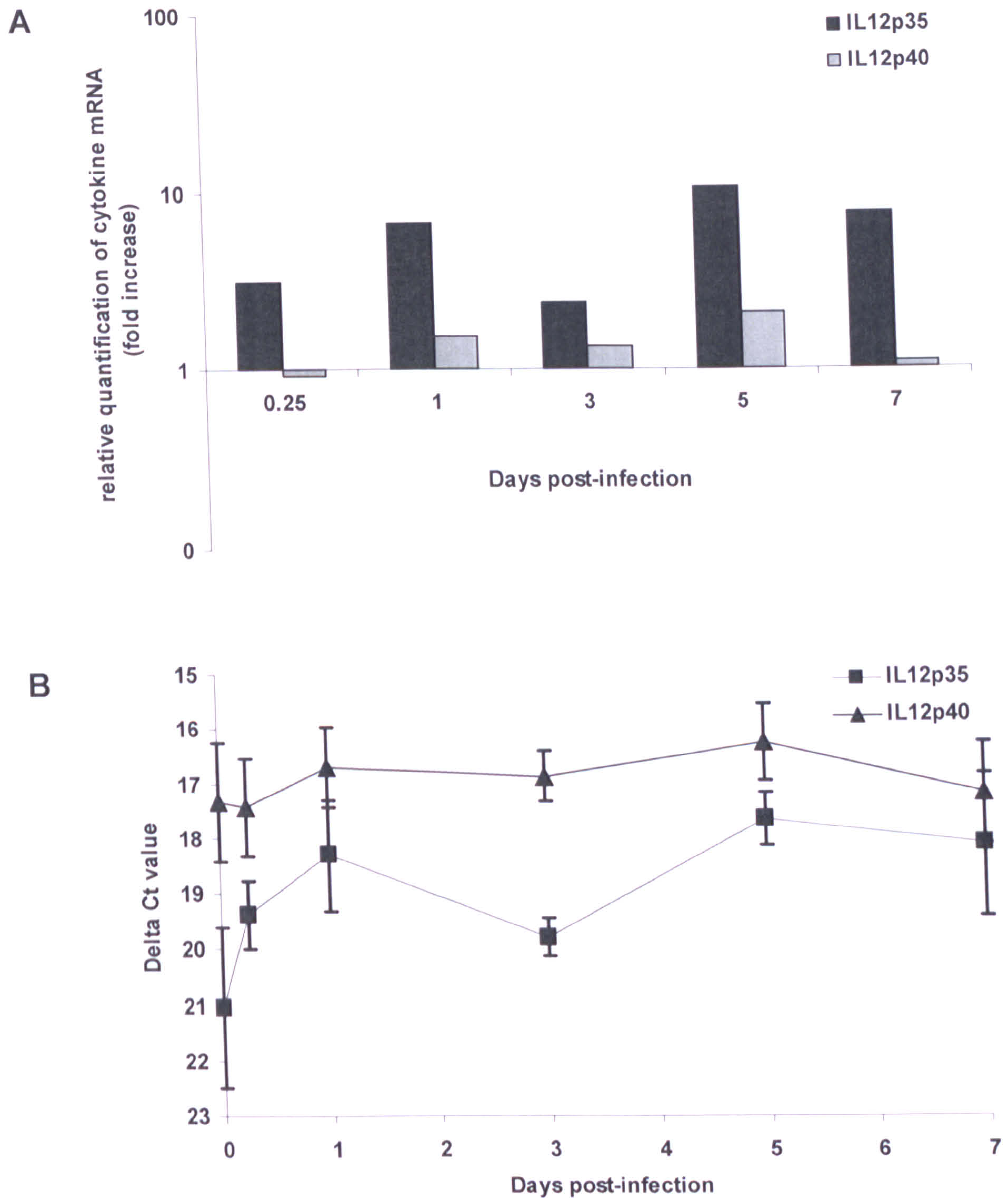


Figure 6.6: Splenic expression of IL-12p35 and IL-12p40 during *B. mallei* infection of BALB/c mice (Day 0: n=7; 5 h: n=6; Day1: n=6; Day 3: n=6; Day 5: n=5; Day 7: n=4). (A) Relative fold increase in expression of IL-12p35 and IL-12p40 compared with uninfected mice; (B) Median delta threshold cycle (Ct) values for IL-12p35 and IL-12p40 over the time course of the infection. Error bars indicate 99% confidence intervals.

Cytokine expression was related to splenic bacterial burdens for most, but not all cytokines, 24 h p.i.. One individual mouse with low splenic bacterial colonisation (<10 cfu) had higher threshold cycle (Ct) values (and therefore lower cytokine expression) for IL-6, IL-27, IFN-γ, TNF-α, IL12p35, GM-CSF and IL-10 (Figure 6.7A & B) but not IL-18, IL-1β and IL12p40 (Figure 6.8) at 24 h p.i. in comparison with the remaining individuals in the group. A notable decrease in the expression of all cytokines, in comparison to the peak in cytokine expression observed 24 h p.i., occurred 72 h p.i.. Expression of IL-27 ($p=0.0124$), IFN-γ and IL-6 remained slightly elevated above control values 72 h p.i. although this was not significant (Figure 6.4 & 6.5). Expression of all other cytokines returned to control levels. At days 5 and 7 p.i., no significant deviation from control values for any cytokine was observed although elevation of IL-27 ($p=0.0182$) and TNFα ($p=0.028$) expression was observed 7 days p.i..

Examination of mRNA expression in spleens of individual mice at day 14 p.i. during Immune Analysis (1), revealed elevated expression of TNFα, IL-1β, IL-6, GM-CSF and IL-10 in abscessed animals in comparison with control values and non-abscessed animals (Figure 6.9A). At day 21 p.i., increased expression of TNFα, IL-10, IFN-γ, IL-6, GM-CSF, IL12p35 and IL-18 and decreased expression of IL12p40 were observed in abscessed mice in comparison with non-abscessed mice (Figure 6.9B).

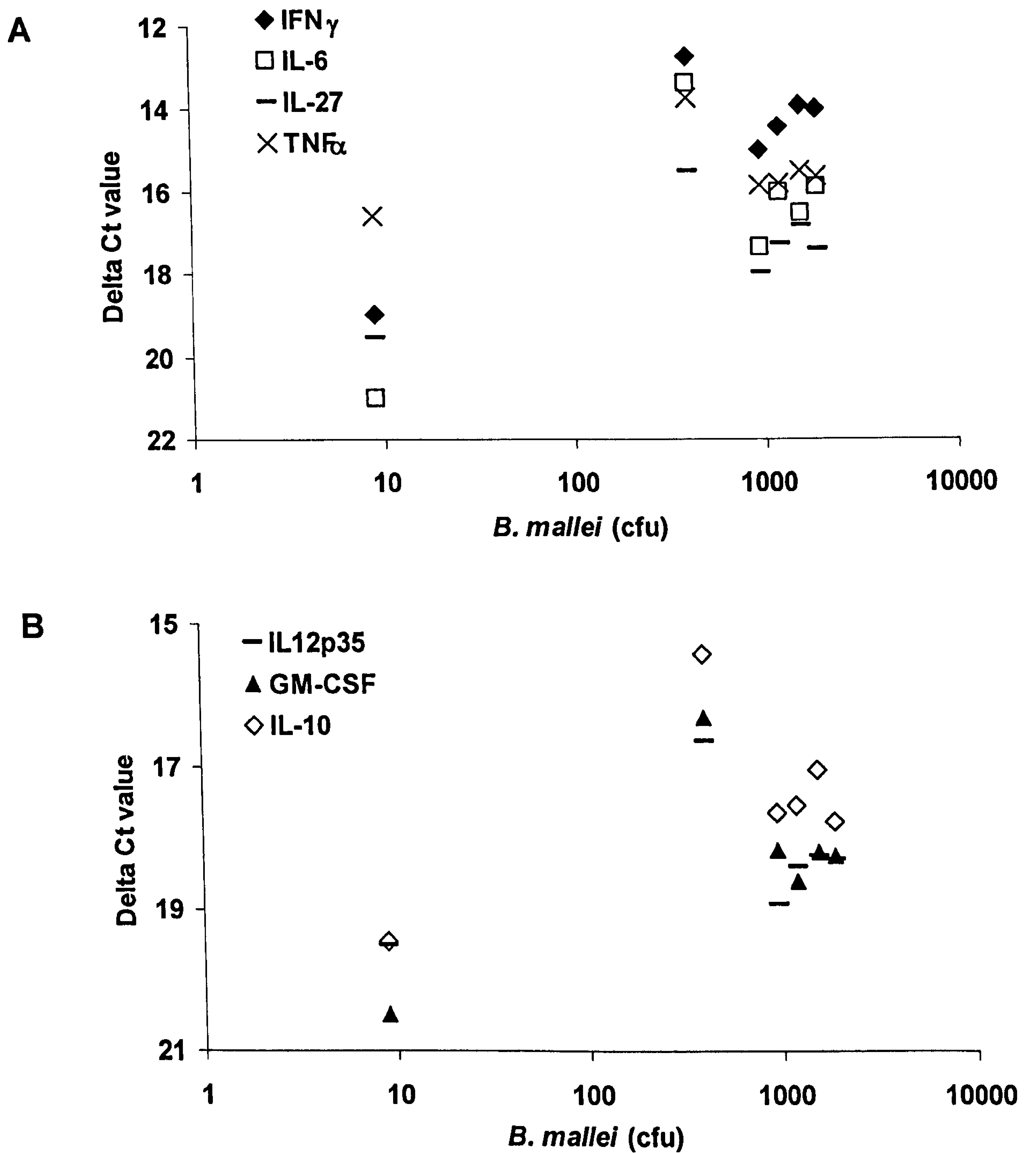


Figure 6.7: Comparison of bacterial colonisation and cytokine expression in the spleen 24 h post-infection. Delta Threshold cycle (Ct) values for A) IFN-γ, IL-6, IL-27 and TNFα; B) IL-12p35, GM-CSF and IL-10 in individual BALB/c mice (n=6) from Immune Analysis 2 are plotted against the number of bacterial colonies (cfu) detected in each individual spleen.

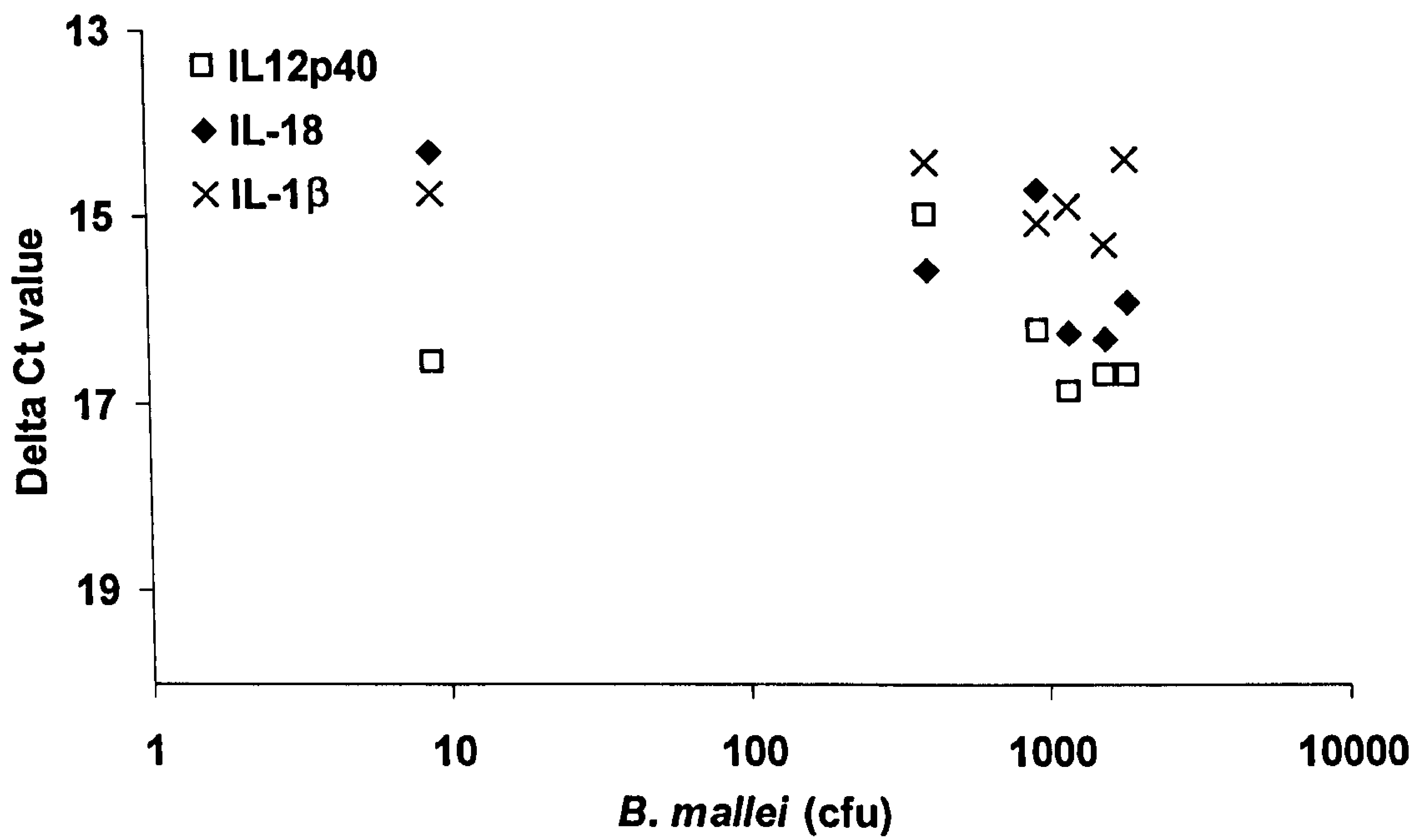


Figure 6.8: Comparison of bacterial colonisation and cytokine expression in the spleen for IL12p40, IL-18 and IL-1β 24 h post-infection. Delta Threshold cycle (Ct) values for IL12p40, IL-18 and IL-1β in individual BALB/c mice (n=6) from Immune Analysis 2 are plotted against the number of bacterial colonies (cfu) detected in each individual spleen.

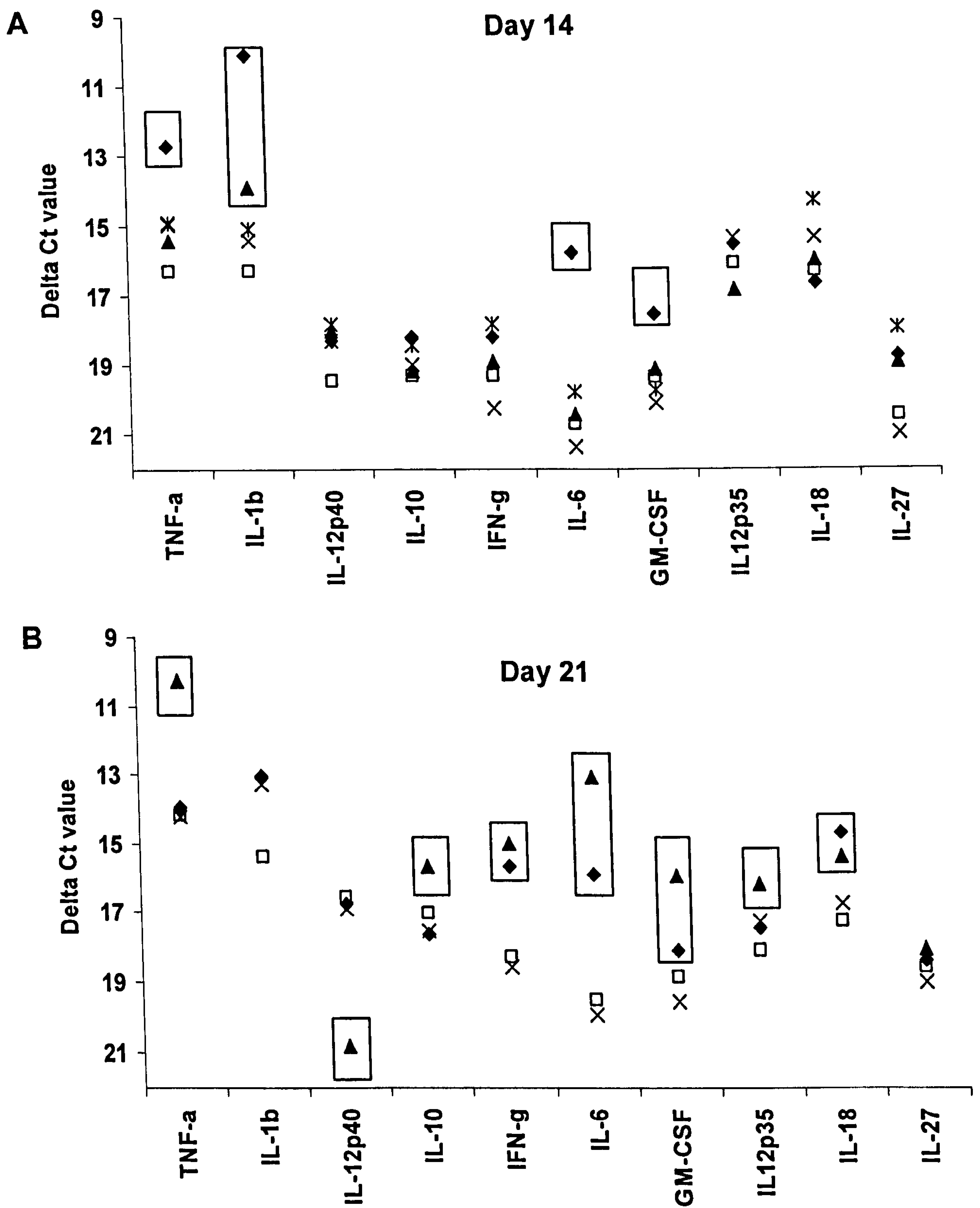


Figure 6.9: Splenic cytokine expression is related to abscess formation during *B. mallei* infection. Delta Threshold cycle (Ct) values for cytokine mRNA expression from individual BALB/c mice (n=5) investigated during Immune Analysis 1 at A) Day 14 and B) Day 21 post-infection with *B. mallei*. Boxes indicate mice with splenic abscesses.

6.3.4 Systemic cytokine responses

The presence of the cytokines (IFN-γ, IL-6, IL-12p70, TNFα, IL-10) and the chemokine, monocyte chemoattractant protein (MCP)-1, were examined in serum from infected and control animals in the first 7 days of infection during Immune Analysis (2). Within 5 h p.i., increases in IFN-γ, MCP-1 and IL-6 were detected in the serum of infected animals compared with uninfected controls ($p<0.01$) (Figure 6.10). At 24 h p.i., circulating IFN-γ, MCP-1 and IL-6 peaked at maximum levels ($p<0.01$) (Figure 6.10). At 72 h p.i. serum levels of IL-6, MCP-1 and IFN-γ had returned to control levels and remained at control levels until day 7. No significant changes in serum levels of TNFα, IL-12p70, IL-10, IL-2, IL-4 and IL-5 were detected at any time-point during infection.

During Immune Analysis (1), similar profiles were observed during the first 7 days of infection in comparison with Immune Analysis (2) with the predominant mediators being IFN-γ, IL-6 and MCP-1 24 h p.i. (Figure 6.11A). From days 14-36, median expression levels of all cytokines was low. At day 14 p.i., MCP-1 and IL-6 were detected only in abscessed mice and serum IFNγ was detected in 3/5 animals – two of which possessed abscesses. At day 36 p.i., MCP-1 and IL-6 were present only in the serum of animals that possessed splenic abscesses (Figure 6.11B).

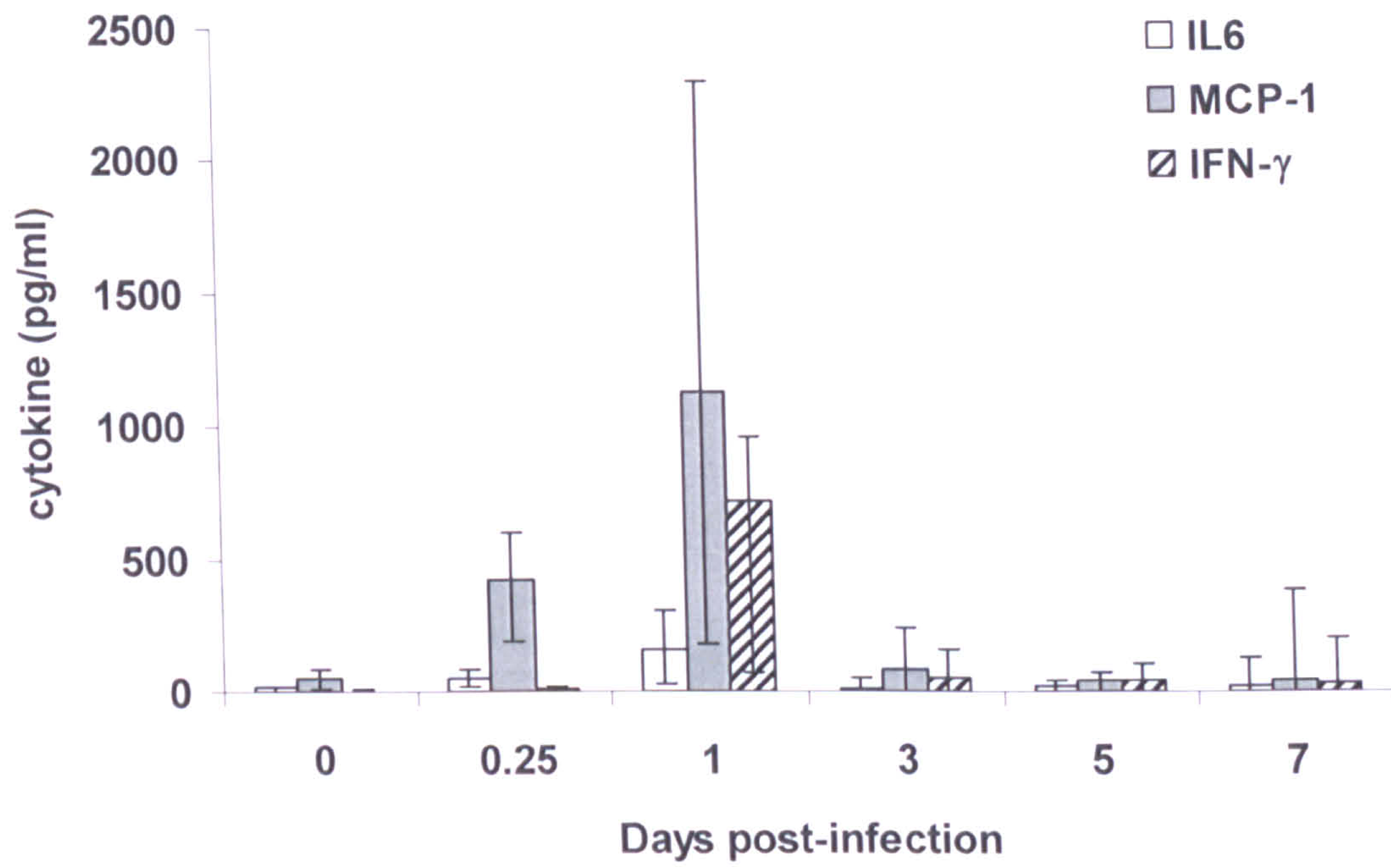


Figure 6.10: Systemic cytokine responses during *B. mallei* infection. Median IL-6, MCP-1 and IFN-γ production in the serum of BALB/c mice (n=8) 0-7 days post-infection with *B. mallei* in Immune Analysis 2. Error bars indicate 99% confidence intervals. Asterisks (*) indicate statistical significance ($p<0.01$) in comparison with day 0 (uninfected).

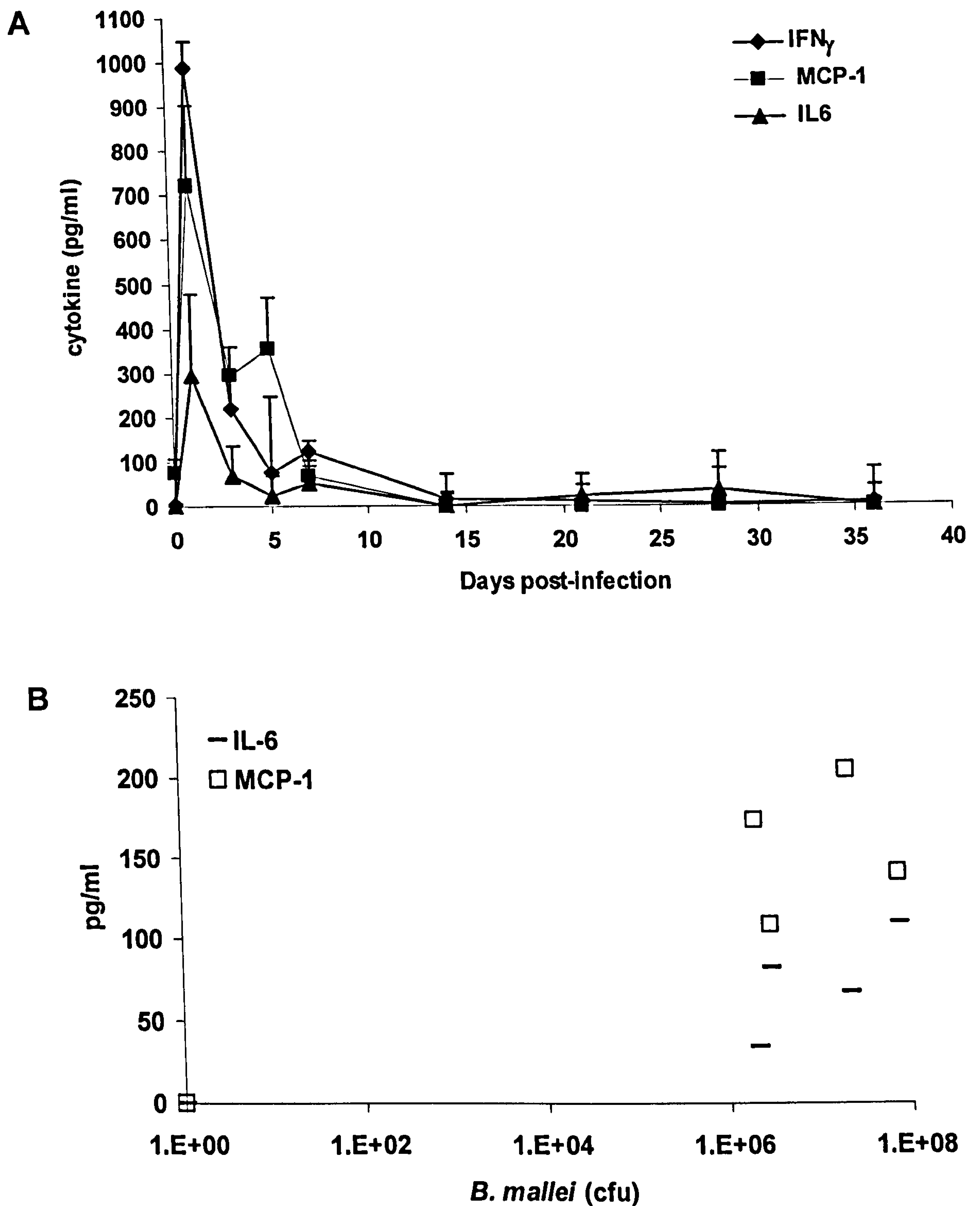


Figure 6.11: Systemic cytokine responses during 36 days of *B. mallei* infection (Immune Analysis 1). A) Median IL-6, MCP-1 and IFN-γ production in the serum of BALB/c mice (n=5) 0-36 days post-infection with *B. mallei* in Immune Analysis 1. Error bars indicate standard deviation. B) Relationship between splenic bacterial colonisation and systemic IL-6 and MCP-1 from individual mice (n=5) 14 and 36 days post-infection.

6.3.5 The role of IFN-γ during B. mallei infection.

The importance of the IFN-γ peak observed 24 h following *B. mallei* infection, both in the spleen and serum of BALB/c mice, was investigated in IFN-γ knockout (KO) mice.

6.3.5.1 Infection of IFN-γ knockout mice with B. mallei

IFN-γ KO mice on both BALB/c and C57BL/6 backgrounds were used to assess IFN-γ-mediated immunity during *B. mallei* infection. BALB/c IFN-γ KO mice (9/10) died 2 days post-infection following challenge with a high dose (1 x 10^6 cfu) of *B. mallei* (Figure 6.12A). IFN-γ KO mice on a C57BL/6 background also died 2 days following challenge with a high dose (4.1 x 10^4 cfu) of *B. mallei* (Figure 6.12B). Challenge with a low dose (1 x 10^2 cfu) of *B. mallei* caused 6/7 IFN-γ KO (BALB/c) mice to die within 5 days p.i. (Figure 6.12A) and IFN-γ KO C57BL/6 mice challenged with a low dose of *B. mallei* (41 cfu) died within 3 days p.i. (Figure 6.12B). Wild-type BALB/c and C57BL/6 mice challenged with both low and high doses survived for the duration of the experiment (Figure 6.12).

6.3.6 The role of TNFα during B. mallei infection

The effect of the pro-inflammatory cytokine TNFα on the progression of *B. mallei* infection was assessed by depleting TNFα from mice using TNFα antibodies. The specificity of TNFα antibodies was assessed *in vitro* prior to infection of antibody-treated mice.

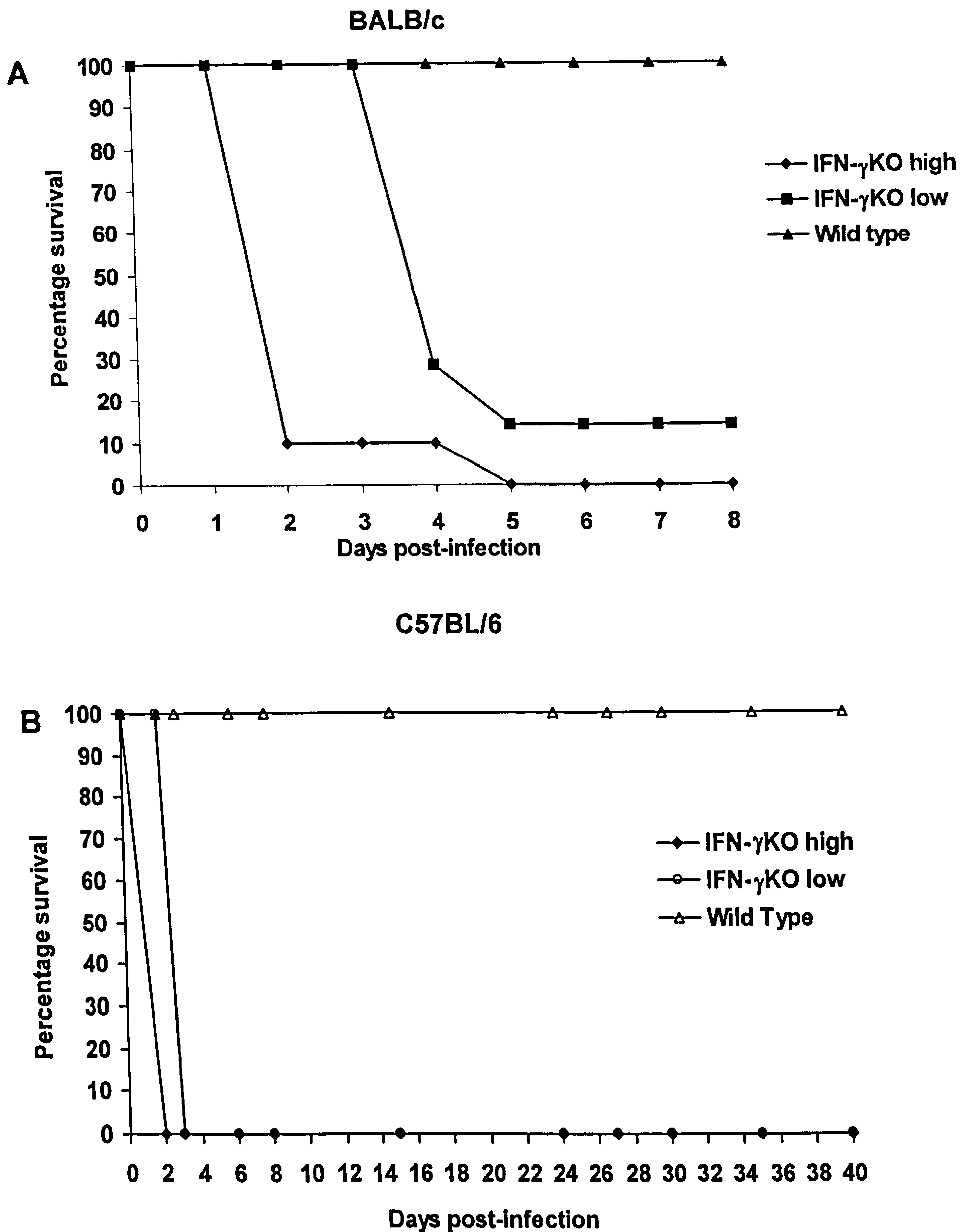


Figure 6.12: IFN-γ is essential for protection against *B. mallei* infection. The percentage survival of A) IFN-γ (BALB/c) knockout mice (n=6/group) challenged with 1 x 10^6 cfu (high) or 1 x 10^2 cfu (low) *B. mallei* and wild type BALB/c mice (n=6) challenged with 1 x 10^6 cfu. B) IFN-γ (C57BL/6) knockout mice (n=6/group) challenged with 4.1 x 10^4 cfu (high) or 41 cfu (low) *B. mallei* and wild type C57BL/6 mice (n=6) challenged with 4.1 x 10^4 cfu. The data from this experiment was supplied by R. Lukaszewski, Dstl, UK.

6.3.6.1 *Specificity of TNFα antibodies*

CD54 expression on J774A.1 macrophages was significantly reduced in the presence of TNFα antibodies at a range of TNFα concentrations following stimulation for 24 h (Figure 6.13). No significant reduction in CD54 expression was observed in the presence of isotype-matched control antibodies in comparison with cultures containing TNFα alone (Figure 6.13) demonstrating that TNFα antibodies were able to neutralise TNFα-specific macrophage activation.

6.3.6.2 *Depletion of TNFα during B. mallei infection*

The role of TNFα during early infection with *B. mallei* was assessed by administering TNFα antibodies to BALB/c mice at several time-points during the first 7 days of infection. Treatment with TNFα antibodies 24 h prior to infection, 24 h p.i. and 4 days p.i. did not result in mortality during the first 7 days of infection regardless of when the first dose of antibody was administered. No mortality was observed in mice treated with control antibodies. Bacteriological analysis of spleens obtained 7 days p.i. from all anti-TNFα dose groups revealed a 100 to a 1000-fold increase in bacterial counts in comparison with infected animals treated with control antibodies (Figure 6.14A & B). Mice treated with TNFα antibodies developed overt signs of disease (measured by ruffling of fur during the 7 days of infection) and abscesses had developed by 7 days p.i..

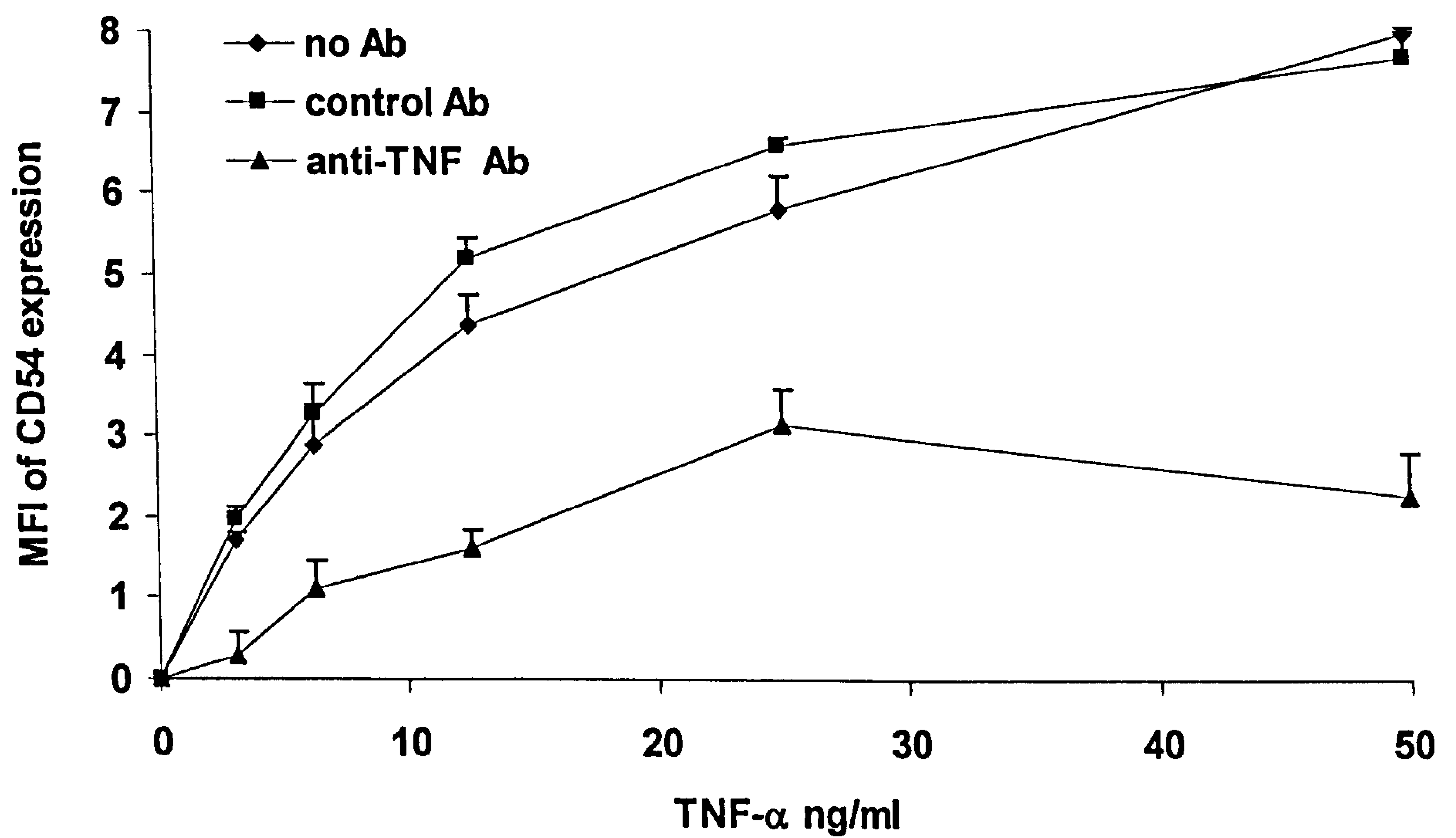


Figure 6.13: TNFα antibodies reduce TNFα-mediated macrophage activation. The mean fluorescence intensity (MFI) of CD54 expression in a J774A.1 macrophage cell line stimulated *in vitro* with a range of doses of TNFα in triplicate for 24 hours in the presence of isotype-matched control antibody (control Ab), TNFα (MP6-XT22) antibody (anti-TNFα Ab) or TNFα alone (no Ab).

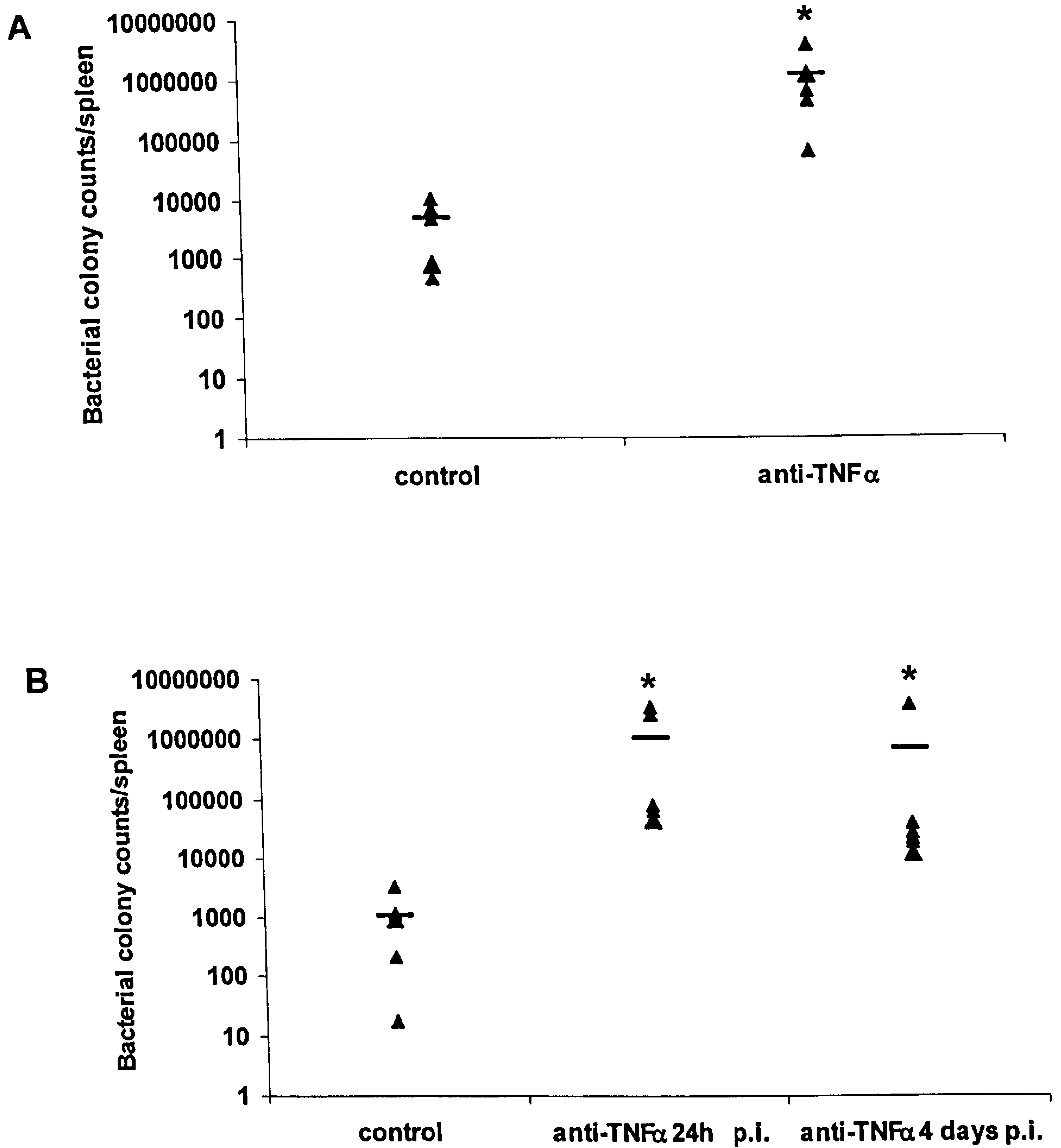


Figure 6.14: Bacterial counts detected in the spleens of BALB/c mice 7 days following infection with *B. mallei* treated with control or TNFα antibodies. Mice (n=6) were treated with control or TNFα (MP6-XT22) antibodies A) 24 hours prior to infection and B) 24 hours post-infection (24h p.i.) or 4 days post-infection (4 days p.i.). Mice (n=6) were dosed with control antibodies at the same time-points as mice treated with TNFα antibodies. Asterisks indicate statistical significance following treatment with TNFα antibodies compared with mice dosed with control antibody ($p<0.05$).

Abscesses did not develop 7 days p.i. and overt signs of disease were not observed in infected control animals. The role of TNFα during the late stages of *B. mallei* infection was investigated by administering TNFα antibodies to BALB/c mice 42 days following infection (Figure 6.15). At day 38 p.i., 1/6 mice in the control group died prior to antibody dosing and at day 41 p.i. 2/6 mice in the anti-TNFα dosing group died prior to administration of the antibody. Following treatment with TNFα antibodies the remaining mice (4/6) died within 3 days. Infected control mice (5/6) did not die as a result of the infection following administration of isotype control antibodies.

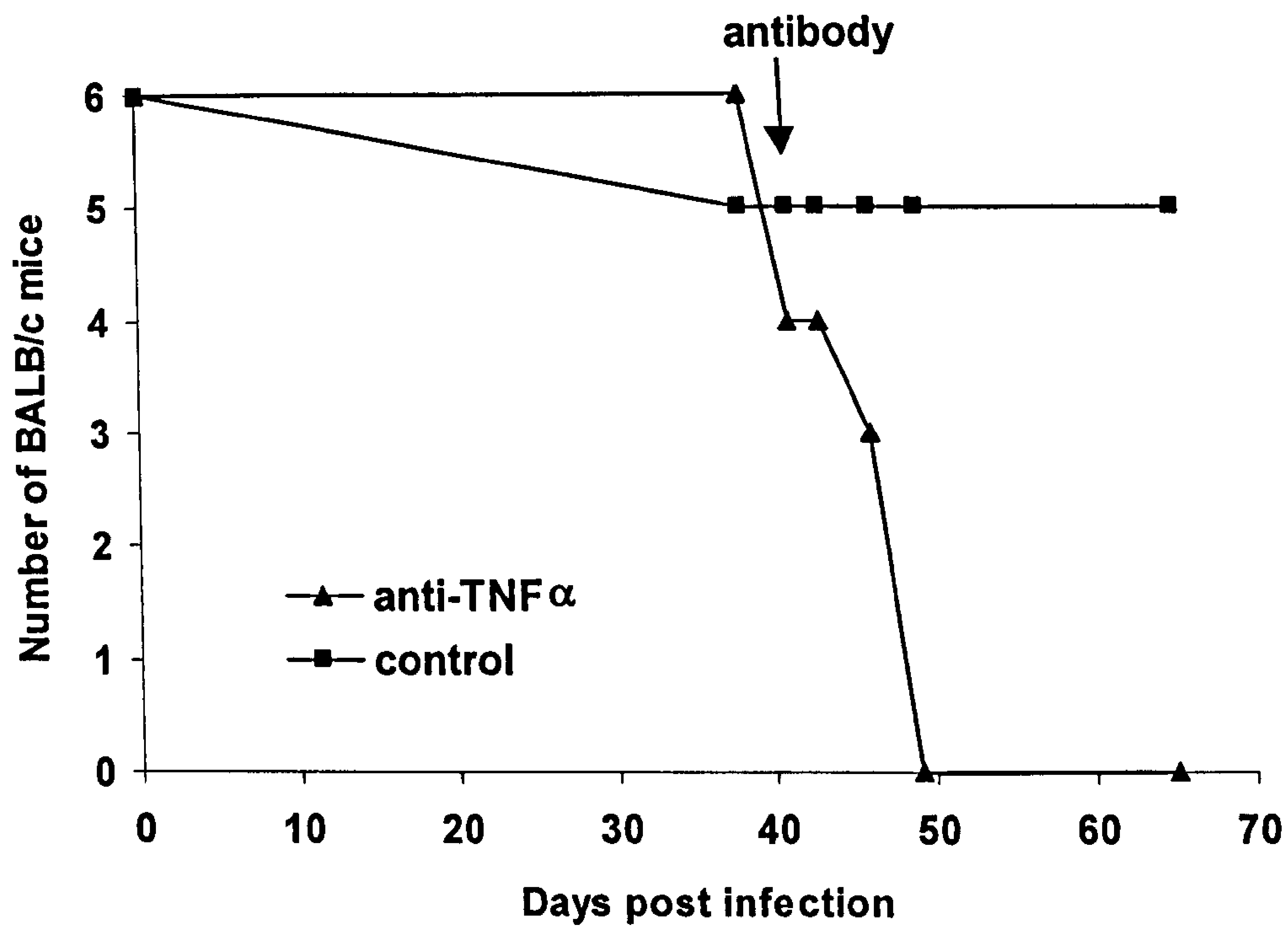


Figure 6.15: Survival of BALB/c mice (n=6/group) treated with TNFα antibodies or control antibodies 6 weeks following infection with *B. mallei.*

6.4 Discussion

Characterisation of splenic and systemic cytokine responses during *B. mallei* infection revealed the induction of a range of inflammatory and type 1 cytokines. Expression of splenic IL-1β and IL-18 was enhanced very early (5 h p.i.) in the immune response. IL-1β is a proinflammatory cytokine and acts both locally and systemically in the induction of fever (Lee *et al*, 2004). It is important in host defence against *L. monocytogenes* infection, possibly by modulating neutrophil responses and upregulating MHCII expression on macrophages (Rogers *et al*, 1994). IL-1β can be induced in neutrophils by granulocyte macrophage-colony stimulating factor (GM-CSF) (Fernadez *et al*, 1996) during infection (Hartung *et al*, 2001) linking the observed increased expression of GM-CSF and IL-1β 24 h p.i. in this study. IL-18 is important in host defence against infection for a number of intracellular pathogens including *B. pseudomallei* (Santanirand *et al*, 1999), *Y. enterocolitica* (Bohn *et al*,1998) and *L. major* (Ohkusu *et al*, 2000). It exerts its effects either via the induction of IFN-γ or in an IFN-γ-independent manner by inducing bacterial killing mechanisms of macrophages eg. nitric oxide and TNFα production (Neighbors *et al*, 2001). This implicates IL-18 in the induction of IFN-γ early in *B. mallei* infection.

Both IL-1β and IL-18 are members of the IL-1 family of ligands (Lee *et al*, 2004) and require proteolytic cleavage by caspase-1 for their activation. Bacteria are able to activate caspase-1 to induce the death of infected macrophages. *B. pseudomallei* (Sun *et al*, 2005) and *F. tularensis* (Mariathasan *et al*, 2005) induce caspase-1-dependent cell death leading to the release of IL-1β and IL-18. The mRNA expression of either of these cytokines was not related to bacterial colonisation 24 h p.i., although significant increases in their expression were observed over the first 24 h p.i.. This suggests that processing the protein form of these cytokines by caspase-1 is important in immunity to *B. mallei* infection.

High levels of splenic IFN-γ mRNA expression and systemic IFN-γ were detected within 24 h p.i. implicating it in immunity to *B. mallei* infection. IFN-γ is a cytokine essential for type 1 responses and is important for host protection against a number of intracellular pathogens including *B. pseudomallei* (Santanirand *et al*, 1999), *M. tuberculosis* (Cooper *et al*, 1993) and *C. trachomatis* (Perry *et al*, 1997). IFN-γ was found to be essential for protection against *B. mallei* infection. Disruption of the IFN-γ gene in IFN-γ KO mice rendered mice highly susceptible to *B. mallei* infection with death occurring 2-3 days post-infection. Removing this rapid, innate IFN-γ response early in infection demonstrates the critical nature of this response for survival against *B. mallei* infection. It is also critical in defence for a number of other intracellular pathogens including the closely related bacterium *B. pseudomallei* (Santanirand *et al*, 1999) and *Listeria monocytogenes* (Huang *et al*, 1993).

IFN-γKO mice on both a BALB/c and C57BL/6 background succumb to the disease within the early stages of infection. This demonstrated that IFN-γ is essential for immunity to *B. mallei* infection in both mouse strains suggesting that genetic background does not affect the host's response to IFN-γ mediated immunity. In addition to this, immunocompetent mice on either a BALB/c or a C57BL/6 background died several weeks following *B. mallei* infection. This suggests that both mouse strains have a similar disease progression in response to *B. mallei* infection. In infection with other pathogens including *B. pseudomallei*, differences in resistance to infection occur between BALB/c and C57BL/6 mice. Infection of BALB/c mice with *B. pseudomallei* leads to an acute disease whereas C57BL/6 mice are relatively resistant to *B. pseudomallei* infection (Leakey *et al*, 1998). In both mouse strains IFN-γ is important for immunity to *B. pseudomallei* although differences in susceptibility appear to be related to the balance in production of other cytokines during infection (Ulett *et al*, 2000).

Increased expression of other cytokines involved in type 1 immunity was also observed during *B. mallei* infection. IL-27 mRNA expression was elevated 24 h p.i. following *B. mallei* infection. IL-27 is a heterodimeric cytokine that binds to the receptor WSX-1 and is involved in development of type 1 responses during infection. It is produced by activated antigen-presenting cells during infection and plays a role in IFN-γ production from naïve mouse $CD4^+$ T cells and human NK cells *in vitro* (Pflanz *et al*, 2002). It is expressed during *M. tuberculosis* infection (Pearl *et al*, 2004) and is important *in vivo* in *L. major* infection as impaired IFN-γ production and increased susceptibility to infection occurs in WSX-1-/- (IL-27 Receptor KO) mice (Yoshida *et al*, 2001). The concurrent expression of IL-27 and IFN-γ during *B. mallei* infection implicated IL-27 as a potential inducer of IFN-γ production.

Increased expression of IL12p35, a cytokine involved in instigation of type 1 responses, was observed over the first 7 days of *B. mallei* infection. IL-12 is a cytokine critical in guiding type 1 responses during intracellular infection (Gazzinelli *et al*, 1994), is produced by macrophages and acts on naïve T cells and NK cells to stimulate IFN-γ production (Yoshimoto *et al*, 1998). IL-12 is composed of two subunits, p35 and p40. Increased expression of IL12p35 but not IL12p40 was observed and IL12p35 expression (but not p40) was associated with bacterial colonisation of the spleen 24 h p.i. suggesting that the p35 subunit is upregulated in response to the infection. Other studies have revealed that the IL-12p40 subunit is important for survival following the first week of *B. mallei* infection (Lukaszewski, 2004) and, therefore, has a role in controlling the infection although mRNA expression of IL12p40 was not detected. IL12p40 is homologous to a subunit from the cytokine IL-23 (Oppmann *et al*, 2000) whereas the IL12p35 is specific for IL-12. Higher levels of IL12p35 expression suggests that IL-12 and not IL-23 may have a more important role in the spleen during *B. mallei* infection although this requires further investigation.

IL-6 is a multi-functional cytokine and high levels of this cytokine both in the spleen and systemically 24 h p.i. suggest it may be important in immune responses to *B. mallei*. IL-6 is essential for surviving infection with *L. monocytogenes* (Dalrymple *et al*, 1995), *M. tuberculosis* (Ladel *et al*, 1997) and *Escherichia coli* (Dalrymple *et al*, 1996) although it is not important for immunity to *L. major* infection (Moskowitz *et al*, 1997). It exerts its activity during the innate immune response by aiding and allowing appropriate neutrophil responses to develop during infection (Dalrymple *et al*, 1995; Romani *et al*, 1996). In addition to this, IL-6 has also been shown to induce IFN-γ during infection with the intracellular bacteria *M. tuberculosis* (Saunders *et al*, 2000) and *L. monocytogenes* (Guilera & Pollard, 2001) as well as following BCG inoculation (Luo *et al*, 2003). High levels of IL-6 and IFN-γ co-expression and production suggested that IL-6 may be involved in IFN-γ responses during *B. mallei* infection. Upregulation of IL-6 in the serum also coincided with high levels of MCP-1. MCP-1 (CCL2) is a chemokine involved in the chemoattraction of monocytes (Lu *et al*, 1998) and T cells (Carr *et al*, 1994) to sites of infection. It is important in protective immune responses during bacterial infection by recruiting cells to the site of infection (Chae *et al*, 2002; Guilera & Pollard, 2001). Following *B. mallei* infection, IL-6 may be inducing MCP-1 which in turn may induce recruitment of monocytes into the spleen as has been observed in other bacterial infections (Bohn *et al*, 1998). In addition to this, detection of systemic IL-6 and MCP-1 responses in the serum of abscessed animals at days 14 and 36 p.i. were associated, suggesting the combined release of these mediators from infected tissues during infection.

The strong immune responses elicited by the host in the first 24 h p.i. represented a peak in cytokine responses. Decreased splenic expression of IFN-γ, IL-1β, IL-6, GM-CSF and IL12p35 occurred 3 days p.i.. This was complemented by decreased serum production of IFN-γ, IL-6 and MCP-1 to control levels. This suggests that *B. mallei* may be modulating host immunity after the first 24 h p.i. by interfering with cytokine responses. IL-6 is

important in protective immune responses during bacterial infection, however, it is a multifunctional cytokine that is also able to downregulate protective immune responses during *M. tuberculosis* infection to allow pathogen survival. IL-6 acts to reduce macrophage responsiveness to IFN-γ, causing down regulation of MHCII expression on macrophages (Nagabhushanam *et al*, 2003). The high levels of IL-6 observed 24 h p.i. with *B. mallei*, in addition to a potential role in protective immunity, implicate it in the downregulation of cytokine responses. IL-10 is an anti-inflammatory cytokine that acts as a negative regulator of the inflammatory response by instigating processes required for down-regulating proinflammatory responses (Weissman, 2001). Although increases in IL-10 24h p.i. were not significant during Immune Analysis 2, IL-10 expression was related to bacterial colonisation of the spleen suggesting that its expression is influenced by the infection. In addition to this, IL-10 expression levels were raised 24 h p.i. in Immune Analysis 1. IL-10 is able to down-regulate the expression of GM-CSF (Lenhoff *et al*, 1998) and may be involved in suppressing expression of the macrophage activators, GM-CSF and IFN-γ, during *B. mallei* infection. Although most responses examined were downregulated by 3 days p.i., IL-27 expression remained elevated suggesting that IL-27 may be important in controlling bacterial numbers which remained constant in the spleen over the first 7 days of infection.

TNFα is involved in protection against a number of intracellular pathogens including *M. tuberculosis* (Scanga *et al*, 1999) and *S. typhimurium* (Kirby *et al*, 2002). TNFα expression was significantly higher than control expression throughout the first 7 days of *B. mallei* infection and was related to bacterial colonisation 24 h p.i. suggesting a role for TNFα in immunity to *B. mallei* infection. Antibody treatment demonstrated that TNFα played an important role in innate immunity to *B. mallei* infection by controlling expansion of bacterial populations in the spleen. Control of bacterial replication was independent of when TNFα was depleted. This suggests that TNFα maintained continual control of

bacterial replication during the early stages of infection, although it was not critical for survival during early infection. TNFα may mediate its effects by a number of mechanisms including upregulation of adhesion molecules including CD54 on the surface of neutrophils, macrophages and endothelial cells to aid migration of cells into the site of infection (Gibson III *et al*, 1998). Early abscess formation occurred 7 days p.i. in the absence of TNFα, suggesting that TNFα may delay abscess formation during infection of immunocompetent mice, possibly by controlling bacterial proliferation. TNFα is implicated in abscess formation and controls the number of abscesses observed during *C. albicans* infection by enhancing the killing capacity of granulocytes (Vonk *et al*, 2002). In a *S. aureus* abscess formation model, TNFα antibodies prevented the formation of abscesses (Song *et al*, 2002) and administration of TNFα antibodies during *B. fragilis* infection also prevented the formation of abscesses (Gibson III et al, 1998) suggesting that TNFα has a role in prevention and control of abscess formation for different pathogens.

Increased splenic expression of TNFα and other proinflammatory cytokines including IL-1β, IL-6 and GM-CSF were associated with abscessed mice at 14 and 21 days p.i.. During this chronic phase of infection, TNFα was also found to be involved in controlling *B. mallei* infection since death occurred rapidly following depletion of TNFα 6 weeks following infection. At this stage of infection, large abscesses existed within the spleen. TNFα appeared to maintain protective responses in the host, possibly by controlling bacterial growth in localised areas of infection in abscesses. In the absence of TNFα, bacteria may have been released into the circulation from the organs leading to a rapid and fatal outcome.

In summary, the expression and production of a number of cytokines was observed during *B. mallei* infection. Pro-inflammatory cytokines were induced 5 h p.i. followed by a strong

splenic and systemic cytokine response, mediated predominantly by IFN-γ, IL-6 and the chemokine, MCP-1 24 h p.i.. This was followed by a decrease in cytokine responses 72 h p.i.. The IFN-γ response was found to be essential for protection against *B. mallei* infection. TNFα was also important in controlling bacterial proliferation in the spleen during innate responses and was involved in protective immunity during the chronic stages of infection.

Chapter 7

Induction and production of IFN-γ in response to heat-killed *B. mallei in vitro.*

7.1 Introduction

The work presented so far in this thesis has demonstrated the essential nature of IFN-γ in the innate immune response to *B. mallei* infection in mice. Therefore, the role of cytokines and cells involved in the IFN-γ response was investigated in more detail. IFN-γ is an essential component of the innate immune response to a number of intracellular pathogens including *B. pseudomallei* (Haque *et al*, 2005) and *L. monocytogenes* (Berg *et al*, 2005). The main cellular sources of innate IFN-γ during infection with *B. pseudomallei* and *L. monocytogenes* are NK cells and T cells (Lertmemongkolchai *et al*, 2001; Berg *et al*, 2005) although other cell types, including macrophages, are involved in production of IFN-γ *in vivo* (Haque *et al*, 2005). IFN-γ is induced by a range of cytokines during infection, which are mainly associated with type 1 immunity to intracellular pathogens, including IL-12, IL-18, IL-23 and IL-27. Other cytokines, including IL-6, have also been implicated in induction of IFN-γ *in vivo* during infection (Guilera & Pollard, 2001). In Chapter 6, upregulated expression of IL12p35, IL-18, IL-27 and IL-6 was described during the first 24 h following *B. mallei* infection. This corresponded with a strong IFN-γ response suggesting a link between IFN-γ production and these cytokines. The aims of this chapter were to determine:

- The role of type 1 cytokines, upregulated early during *B. mallei* infection, in induction of IFN-γ *in vitro* in response to heat-killed *B. mallei*.
- The role of T cells and NK cells in IFN-γ production *in vitro* in response to heat-killed *B. mallei* to provide a comparison with other intracellular pathogens.
- The role of Gr-1$^+$ cells in IFN-γ production *in vitro* in response to heat-killed *B. mallei* due to the importance of both IFN-γ and Gr-1$^+$ cells in protection during the early phases of *B. mallei* infection.

7.2 Methods

The secretion of IFN-γ and other cytokines (TNFα, IL-10, MCP-1, IL-6 and IL-12p70) was detected in spleen cell culture supernatants from wild type and knockout mice, with or without antibody treatment, following stimulation with 10^6 cfu/ml heat-killed *B. mallei* (Table 7.1). This allowed investigation of the contribution of IL12p35, IL12p40, IL-18, IL-6, IL-27 and Gr-1^+ cells to the IFN-γ response *in vitro*.

Intracellular IFN-γ was detected in $CD3^+$ T cell and $DX5^+$ NK cell populations by flow cytometry in response to heat-killed *B. mallei in vitro*. Splenic cell cultures were stimulated with 10^6 cfu/ml heat-killed *B. mallei* and IFN-γ secretion was prevented 3 h prior to intracellular staining using Brefeldin A. Intracellular IFN-γ was assessed in spleens from wildtype (C57BL/6 and BALB/c) mice in the presence or absence of anti-IL-18R antibodies and IL-12KO mice to determine the effects of IL-12 and IL-18 on IFN-γ production from different cell types.

Mouse strain	Antibody treatment	Stimulant
IL-6 Knockout C57BL/6	-	Heat-killed *B.mallei*
WSX-1 (IL-27R) Knockout C57BL/6	-	Heat-killed *B.mallei*
IL12p35 Knockout C57BL/6	-	Heat-killed *B.mallei*
IL12p40 Knockout C57BL/6	-	Heat-killed *B.mallei*
C57BL/6	± IL-18R or Mac5 antibody (*in vitro*)	Heat-killed *B.mallei*
BALB/c	Gr-1 (RB6-8C5) or Mac5 antibody (*in vivo*)	Heat-killed *B.mallei*

Table 7.1: Summary of the mouse strains and antibodies used to determine the role of IL-6, IL-27, IL12p35, IL12p40, IL-18 and Gr-1^{+} cells on IFN-γ production in response to heat-killed *B. mallei in vitro.*

7.3 Results

7.3.1 *Induction of IFN-γ in response to B. mallei in vitro*

The role of the cytokines IL12p35, IL12p40, IL-18, IL-27 and IL-6 in inducing IFN-γ production was investigated to determine the significance of the elevation and co-expression of these cytokines with IFN-γ during the first 24 h of *B. mallei* infection.

7.3.1.1 *IL12p35 and IL12p40*

Following stimulation of splenic cultures from wildtype C57BL/6 mice with heat-killed *B. mallei*, 3-5ng/ml of IFN-γ was detected. In response to stimulation with heat-killed *B. mallei*, no IFN-γ was detected in splenic cultures from either IL12p35 or IL12p40 KO mice (Figure 7.1A). In wildtype cultures, significantly higher amounts of IL-12p70 were detected in response to heat-killed *B. mallei* in comparison with IL12p35KO and IL12p40 KO spleen cultures ($p<0.05$) (Figure 7.1B). A reduction in IL-6, MCP-1 and IL-10 was also observed in both IL-12KO mouse strains in comparison with wildtype cultures ($p<0.05$) (Figure 7.1 A&B).

7.3.1.2 *IL-18*

Following stimulation of spleens from wild-type C57BL/6 mice treated *in vitro* with neutralising antibodies against the IL-18 receptor, a 3-fold reduction in IFN-γ production compared with wild-type mice (without antibody) was observed ($p<0.05$)

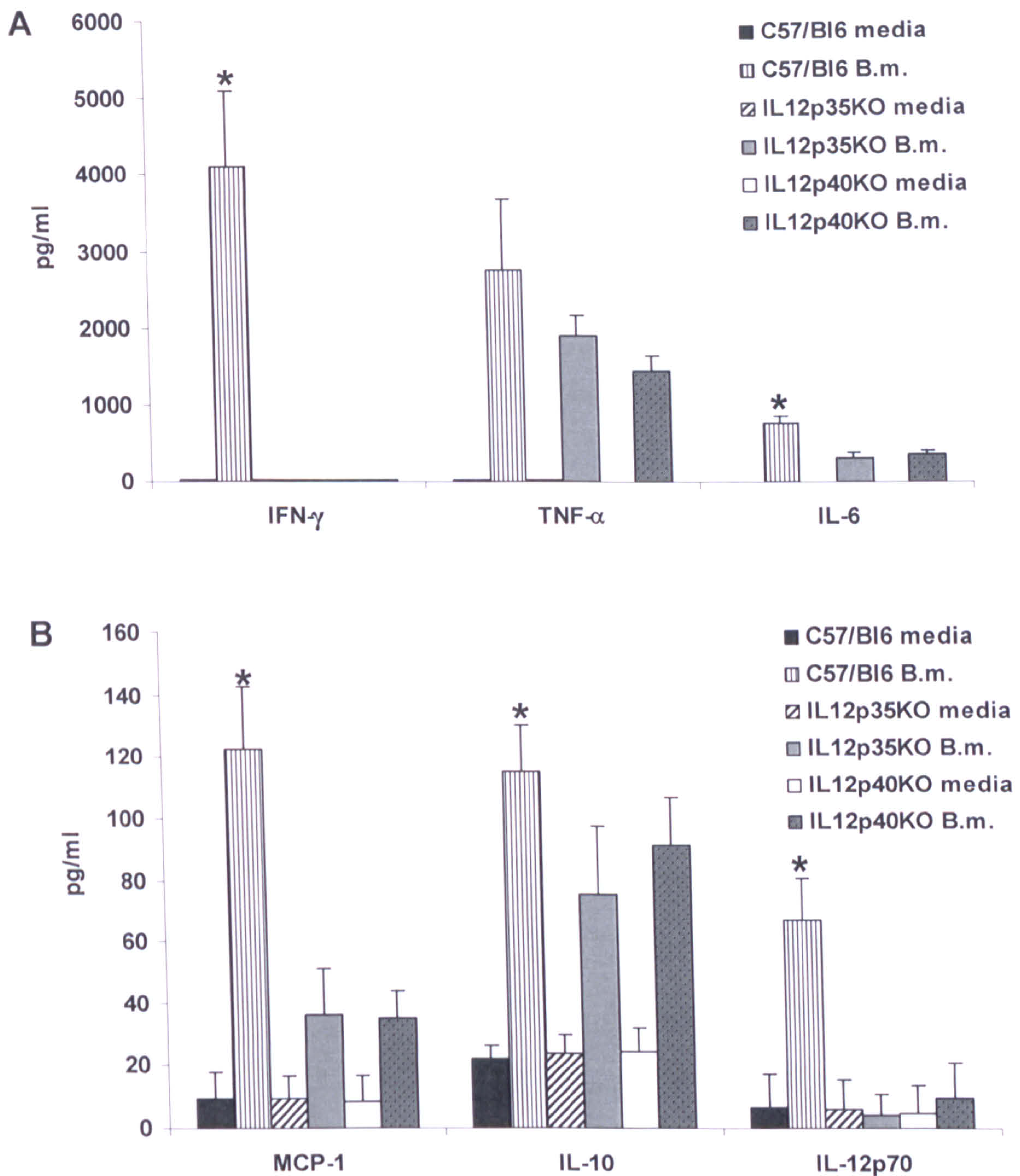


Figure 7.1: Mean cytokine production in cell culture supernatants following *in vitro* stimulation of spleens from IL12p35KO, IL12p40KO or control C57BL/6 mice with heat-killed *B. mallei* (B.m.) or media for 24 hours. A) Mean amount (pg/ml) of IFN-γ, TNFα and IL-6; B) Mean amount of MCP-1, IL-10 and IL-12p70 detected in culture supernatants. Error bars indicate standard deviation. Asterisks represent statistical significance ($p<0.05$) in comparison with IL12p35 and IL12p40 spleen cultures stimulated with B.m.. This data is representative of two experiments with groups of 4 mice.

(Figure 7.2A). No differences between wild-type and treated cultures in IL-12p70, TNFα, MCP-1, IL-10 or IL-6 production occurred (Figure 7.2A&B).

7.3.1.3 IL-27

No significant differences in IFN-γ secretion in cell culture supernatants from WSX-1 (IL-27 Receptor) KO mice occurred in comparison with control mice following stimulation with heat-killed *B. mallei* (Figure 7.3A). Decreased MCP-1 and IL-10 production was observed in wildtype cultures in comparison with WSX-1KO cultures ($p<0.05$) (Figure 7.3A&B).

7.3.1.4 IL-6

No differences in IFN-γ secretion were observed between IL-6KO or wildtype cultures in response to heat-killed *B. mallei in vitro* (Figure 7.4A). Decreased production of TNFα, IL-10 and MCP-1 occurred in IL-6KO cultures in comparison with wildtype cultures (Figure 7.4A). IL-6 could not be detected in cultures from IL-6KO mice although it was detected in wildtype cultures in response to heat-killed *B. mallei*. Statistical analysis could not be performed on this data as only two IL-6KO mice were available for this experimental procedure. To determine whether IL-6 could alone induce IFN-γ *in vitro* in spleen cultures, recombinant IL-6 was added to cultures at a range of concentrations (0-40ng/ml). No IFN-γ was detected in cell culture supernatants at any dose of IL-6 in either BALB/c or C57BL/6 mice, although addition of IL-12 and IL-18 to cultures induced a strong IFN-γ response (Figure 7.5).

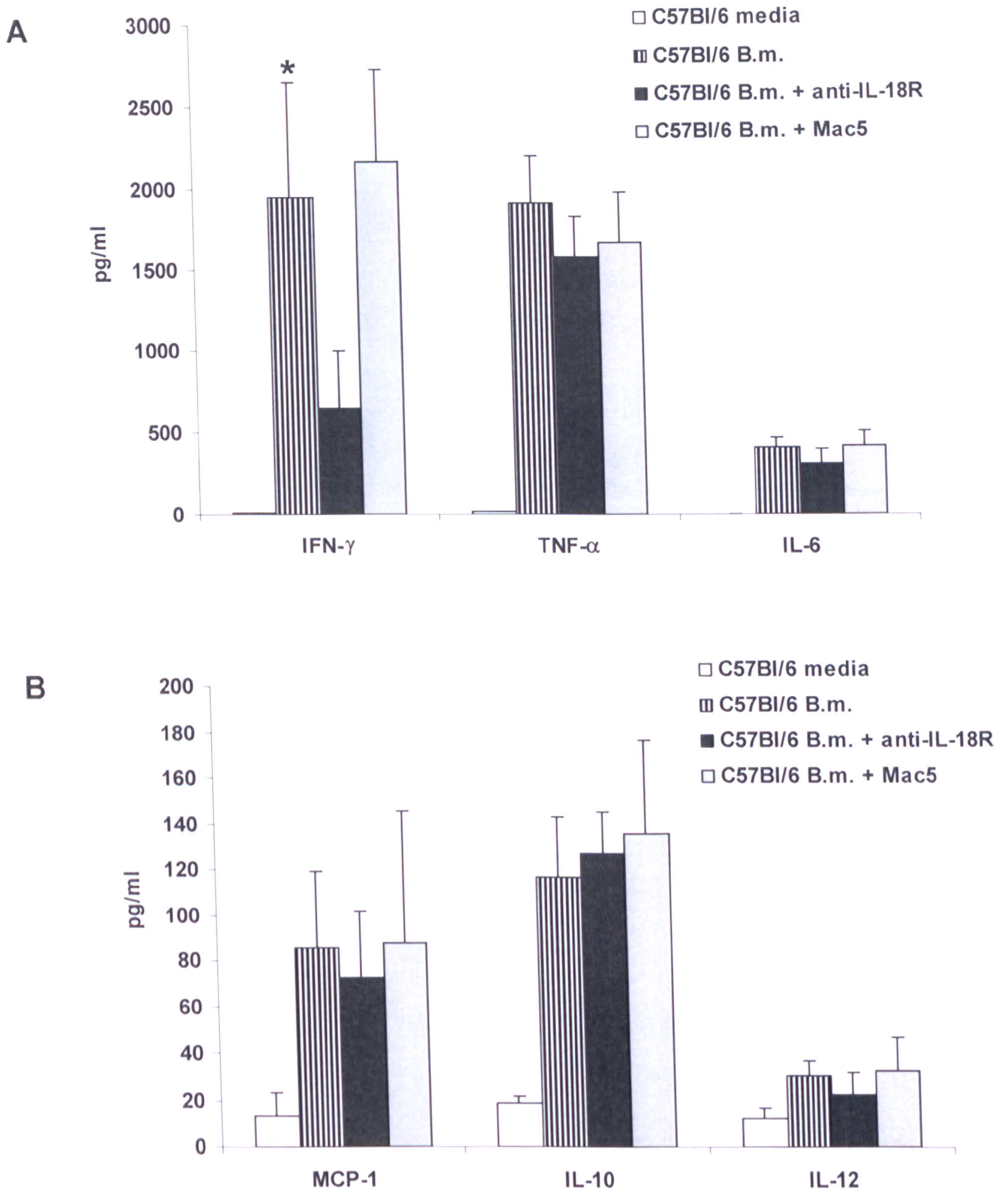


Figure 7.2: Mean cytokine production in the supernatant following *in vitro* culture of C57BL/6 spleens (n=3) in triplicate with or without IL-18R or Mac5 control antibody and heat-killed *B. mallei* (B.m.) or media for 24 hours. A) Mean amount (pg/ml) of IFN-γ, TNFα and IL-6; B) Mean amount of MCP-1, IL-10 and IL-12p70 detected in culture supernatants. Error bars indicate standard deviation. Asterisks represent statistical significance between C57BL/6 + B.m. and C57BL/6 + B.m. + anti-IL-18R cultures ($p<0.05$).

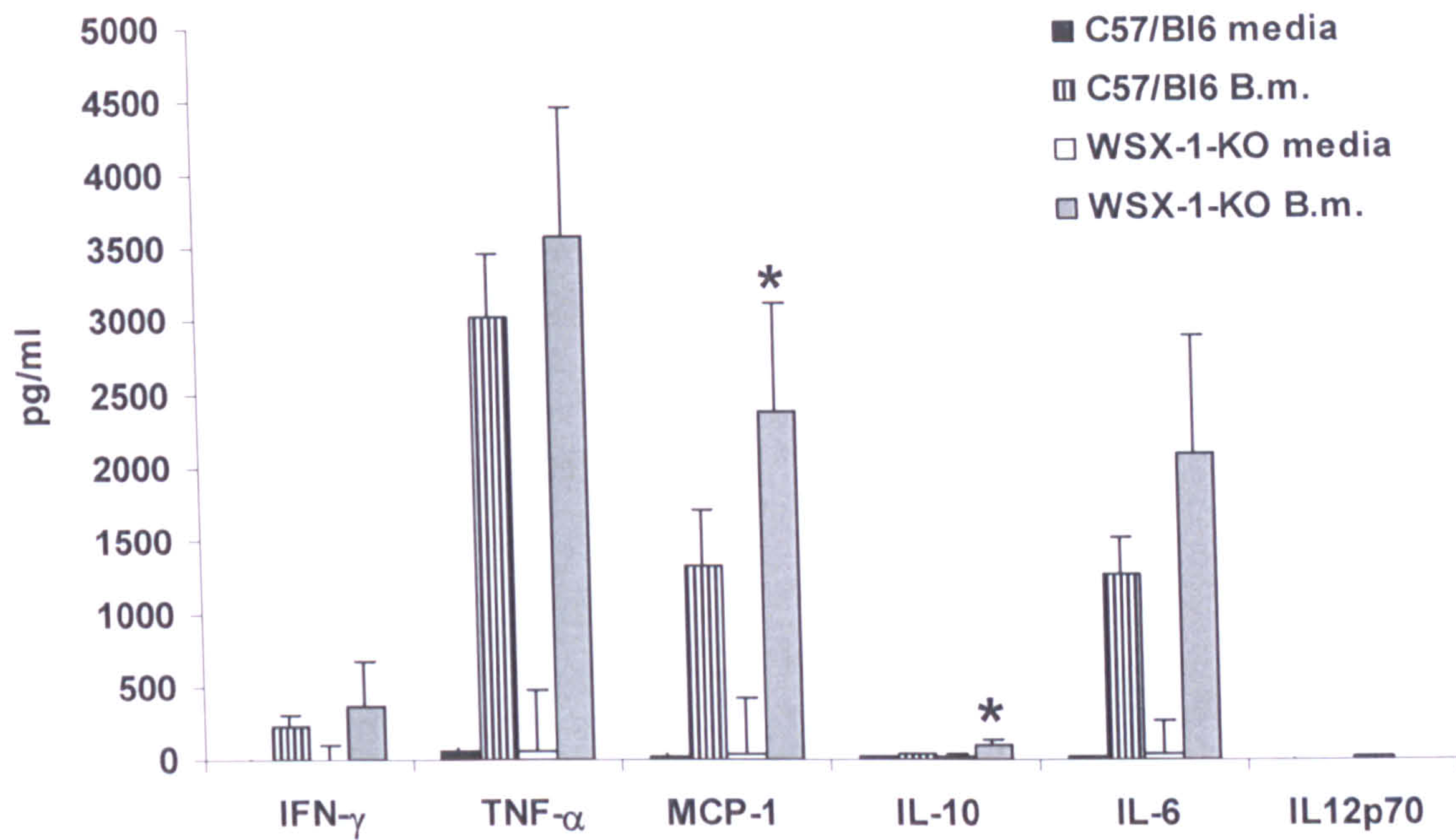


Figure 7.3: Mean cytokine production in the supernatant following *in vitro* culture of spleens from WSX-1KO (IL-27 receptor) mice (n=5) or control C57BL/6 mice (n=5) stimulated with heat-killed *B. mallei* (B.m.) or media for 24 hours. Error bars indicate standard deviation. Asterisks indicate statistical significant differences between WSX-1KO + B.m. and C57BL/6 + B.m. cultures ($p<0.05$).

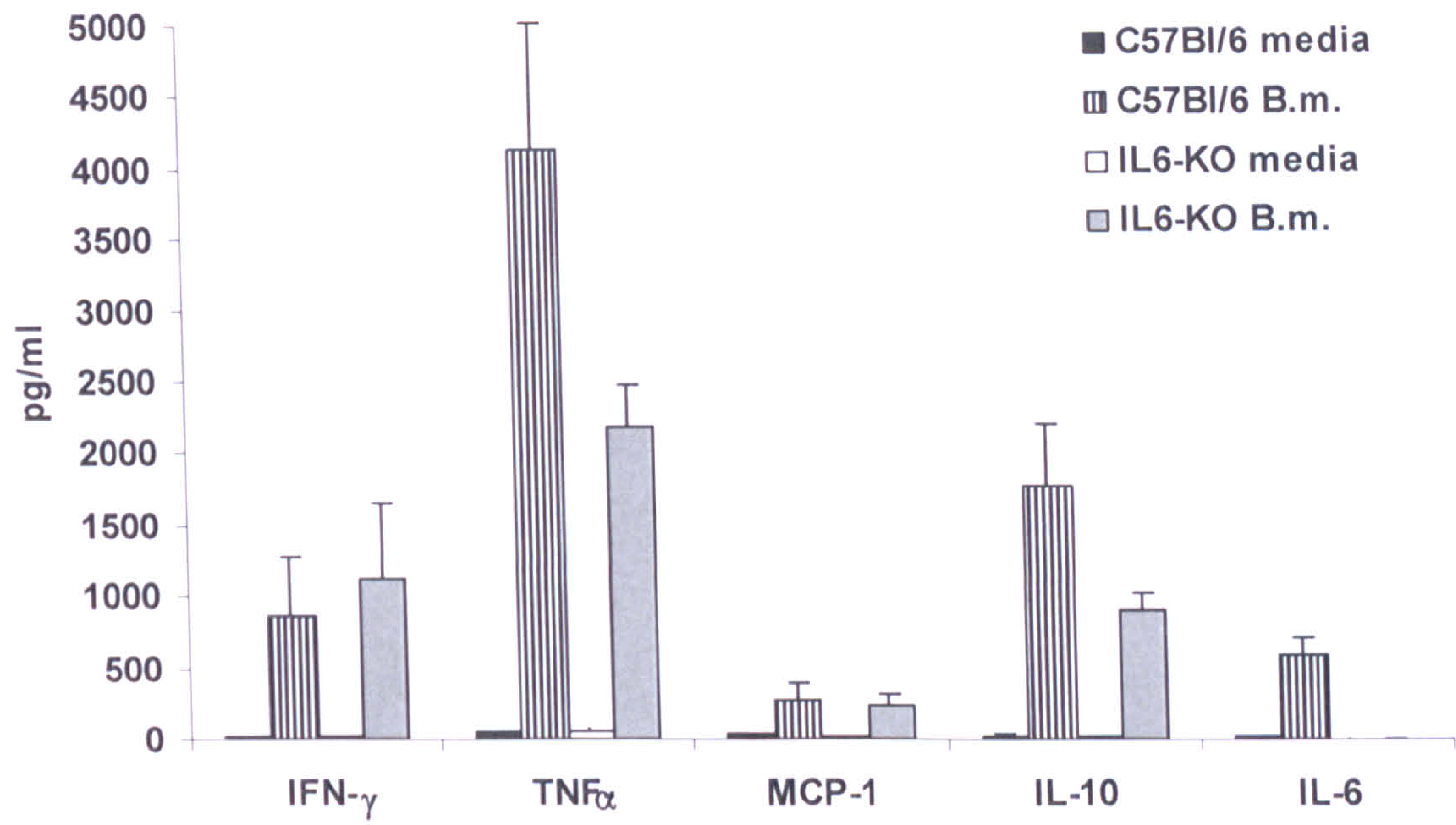


Figure 7.4: Mean cytokine production in the supernatant following *in vitro* culture of spleens from IL-6KO mice (n=2) or control C57BL/6 mice (n=5) stimulated with heat-killed *B. mallei* (B.m.) or media for 24 hours. Error bars indicate standard deviation.

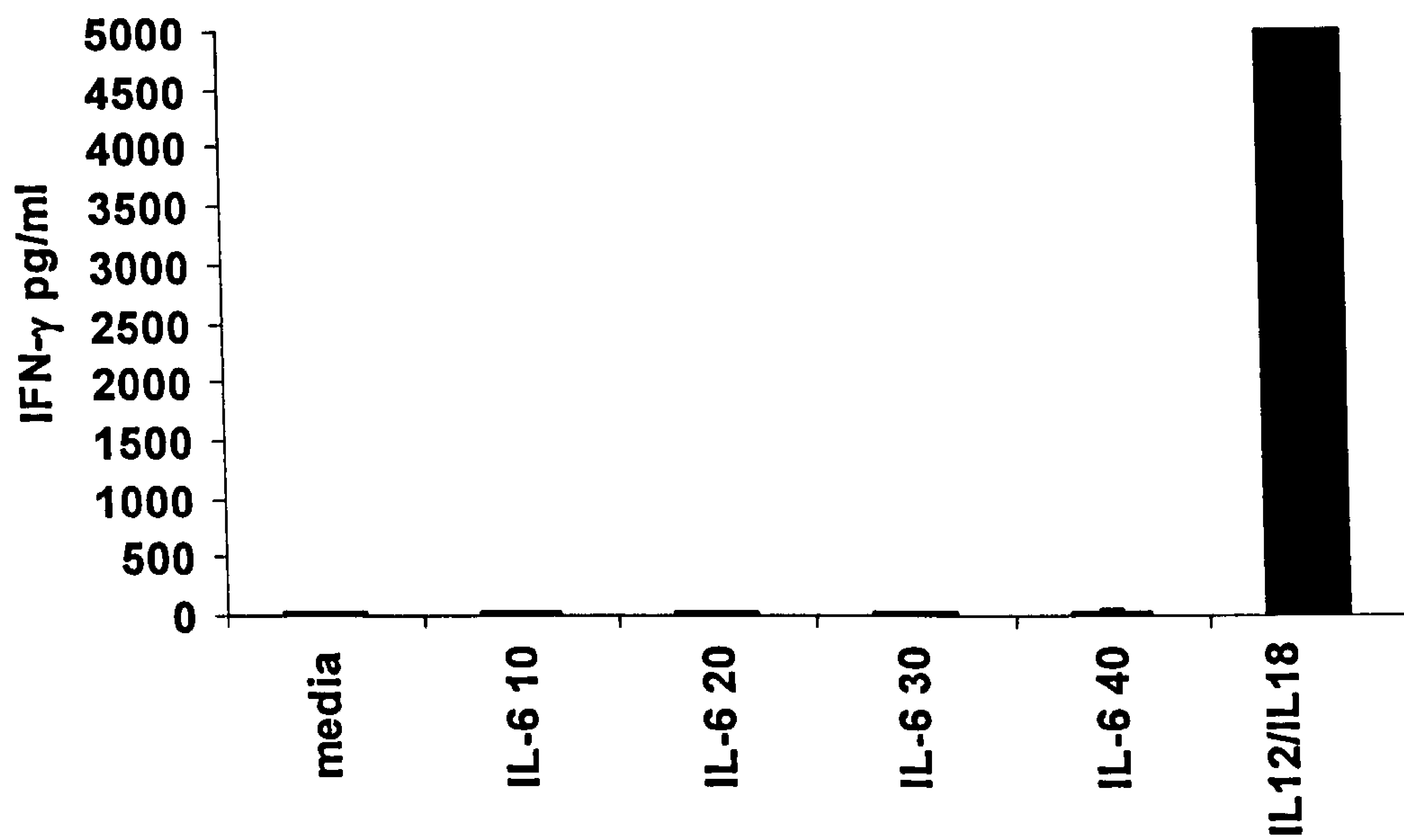


Figure 7.5: Mean IFN-γ production in the supernatant following *in vitro* stimulation of splenic cell cultures from C57BL/6 (n=3) and BALB/c (n=3) mice with IL-6. IFN-γ production in response to recombinant IL-6 (10-40 ng/ml), a combination of IL-12 (5 ng/ml) and IL-18 (5 ng/ml) or media alone for 24 hours.

7.3.2 Cellular sources of B. mallei-induced IFN-γ in vitro

The lymphocyte populations responsible for IFN-γ production *in vitro* were examined by intracellular flow cytometry. The predominant producers of IFN-γ in wildtype C57BL/6 mice in response to *B. mallei in vitro* were $DX5^+$ NK cells with $CD3^+$ T cells also contributing to the IFN-γ response (Figure 7.6). Of the NK cell population, 12-22% were IFN-γ^+ and 1-2% of the T cell population were IFN-γ^+ (Figure 7.7).

Intracellular analysis of IFN-γ production following *B. mallei* stimulation of IL12p35KO and IL12p40 KO confirmed the absence of an IFN-γ response in cell culture supernatants. Intracellular IFN-γ was not detected in either T cells or NK cells in the absence of IL-12 (Figure 7.8). The viability of spleen cell suspensions from IL12p35KO and IL12p40KO mice was examined by adding a combination of IL-12 and IL-18 to splenic cultures. IFN-γ was produced from both T cells and NK cells in spleens from both KO mice in response to IL-12/IL-18 demonstrating their ability to respond under these experimental conditions (Figure 7.9). The reduced IFN-γ response observed in cell culture supernatants in the absence of the IL-18R was investigated further by intracellular analysis of IFN-γ. A decrease in IFN-γ production from the NK cell population was observed in cell culture supernatants treated with IL-18R antibody *in vitro* (Figure 7.10).

In order to determine whether genetic background affected the cellular source of IFN-γ, IFN-γ production was assessed in splenic NK and T cells from BALB/c mice and like C57BL/6 mice NK cells were the predominant producers of IFN-γ in response to heat-killed *B. mallei* with 2.5 - 4.5% of NK cells producing IFN-γ (Figure 7.11). The $CD3^+$ T cell

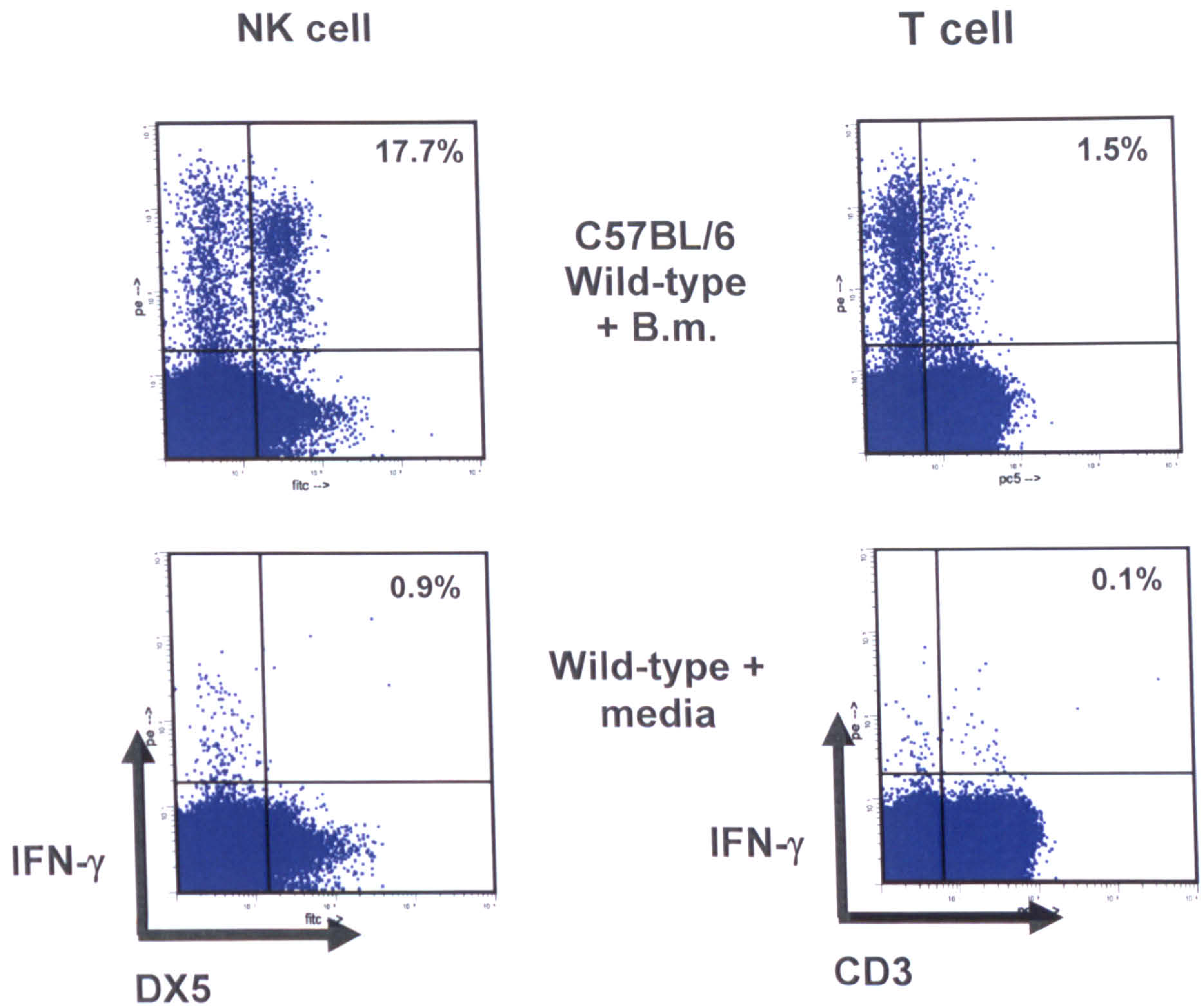


Figure 7.6: Intracellular IFN-γ production in T cells and NK cells in response to heat-killed *B. mallei* or media *in vitro*. Intracellular IFN-γ detected in NK ($DX5^+$) cells and T ($CD3^+$) cells following stimulation of spleens from wild type C57BL/6, *in vitro* with heat-killed *B. mallei* (B.m.) or media alone. Percentage of IFN-γ-positive NK cells or T cells is shown in right-hand quadrant. This data is representative of two experiments with groups of 4 mice.

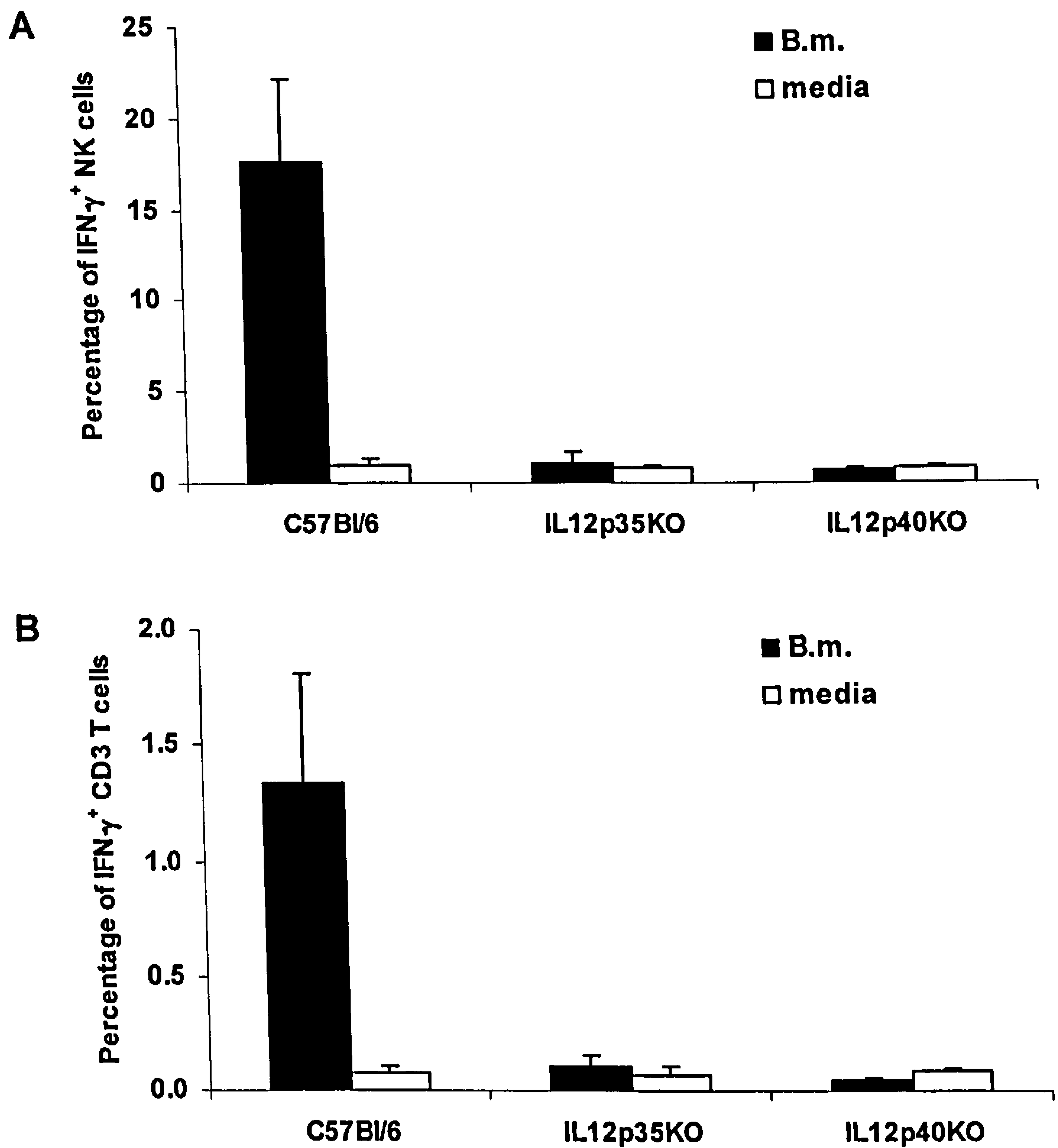


Figure 7.7: Mean percentage of IFN-γ^+ T cells and NK cells produced in response to heat-killed *B. mallei* or media *in vitro*. Intracellular IFN-γ detected in A) NK ($DX5^+$) cells and B) T ($CD3^+$) cells following stimulation of spleens from wildtype C57BL/6, IL12p35KO or IL12p40KO mice *in vitro* with heat-killed *B. mallei* (B.m.) or media alone. Error bars indicate standard deviation. This data is representative of two experiments with groups of 4 mice.

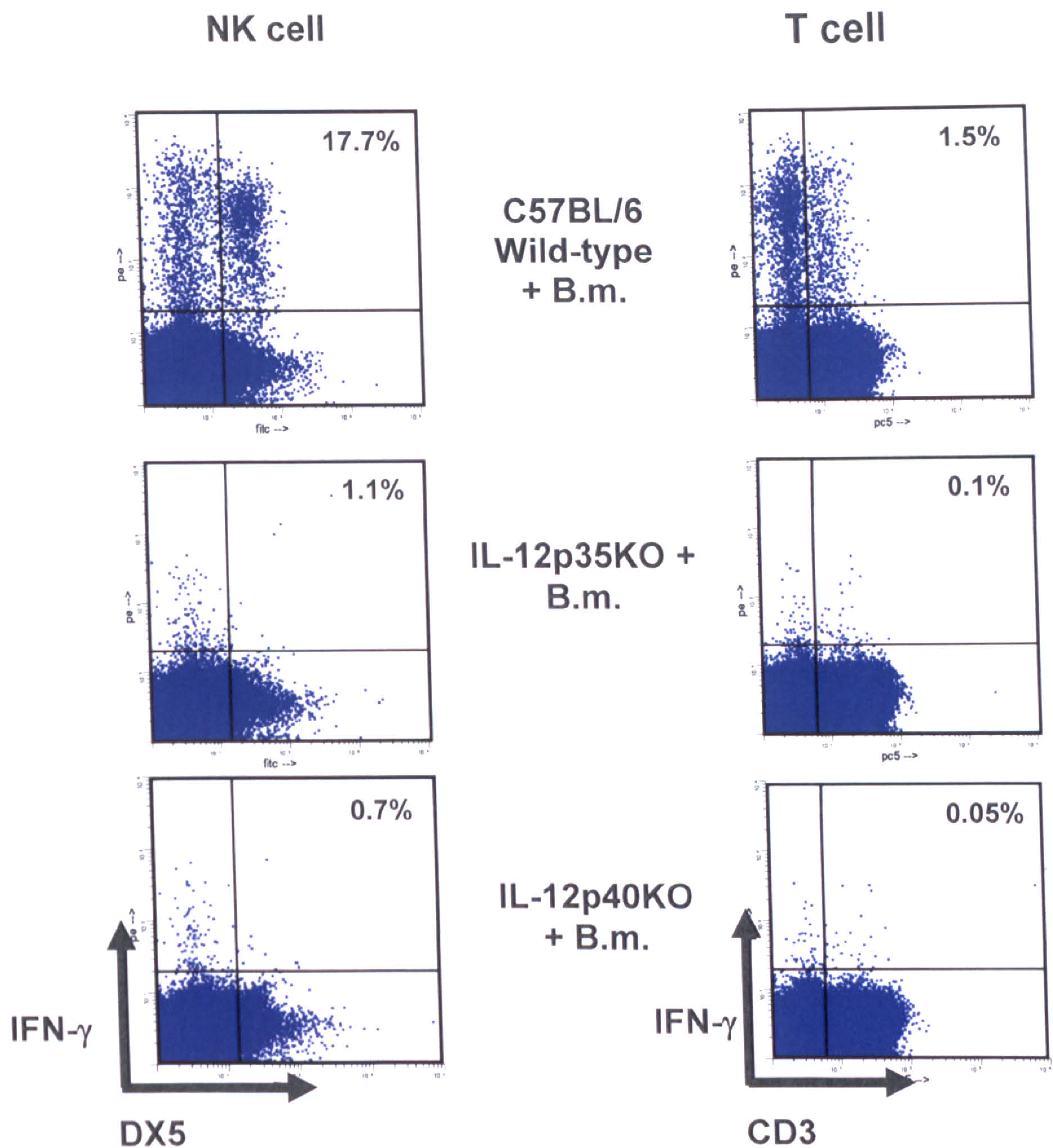


Figure 7.8: Intracellular IFN-γ production in T cells and NK cells in response to heat-killed *B. mallei* or media *in vitro*. Intracellular IFN-γ detected in NK ($DX5^+$) cells and T ($CD3^+$) cells following stimulation of spleens from C57BL/6 wildtype, IL12p35KO or IL12p40KO mice *in vitro* with heat-killed *B. mallei* (B.m.). Percentage of IFN-γ-positive NK cells or T cells is shown in right-hand quadrant. This data is representative of two experiments with groups of 4 mice.

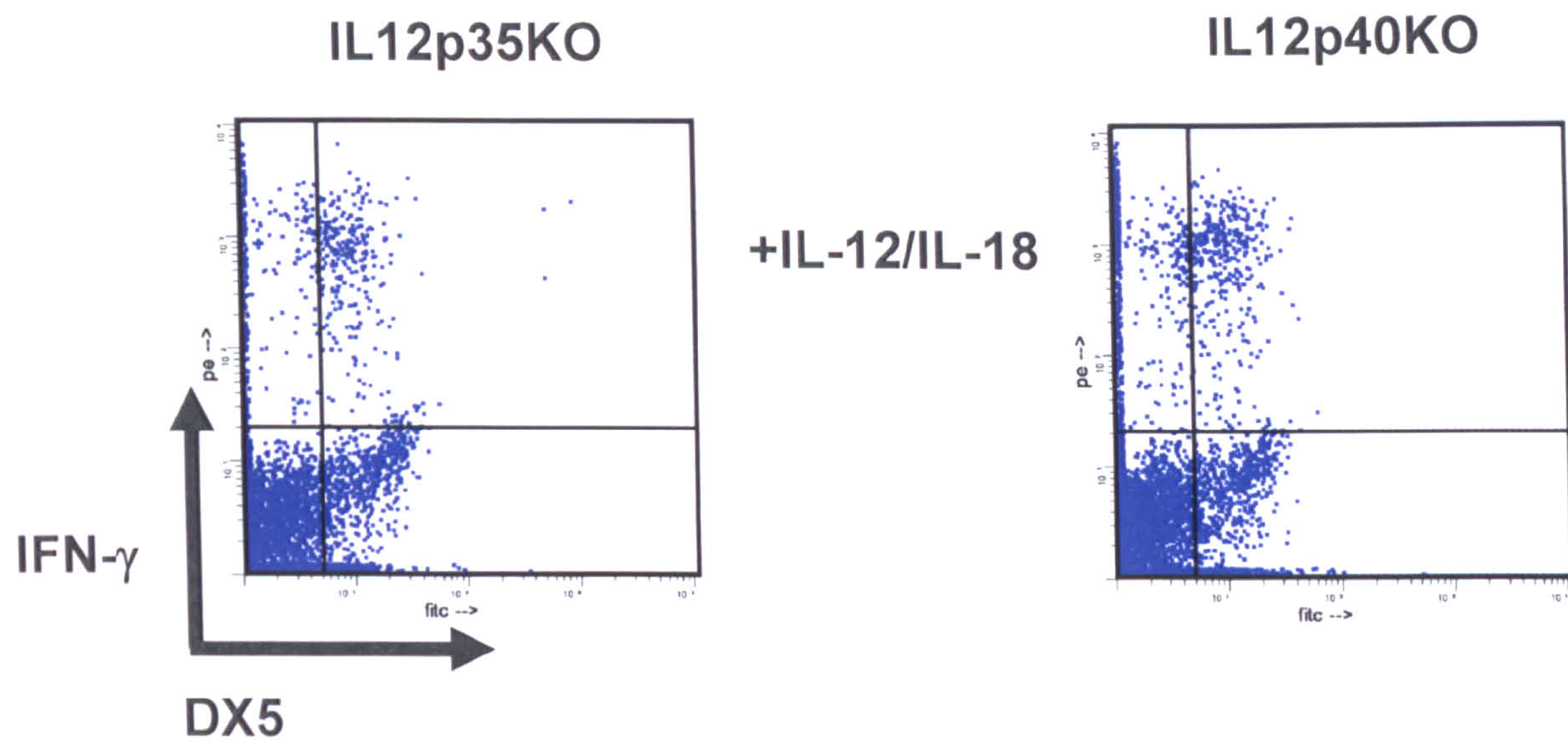


Figure 7.9: Intracellular IFN-γ production in NK cells in response to a combination of IL-12 and IL-18 *in vitro*. Intracellular IFN-γ detected in NK ($DX5^+$) cells following stimulation of spleens from IL12p35KO mice (n=3) and IL12p40KO mice (n=3) *in vitro* with IL-12 and IL-18.

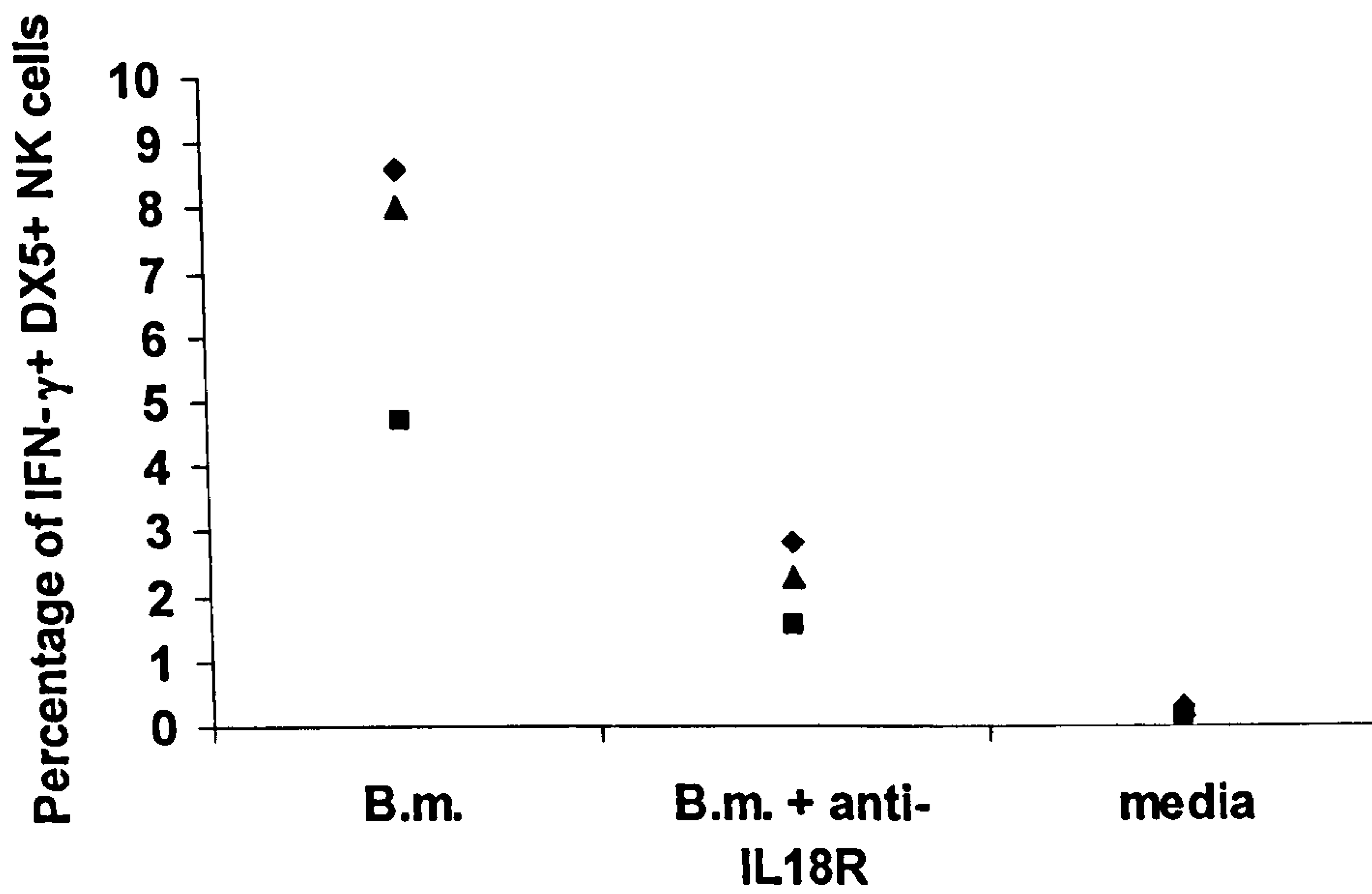


Figure 7.10: Percentage of IFN-γ^+ NK cells detected in response to heat-killed *B. mallei* following IL-18R neutralisation *in vitro*. Intracellular IFN-γ detected in NK ($DX5^+$) cells following stimulation of spleens from wildtype C57BL/6 mice (n=3) *in vitro* with heat-killed *B. mallei* with (B.m. + anti-IL-18R) or without (B.m.) IL-18R antibody neutralisation or media alone. Data points represent spleen cultures from individual mice.

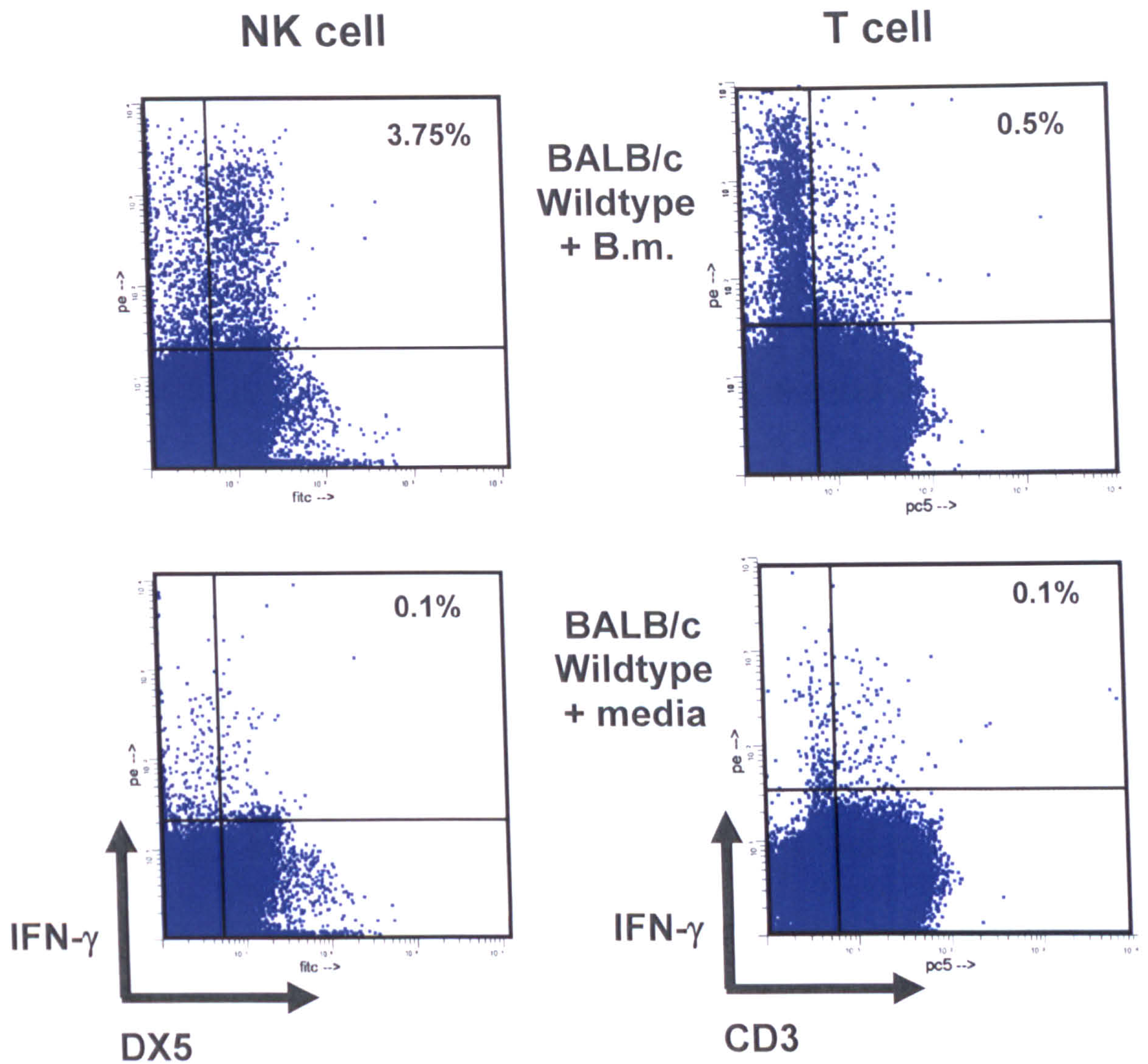
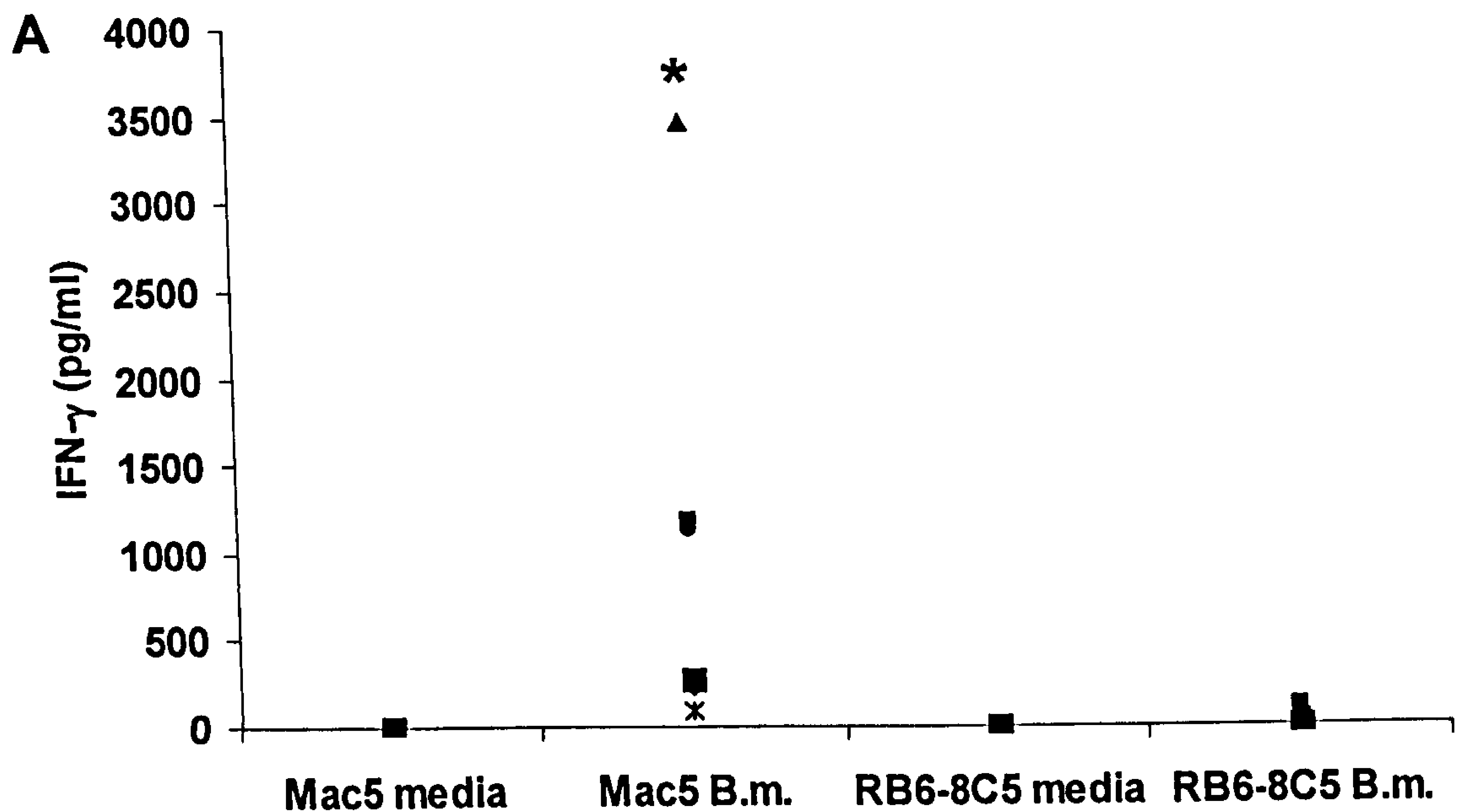


Figure 7.11: Intracellular IFN-γ production in T cells and NK cells in response to heat-killed *B. mallei in vitro*. Intracellular IFN-γ detected in NK ($DX5^+$) cells and T ($CD3^+$) cells following stimulation of spleens from BALB/c mice (n=4) *in vitro* with heat-killed *B. mallei* (B.m.) or media alone. Percentage of IFN-γ-positive NK cells or T cells is shown in right-hand quadrant.

population also contributed to this response with between 0.4-0.6% T cells producing IFN-γ (Figure 7.11).

7.3.2.1 *Effects of Gr-1 depletion on B. mallei induced IFN-γ*

The role of Gr-1$^+$ cells in IFN-γ production *in vitro* was assessed due to the essential requirement for Gr-1$^+$ cells in protection against *B. mallei* infection *in vivo*. IFN-γ production was significantly reduced in spleen cell suspensions from RB6-8C5 depleted mice in comparison with Mac5-treated control spleens ($p<0.05$) (Figure 7.12A). IFN-γ was produced in all Mac5 treated control spleen suspensions in response to heat-killed *B. mallei* although inter-individual variability existed in the strength of this response (Figure 7.12A). Production of IL-6, TNFα and IL-10 was significantly lower in RB6-8C5 treated mice than Mac5 treated controls ($p<0.05$) and MCP-1 levels were comparable with Mac5 controls in spleens from RB6-8C5 depleted mice (Figure 7.12B).



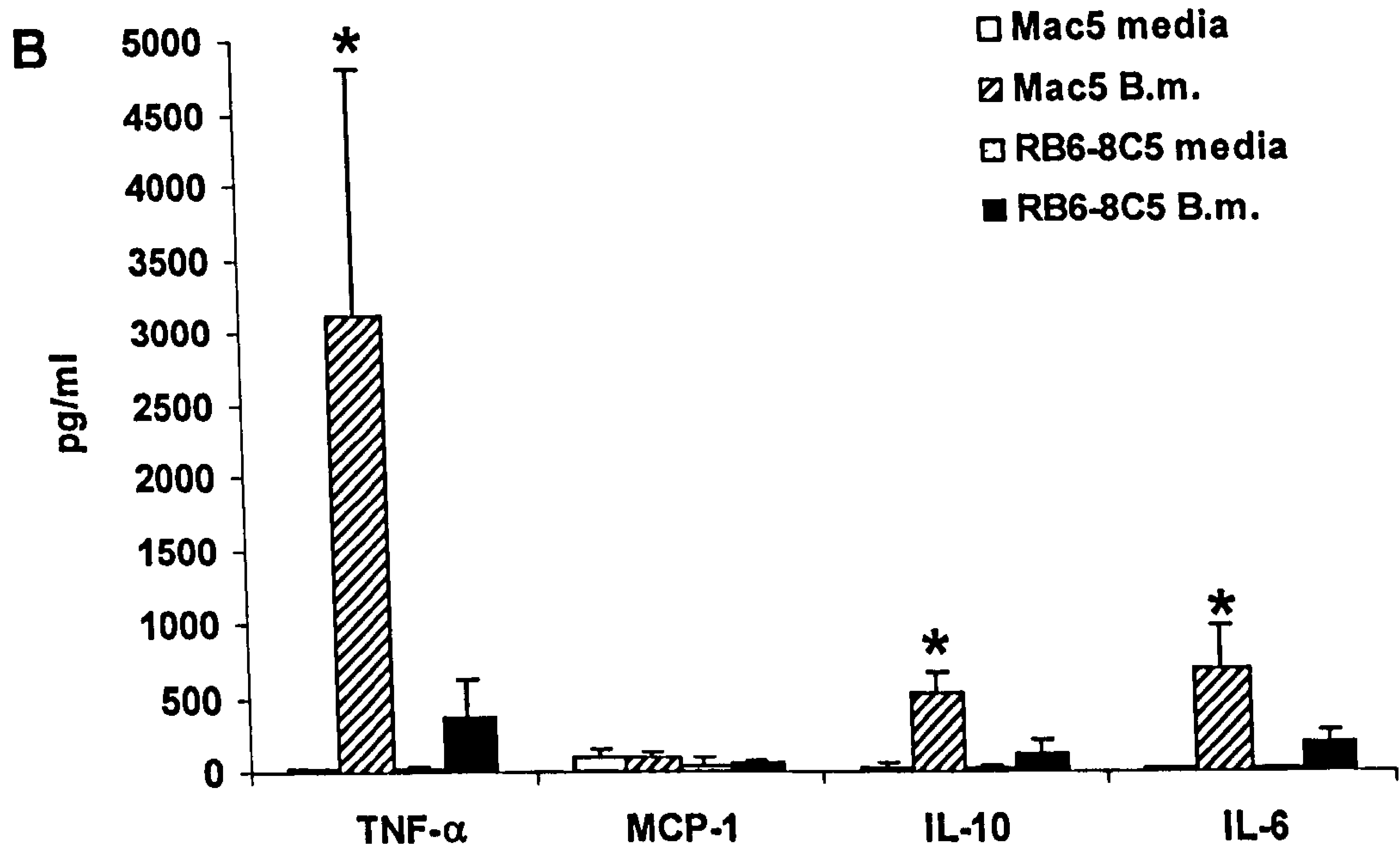


Figure 7.12: The role of Gr-1^{+} cells in cytokine production in response to heat-killed *B. mallei in vitro*. A) IFN-γ production in spleen cell suspensions from individual mice; B) Mean cytokine production of TNFα, MCP-1, IL-10 and IL-6 in the supernatant following *in vitro* culture of spleens from BALB/c mice (n=6) treated with Gr-1 (RB6-8C5) antibody or control (Mac5) antibody (n=6) and heat-killed *B. mallei* (B.m.) or media for 24 hours. Error bars indicate standard deviation. Asterisks indicate statistical significance ($p<0.05$) in comparison with RB6-8C5 spleen cultures stimulated with B.m..

7.4 Discussion

The aim of this chapter was to investigate the cytokine-mediated induction of IFN-γ in response to heat-killed *B. mallei* and to determine which cell types are involved in IFN-γ production *in vitro*. IL12p35 and IL12p40 were found to be essential for IFN-γ production in the presence of heat-killed *B. mallei in vitro*. IL-12 is essential for development of type 1 responses during intracellular infection and is involved in IFN-γ production during infection with *M. tuberculosis* (Nolt & Flynn, 2004) and *C. trachomatis* (Perry *et al*, 1997). IL-23 may also be involved in production of IFN-γ responses during infection in the absence of IL-12 (Khader *et al*, 2005). The requirement of both the p35 and p40 subunits for IFN-γ production suggests that IL-12 (p35 + p40) itself and not IL-23 (p40 but not p35) is responsible for the IFN-γ response.

IL-18 was found to be involved in IFN-γ production in response to *B. mallei*. IL-18 is important in host defence against intracellular infection with *B. pseudomallei* (Haque *et al*, 2005) and *L. monocytogenes* (Neighbors *et al*, 2001) by inducing IFN-γ, in synergy with IL-12, from T cells (Yoshimoto *et al*, 1998) and NK cells (Garcia *et al*, 1999). IL-18 is known to induce IFN-γ production from NK cells during infection with *Cryptococcus neoformans* (Kawakami *et al*, 2000) and was involved in production of IFN-γ from NK cells in response to *B. mallei in vitro*. IL-12 was absolutely required for IFN-γ production *in vitro* although both IL-12 and IL-18 were involved in IFN-γ production from NK cells suggesting a synergistic effect of these 2 cytokines on IFN-γ induction. However, in the absence of IL-12, the IFN-γ response from both NK cells and T cells is eradicated suggesting that IL-12 may act independently as well as in synergy with IL-18 in IFN-γ induction.

IL-27 was implicated as a potential inducer of IFN-γ production during *B. mallei* infection due to their co-expression 24 h p.i.. IL-27 plays a role in IFN-γ production from naïve mouse $CD4^{+}$ T cells and human NK cells *in vitro* (Pflanz *et al*, 2002) and is also important *in vivo* in *L. major* infection as impaired IFN-γ production occurs in WSX-1-/- (IL-27 Receptor) mice (Yoshida *et al*, 2001). Other studies with intracellular pathogens eg. *T. gondii* suggest that IL-27 is not required for the development of IFN-γ mediated immunity (Villarino *et al*, 2003). The discrepancy between the requirement for IL-27 in development of IFN-γ mediated immunity appears to be due to the production of IL-4 during infection. High levels of IL-4 are produced early during *L. major* infection where IL-27 appears to be important in IFN-γ production, however, in infections with low IL-4 involvement (eg. *T. gondii, T. cruz*), IL-27 is not important during the developmental phases of a Th1 response (Artis *et al*, 2004). IL-4 was not detectable during the early stages of *B. mallei* infection suggesting that IL-27 may have an independent role in Th1 mediated immunity during *B. mallei* infection. *In vitro* studies suggested that IL-27 was not involved in IFN-γ production in response to *B. mallei*. However, lower levels of IFN-γ were found in control mice in these studies in comparison with control mice in IL-12KO and IL-18R neutralisation studies. Therefore, it would be useful to repeat these studies to determine whether the similar IFN-γ responses observed between control and IL-27KO mice were a valid finding.

IL-6 plays a role in IFN-γ production during *M. tuberculosis* infection (Saunders *et al*, 2000a) and is also important in inducing IFN-γ responses during *L. monocytogenes* infection, possibly in synergy with IL-12 (Guleria & Pollard, 2001). In other studies IL-6 was found to have immunosuppressive effects by suppressing IFN-γ-mediated responses during *M. tuberculosis* infection (Fortune *et al*, 2004). The co-expression and production of IL-6 and IFN-γ during the early stages of *B. mallei* infection suggested a link between the two cytokines. In the limited studies performed, IL-6 was found not to be responsible

for IFN-γ production in response to heat-killed *B. mallei in vitro* and the role of IL-6 in induction or suppression of IFN-γ-mediated responses during *B. mallei* infection requires further investigation.

Previous studies with heat-killed *B. pseudomallei* demonstrated that IFN-γ is produced by NK cells and $CD8^+$ memory T cells *in vitro* (Lertmenmongkolchai *et al*, 2001). Therefore, the contribution of these cell types to IFN-γ production in response to heat-killed *B. mallei* was investigated. NK cells and $CD3^+$ T cells were found to provide most of the IFN-γ in response to *B. mallei*. NK cells are essential in host defence against a number of intracellular pathogens through their production of IFN-γ e.g. *L. major* (Scharton & Scott, 1993) and *B. pseudomallei* (Haque *et al*, 2005) and, as the main contributors of IFN-γ in response to *B. mallei in vitro,* these cells may be important in the generation of type 1 cell-mediated immune responses to *B. mallei*. T cells also produced IFN-γ in a non-antigen specific manner in response to *B. mallei* although the subset responsible for this production was not identified in these studies. In other studies performed with heat-killed *B. mallei*, $CD8^+$ T cells and γδ T cells were the main T cell contributors to the antigen-independent IFN-γ response, however, $CD4^+$ T cells did not contribute to this response (Lertmemongkolchai, 2005; Personal communication).

$Gr\text{-}1^+$ cells were also involved in the secretion of IFN-γ in response to heat-killed *B. mallei*. $Gr\text{-}1^+$ cells are implicated in IFN-γ production as a direct source of IFN-γ or by producing the cytokines (i.e. IL-12 and IL-18) responsible for induction of IFN-γ. Certain $Gr\text{-}1^+$ cell types are able to produce IFN-γ in response to bacteria. Neutrophils secrete IFN-γ during infection with *S. typhiumurium* (Kirby *et al*, 2002) and in response to cytokines and LPS (Yeaman *et al*, 1998) and $Gr\text{-}1^+$ T cells also secrete IFN-γ (Matsuzaki *et al*, 2003). Neutrophils (Bliss *et al*, 2000) and $Gr\text{-}1^+$ macrophage populations (Mordue & Sibley, 2003) secrete IL-12 during *T. gondii* infection suggesting that they may play an

indirect role in induction of IFN-γ responses. Other cells types may also contribute to the IFN-γ response during *B. mallei* infection. During *B. pseudomallei* and *S. typhimurium* infection, macrophages are important in providing a protective IFN-γ response (Haque *et al*, 2005; Kirby *et al*, 2002). Therefore, the role of other cell populations in IFN-γ production during *B. mallei in vivo* cannot be discounted and should be investigated further.

In summary, IL-12 was found to be essential for IFN-γ production from NK cells and T cells *in vitro* in response to heat-killed *B. mallei*. IL-18, but not IL-27 or IL-6, was also involved in this response. Gr-1$^+$ cells also played a role in IFN-γ production although the mechanism for this induction is unknown.

Chapter 8 General Discussion

8.1 Introduction

Burkholderia mallei is a pathogen which is currently considered to be a bioterrorism threat (Bossi *et al*, 2004) and has historically been used in biological warfare (Christopher *et al*, 1997). No post-exposure therapies or vaccines are currently available for *B. mallei* and, therefore, the development of such treatments is important in protecting against this threat. Previous research on *B. mallei* has been limited to a few studies investigating the pathogenesis of disease in several animal species. Limited examination of immune responses has focused on histopathological investigation of tissue, however, the cellular and cytokine responses involved in immunity to *B. mallei* infection have not previously been investigated. Characterising cellular and cytokine responses allows the protective aspects of the immune response to be identified thus leading to the development of appropriate immunotherapies and vaccines. The work presented in this thesis has characterised the host immune response over the first five weeks of infection in BALB/c mice, providing valuable information into the interactions of *B. mallei* with the host immune system.

8.2 The early, innate response to B. mallei infection: 5-24h p.i.

The spleen was colonised by *B. mallei* 5 h following i.p. infection. This was accompanied by activation and influx of neutrophils and macrophages and increased expression of the pro-inflammatory cytokines IL-1β and IL-18 demonstrating a rapid response to the infection. Potential infection of macrophages by *B. mallei* may cause activation and caspase-1-dependent release of IL-18 (and IL-1β) known to be important in induction of IFN-γ in response to *B. mallei.* Low levels of serum IL-6, IFN-γ and MCP-1 were also present suggesting systemic immune responses were active. Both splenic and serum

cytokine responses peaked 24 h p.i. and were accompanied by increased macrophage activation. These responses were initially able to control, but not eradicate, bacterial colonisation in the spleen and are illustrated in Figure 8.1.

IFN-γ was found to be essential in controlling the innate response to *B. mallei* infection. IFN-γ is essential in immunity to a number of intracellular pathogens including *B. pseudomallei* (Santinarand *et al*, 1999) and *L. monocytogenes* (Portnoy *et al*, 1993) and is key in the development of type 1 immune responses. IL-12 was found to be essential for IFN-γ production (*in vitro*) and IL-18 was involved in the induction of IFN-γ in response to *B. mallei*. IL-12 is essential in guiding the differentiation of Thl subsets (Heinzel & Rerko, 1999) and can work independently or in synergy with IL-18 to stimulate IFN-γ during infection (Garcia *et al*, 1999). The requirement for IFN-γ, IL-12 and IL-18 demonstrates that type 1 immune responses are mounted in response to *B. mallei* infection and are characteristic of those required to fight infection with intracellular pathogens.

IL-27 is a recently discovered cytokine involved in the development of type 1 immunity and its expression *in vivo* has only previously been described during *M. tuberculosis* infection (Pearl *et al*, 2004). High levels of IL-27 expression occurred in response to *B. mallei* infection, although it was not responsible for IFN-γ production *in vitro*. The upregulation of CD54 on T cells 24 h p.i. corresponded with elevated IL-27 expression in the spleen. IL-27 can induce CD54 on naïve T cells which is important in Thl differentiation (Owaki *et al*, 2005) suggesting that IL-27 may have IFN-γ-independent effects in inducing type 1 immune responses to *B. mallei* infection.

TNFα is important in protection against infection with *M. tuberculosis* (Scanga *et al*, 1999) and *B. pseudomallei* (Santanirand *et al*, 1999) and was important in maintaining control of bacterial replication over the first 7 days of *B. mallei* infection. TNFα may mediate its

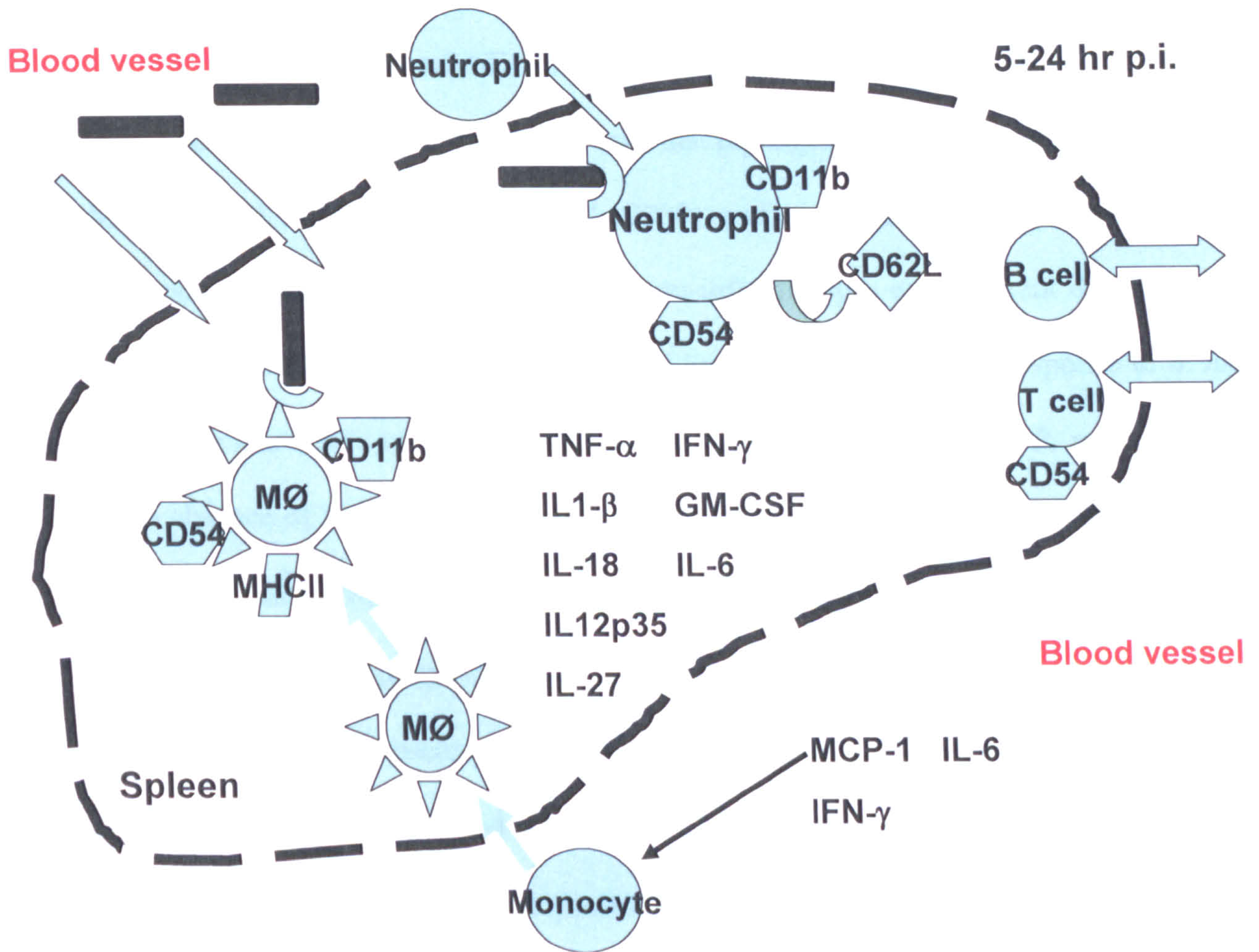


Figure 8.1: Summary of host immune responses occurring in the spleen during the first 24 hours of *B. mallei* infection in BALB/c mice.

protective effects during infection by aiding the development of type 1 responses by stimulating $CD8^+$ T cells and NK cells to produce IFN-γ (Lertmemongkolchai *et al*, 2001). TNFα can also activate neutrophils and macrophages causing upregulation of CD54 (Mandi *et al*, 1997; Tomioka *et al*, 2000) and may be important for establishing macrophage-lymphocyte interactions in a pro-inflammatory environment during infection (Ulett *et al*, 1998). TNFα was also produced by $Gr\text{-}1^+$ neutrophils in response to *B. mallei* suggesting that TNFα may be involved in Gr-1-mediated immunity to *B. mallei* infection. In the absence of TNFα, abscesses developed several days early during *B. mallei* infection suggesting that TNFα played a role in the control of abscess formation. TNFα mRNA expression and macrophage CD54 expression were elevated 7 days p.i., possibly at a time when the process of abscess formation was beginning. CD54 and TNFα expression was also associated with abscessed spleens in the later stages of infection. TNFα and CD54 have been implicated in the development of abscesses in other bacterial infections (Gibson III *et al*, 1998) and maybe involved in the development or propagation of abscesses during *B. mallei* infection.

Macrophage activation is important in immunity to intracellular pathogens. Macrophages are stimulated by type 1 cytokines secreted following the interaction of pathogens with NK cells and T cells. Macrophage activation during early *B. mallei* infection suggested that IFN-γ may mediate its protective effects in part by activating macrophage responses. IFN-γ is a potent macrophage activator that can specifically initiate gene expression of MHCII by signalling via the IFN-γ receptor on the macrophage surface (Schroder *et al*, 2004). High levels of MHCII expression on macrophages observed 24h p.i. corresponded with a peak in splenic and serum IFN-γ production. This suggests that IFN-γ can induce macrophages to initiate bacterial killing mechanisms for intracellular bacteria including the induction of IL-12 (Yoshida *et al*, 1994) and nitric oxide production (Dalton *et al*, 1993). The high levels of MHCII expression also suggests that macrophages are presenting antigen to T cells and

are thus involved in the development of adaptive immune responses. In addition to IFN-γ, increases in GM-CSF expression also coincided with increased macrophage activation 24h p.i.. GM-CSF is an effective macrophage activator and controls infection with *T. cruzi* (Reed *et al*, 1987) and *P. Carinii* (Paine III *et al*, 2000) suggesting it may have a similar role in macrophage activation during *B. mallei* infection. Macrophages also migrated to the spleen within the first 24h p.i. coinciding with elevated serum levels of the macrophage chemoattractant, MCP-1 (Lu *et al*, 1998), suggesting that this chemokine is involved in macrophage migration during *B. mallei* infection. Macrophages, therefore, are implicated in both pathogen killing and development of antigen-specific responses during *B. mallei* infection. The development of antigen-specific responses in a milieu of type 1 cytokines (IFN-γ, IL-27, IL-12 and IL-18) suggests that T cell mediated immunity is likely to be driven by Th1 and Tc1 subsets.

NK cells are important in the development of type 1 immune responses by secreting IFN-γ in the innate response to infection. This allows activation of macrophages and the development of type 1 T cell subsets. The main cellular sources of IFN-γ in response to *B. mallei in vitro* were NK cells. Although it was not possible to study the role of NK cells *in vivo* during *B. mallei* infection, they are likely to be important in the development of cell-mediated immune responses to *B. mallei*. Differentiated T cell subsets are also able to secrete IFN-γ during innate responses and contributed to the IFN-γ response *in vitro* in a non-specific fashion. However, removal of T cells during innate responses to *B. mallei* infection did not affect survival. This suggests that $CD4^+$ and $CD8^+$ T cell subsets were not required for the essential, innate IFN-γ response observed *in vivo* during *B. mallei* infection. A similar scenario has been found to exist for *B. pseudomallei* infection where *in vitro* data suggested a requirement for T cells (and NK cells) in innate IFN-γ production. However, *in vivo* studies suggested that this requirement was dispensable and may be due to compensatory IFN-γ production by other cell types including macrophages (Haque *et al*,

2005). The contribution of $CD4^-CD8^-$ T cell subsets, remaining following depletion by CD4 and CD8 antibodies, requires further investigation. These cells potentially include γδT cells which have been found *in vitro* to produce IFN-γ in response to *B. mallei* and may be important in innate immune responses to infection.

Neutrophils are important in innate immunity to pathogens including *B. pseudomallei* (Easton & Bancroft, 2005). Neutrophils became activated early in *B. mallei* infection, migrated into the spleen and were the major $Gr\text{-}1^+$ population depleted following anti-Gr-1 treatment. Therefore, it is likely that they played a major role in protection in early immunity to *B. mallei* infection. It is possible that neutrophils were involved in protection as sources of IFN-γ, the IFN-γ inducing cytokines IL-12 and IL-18 or TNFα and, therefore, were important in contributing to the development of type 1 immunity during *B. mallei* infection. The effect of neutrophils in Gr-1 mediated immunity may have been complimented by the production of IL-12 by $Gr\text{-}1^+$ macrophages and pDCs. However, $Gr\text{-}1^+$ T cells and NK cells did not contribute to Gr-1-mediated immunity as T cells were not responsible for early protection against *B. mallei* infection and NK cells did not express Gr-1.

8.3 Downregulation of immune responses: Day 3 p.i.

The strong immune responses elicited by the host 24 h p.i. represented a peak in cytokine responses and the end of an initial peak in neutrophil and macrophage activation. A notable decrease in the immune response occurred 3 days p.i. which did not result from decreased bacterial numbers reducing stimulation of immune responses. A reduction in IFN-γ (and other type 1 cytokines), essential for controlling early *B. mallei* infection, was observed.

Macrophage and neutrophil activation was also diminished and was associated with reduced expression of cytokines involved in their activation (IL-1β and GM-CSF).

Phagocytic cells such as macrophages and neutrophils are vital in protection against intracellular infections, however, some bacteria including *B. pseudomallei* have evolved mechanisms to modulate host immune responses to allow them to survive and multiply within these cells (Pruksachartvuthi *et al*, 1990). CD11b upregulated on neutrophils and macrophages during the first 24h p.i. may provide an entry route for complement-fixed *B. mallei* into host cells to allow it to survive and replicate intracellularly (Egan & Gordon, 1996; Clemens *et al*, 2005). The capsule of *B. mallei* is a known virulence factor (DeShazer *et al*, 2001) and although its mechanism of interference with host immune responses is unknown, the capsular protein from another pathogen, *B. fragilis,* impairs phagocytic killing and bacterial clearance during infection (Onderdonk *et al*, 1990). *B. mallei* also expresses a type III secretion system which may contribute to its pathogenicity (Ulrich & DeShazer, 2004). This type III secretion system is also a virulence determinant of *B. pseudomallei* and acts *in vitro* by allowing replication of the bacterium within macrophages (Stevens *et al*, 2002). *B. pseudomallei* is also able to multiply within resting or unactivated macrophages, in the absence of IFN-γ (Utaisincharoen *et al*, 2001). The increased presence of unactivated macrophages and the lack of macrophage activating cytokines (IFN-γ and GM-CSF) 3 days p.i., may allow intracellular *B. mallei* to replicate thus shielding the infection from host immune responses.

Bacteria can also affect host immunity by interfering with cytokine responses. During *M. tuberculosis* infection, IL-6 is able to downregulate protective immune responses to allow pathogen survival. IL-6 acts on macrophages to reduce their responsiveness to IFN-γ, causing down regulation of MHCII expression on macrophages (Nagabhushanam *et al*, 2003). The high levels of IL-6 observed 24 h p.i. and the decrease in MHCII expression

observed 72 h p.i. implicate this cytokine in the downregulation of *B. mallei*-induced immune responses. IL-10 is an anti-inflammatory cytokine that acts as a negative regulator of the inflammatory response by instigating processes required for down-regulating proinflammatory responses (Weissman, 2001). IL-10 is able to down-regulate the expression of GM-CSF (Lenhoff *et al*, 1998). Correlation of its expression with bacterial colonisation of the spleen and upregulated expression during Immune Analysis 1, implicate IL-10 in suppressing GM-CSF and IFN-γ expression during *B. mallei* infection.

8.4 The intermediate phase of infection: Days 5-14 p.i.

B. mallei infection was controlled by the immune response over the first 7 days of infection, however, by day 14 p.i. high bacterial loads were observed in the spleens of infected mice suggesting that host immunity was compromised. Host immune responses appeared to enter a new phase 5-7 days p.i. with increased macrophage and neutrophil numbers accompanied by renewed macrophage and neutrophil activation, in contrast to the downregulation observed 3 days p.i.. This suggests that the interaction between the host and the pathogen changed during this period. *B. mallei* was potentially taken up into, and replicated in, host cells over the first 5 days p.i., as occurs in *B. pseudomallei* infection. The potential release of intracellular *B. mallei* from host cells may cause the increase in macrophage and neutrophil activation observed 5-7 days p.i.. The absence of serum MCP-1 also suggests that increasing splenic macrophage numbers may be a result of local proliferation within the spleen rather than continuing migration. Other systemic cytokine responses from day 3 p.i. were also absent suggesting that the infection is contained, and that cytokine responses are compartmentalised, within the tissues.

It is likely that antigen-specific T cell responses are also developing at this stage of infection. Increased expression of macrophage CD54 and MHCII, both involved in interactions with effector or naïve T cells during antigen presentation, suggests that

antigen-specific immune responses are being propagated. However, the potential destruction of the white pulp in the spleen observed during previous analyses of intraperitoneal infection in BALB/c mice (Fritz *et al*, 1999) may be responsible for the decrease in T cell and B cell numbers observed 5-7 days p.i.. This has obvious implications for the development of protective adaptive immune responses and may be crucial in the breakdown of immunity to *B. mallei* infection within the spleen.

The pathogenesis of disease between 7 and 14 days is unknown; however, interactions between the immune response and bacteria during this time period appear to be essential for determining the course of infection. During this period, bacterial numbers grow uncontrollably within the spleens of infected mice, abscesses form and death ensues or mice are able to apparently able to clear the infection. Although infection could not be detected in these spleens, it is possible that latent infection exists with very low numbers of bacteria surviving. However, the immune mechanisms for this divergence in control of infection are as yet unknown.

8.5 The chronic phase of infection: Days 14-36

In diseases with certain intracellular pathogens, including *B. pseudomallei* and *M. tuberculosis*, the early immune response may not be adequate to clear the initial infection. The disease progresses into a chronic phase, where the infection is contained within the tissues and may eventually overcome the host or remain latent for many years prior to reactivation. During *M. tuberculosis* infection, granulomas form in the tissues which contain the bacteria, thus preventing the spread of infection systemically (Saunders & Cooper, 2000b). In other infections with pathogens including *B. pseudomallei* (Leakey *et al*, 1998) and anaerobic bacteria (Finlay-Jones *et al*, 1999), abscesses form which contain

bacteria in localised areas during infection. During *B. mallei* infection, abscesses formed in the tissues. In addition to confining infection, abscesses appeared to provide a microenvironment for bacterial growth, as bacterial numbers were very high in abscessed spleens. The persistence of high bacterial loads within the spleen (and probably other organs) means that the host could become overwhelmed with the infection leading to bacteria 'leaking' from abscesses to cause a systemic and ultimately fatal infection. This may explain why mice survive for several weeks following the development of large abscesses before developing a fatal systemic illness.

Active inflammatory responses occurred both in the spleen and systemically during this phase of infection and were related to bacterial colonisation of the spleen. Splenic expression of proinflammatory cytokines, including TNFα and IL-6, and neutrophil and T cell activation were related to abscess formation from days 14 – 28. TNFα and T cells were important in controlling *B. mallei* infection following the formation of abscesses although this was not effective in preventing high bacterial loads or eventual lethality of infection. The importance of T cells in adaptive immune mechanisms during this phase of infection suggest they may be involved in killing infected host cells or producing IFN-γ to try and maintain control of the infection. This demonstrates that although *B. mallei* is able to override host immune responses, some immune mechanisms still exist which are able to delay lethality for several weeks.

Systemic production of IL-6 and MCP-1, only in abscessed animals, suggested the release of mediators from infected tissues. IL-6 is one of the main activators of acute phase responses in the liver causing release of acute phase proteins (Kopf *et al*, 1994) which are involved in fever and sickness behaviour (van der Poll & van Deventer, 1999). This corresponds with the ruffling that was observed in some animals at day 14 and all animals from day 21 onwards suggesting that IL-6 may be responsible for overt symptoms of

sickness observed in these animals. Serum levels of IL-6 have also been shown to be a good predictor of severe bacterial infection in human patients (Groenveld *et al*, 2001) suggesting that IL-6 serum levels may also be related to the severity of *B. mallei* infection. IL-6 may also be involved in the induction of MCP-1 observed (Bohn *et al*, 1998). The presence of MCP-1 in the blood suggests that monocytes are still being recruited into sites of infection possibly in an attempt to control bacterial replication in abscessed spleens. The systemic release of these mediators appears to be implicated in instigating development of systemic inflammatory responses which may eventually lead to uncontrolled inflammation or sepsis causing death of the host.

8.6 Conclusions

The protective immune responses identified during early *B. mallei* infection suggest that type 1 immunity is essential for protection against infection as summarised in Figure 8.2. Following intraperitoneal infection, *B. mallei* enters the spleen and interacts with neutrophils and macrophages leading to their activation. Induction of cytokine expression, principally IFN-γ, IL-6 and IL-27, occurs and MCP-1 is released to further propagate macrophage responses. IFN-γ is fundamental for survival against *B. mallei* infection, possibly by activating macrophages to kill intracellular *B. mallei* bacilli. Gr-1$^+$ cells, predominantly neutrophils, are also involved in protective *B. mallei* immunity and the induction of IFN-γ and TNFα responses. These cells potentially mediate their protective effects by producing the type 1 cytokine IL-12 essential for IFN-γ production by T and NK cells, in conjunction with IL-18. TNFα, is involved in controlling bacterial replication, possibly by activating neutrophil and macrophage responses, and containing the infection within abscesses. IL-27 is also implicated in the development of type 1 immunity by potentially upregulating CD54 expression on T cells. T cells are important in adaptive immune responses and may exert their effects by killing infected host cells or maintaining type 1 immune responses by producing IFN-γ.

The requirement of IFN-γ for innate survival of *B. mallei* infection suggests that any potential vaccine or immunomodulatory therapy should preferentially stimulate type 1 cell-mediated immune responses. By maintaining the type 1 cell-mediated immune responses initially instigated, and then downregulated during natural infection, the immune system may be modulated to overcome *B. mallei* infection. Immunomodulation of innate responses may be mediated by bacterial components or cytokines including synthetic bacterial DNA sequences (CpGs) or treatment with exogenous IFN-γ. CpGs have been used effectively during *B. pseudomallei* infection *in vivo* (Wongratanacheewin *et al*, 2004).

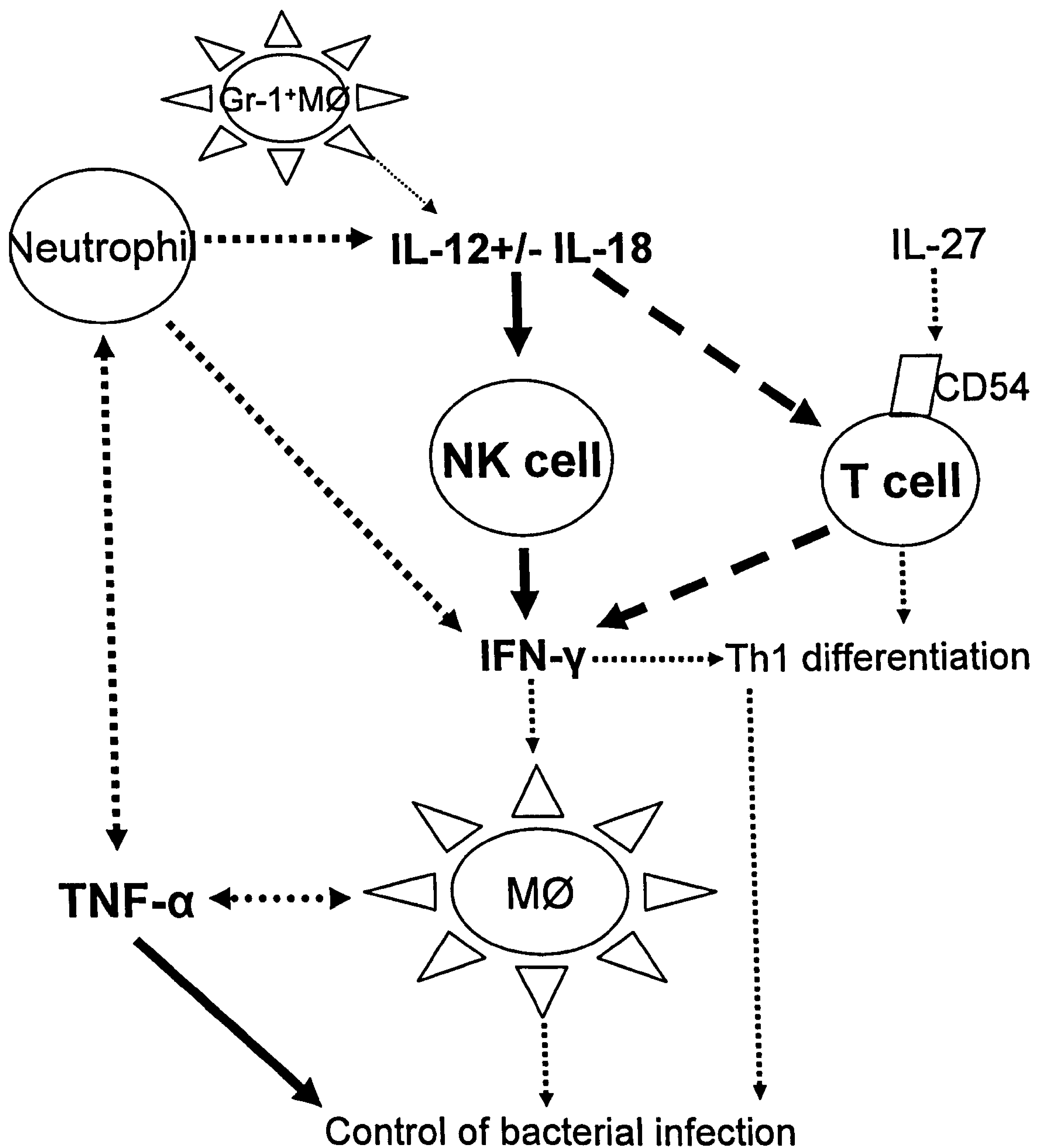


Figure 8.2: Proposed mechanism of protective immunity to *B. mallei* infection. IL-12 and/or IL-18 produced by Gr-1$^+$ populations (including neutrophils) acts on NK cells and T cells to induce IFN-γ production. IFN-γ then acts on macrophages and influences development of type 1 T cell responses with the aid of IL-27. Neutrophil and macrophages releasing and being activated by TNFα allows control of bacterial infection in addition to other macrophage killing mechanisms. MØ: macrophage.

Preliminary studies with *B. mallei* have also shown that some CpGs are able to provide protection against *B. mallei* infection (Lukaszewski, 2004) by upregulating macrophage and neutrophil responses (data not shown).

In conclusion, the work presented in this thesis provides valuable information on host immune responses to *B. mallei* infection that contributes to the field of immunity to intracellular pathogens. The immune response to *B. mallei* shows many similarities with that of other intracellular pathogens including its close relative *B. pseudomallei.* The similarities in immunity to infection with these two pathogens may lead to the development of a vaccine and/or an immunomodulatory therapy that targets type 1 responses and is able to treat both *B. mallei* and *B. pseudomallei* infection.

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