

Interactions of Natural Killer Cells, Dendritic Cells and Chemokines in *Mycobacterium bovis* Infection

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Declaration

I, Nazneen Siddiqui hereby declare that this thesis entitled 'Interactions of Natural Killer Cells, Dendritic Cells and Chemokines in *Mycobacterium bovis* Infection' is my own work and has been generated solely by myself as the result of my own original research. All other sources of information have been appropriately referenced.

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Bovine Tuberculosis (TB), caused by *Mycobacterium bovis* (*M.bovis*), has seen a significant rise in incidence in recent years, posing a considerable burden to the UK economy. Protective immunity is associated in part, with rapid IFN γ production and early Th1 polarisation. Natural Killer (NK) cells are known to be a significant source of IFN γ and may therefore play a pivotal role in the innate immune response to mycobacterial infection. This study hypothesised that bovine NK cells participate in the immune response to bovine TB by migrating towards and reciprocally interacting with *M.bovis* infected dendritic cells (DCs).

Bovine NK cells comprise a heterogeneous population characterised by the expression of NKp46 and CD2. Quantitative PCR analyses revealed that blood derived NKp46+ CD2- NK cells transcribed higher levels of important inflammatory and lymphoid homing chemokine receptors and preferentially migrated towards *M.bovis* infected DCs within *in vitro* chemotaxis assays when compared with NKp46+ CD2+ NK cells. Furthermore, within co-culture assays, *M.bovis* infected DCs selectively induced the release of IFN γ from NKp46+ CD2- NK cells with no detectable levels of IFN γ produced by NKp46+ CD2+ cells. This was partially reliant upon IL-12 secreted by *M.bovis* infected DCs as well as direct cell-cell contact. In addition, NKp46+ CD2- NK cells were able to reciprocally activate infected DCs resulting in up-regulation of cell surface MHC class II. Furthermore, IFN γ expressing NKp46+ CD2- NK cells were detected in the peripheral circulation of cattle as early as two days following experimental infection with *M.bovis*.

This study has provided evidence of reciprocal interactions between CD2- NK cells and mycobacterially infected DCs that could augment both antigen presentation and Th1 biased immune responses. Therefore, this minor blood derived CD2- NK cell subset may be selectively responsible for promoting a protective immune response during the early phases of *M.bovis* infection and represents a potential target for future vaccination strategies.

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Abbreviations

ACDP	Advisory committee for the control of dangerous pathogens
APC	Antigen-presenting cell
Az	Sodium azide
BCG	Bacille Calmette Guérin
BFA	Brefeldin A
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
bTB	bovine tuberculosis
BU	Biological unit
CFDA-SE	Carboxy-fluorescein diacetate, succinimidyl ester
CFU	Colony forming unit
CR	Complement receptor
ConA	Concanavalin A
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific ICAM-3 grabbing non-integrin
ESAT-6	Early secreted antigenic target 6
ELISA	Enzyme-linked immunosorbent assay
FCS	Foetal calf serum
FSC	Forward Scatter
gfp	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony stimulating factor
HEV	High endothelial venules
IC	Isotype control
iDC	Immature dendritic cell
Ig	Immunoglobulin
IFN	Interferon
IL	Interleukin
LN	Lymph node
mAb	Monoclonal antibody
<i>M.bovis</i>	<i>Mycobacterium bovis</i>
MCP-1	Monocyte chemotactic protein 1
MDR	Multi-drug resistant
MFI	Mean fluorescent intensity
MHC	Major histocompatibility complex
MIIC	MHC-II compartment
MNC	Mononuclear cells
MOI	Multiplicity of infection
MR	Mannose receptor

<i>M.tb</i>	<i>Mycobacterium tuberculosis</i>
MVA	Modified vaccinia virus ankara
NK	Natural killer cell
NMS	Normal mouse serum
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PBL	Peripheral blood leukocytes
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PMA	Phorbol-myristate-acetate
PPD	Purified Protein derivative
PRR	Pattern recognition receptor
PSLN	Pre-scapular lymph node
RANTES	Regulated on activation normal T cell expressed and secreted
Rbo	Recombinant bovine
RD1	Region of difference 1
Rhu	Recombinant human
Rmu	Recombinant murine
ROI	Reactive oxygen intermediates
RT	Room temperature
SSC	Side Scatter
TB	Tuberculosis
TCM	Tissue culture medium
TCR	T cell receptor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
WC	Workshop cluster
XDR	Extensively drug resistant

1.1 Tuberculosis

Tuberculosis (TB), a chronic respiratory illness caused by mycobacterial pathogens afflicts both humans and animals and may be fatal if left untreated. Despite being the focus of substantial global investment and scientific research for a number of years, TB is still the leading cause of mortality from a bacterial infection (WHO, 2010). Furthermore, infection of wildlife and domesticated animals inflicts significant economic damage, particularly apparent within the UK and may still pose a public health risk if appropriate preventative strategies are not employed (Cosivi et al., 1998, Thoen et al., 2006). In light of the global burden inflicted by TB, there is a fundamental need for research to elucidate immunological responses to mycobacterial infection which may underpin the future development of prophylactic, diagnostic and treatment strategies.

1.1.1 The *Mycobacterium tuberculosis* (*M.tb*) complex

In 1882 following experimental examination of tuberculous lesions from a range of hosts, Robert Koch first identified a slow replicating, acid fast bacillus as the causative agent of TB. At first believed to be only one species, later comparisons of isolates from humans and cattle suggested minor phenotypic differences may exist between the bacilli (de la Rua-Domenech, 2006). It was not until the advent of genomic sequencing that the multitudes of species were fully defined differing only in important deletions of genes found within the region of difference 1 (RD1) (Mostowy et al., 2005, Brosch et al., 2002). The *M.tb* complex therefore, identifies a group of mycobacterial species and sub-species which possess the capacity to induce TB. These include, *M.tuberculosis*, *M.bovis*, *M.bovis BCG*, *M.africanum*, *M.microti*, *M. canettii*, *M. caprae*, *M. pinnipedii* (Wirth et al., 2008, Mostowy et al., 2005). TB within humans is most commonly as a result of infection with *M.tuberculosis*, whilst comparatively, *M.bovis* a zoonotic pathogen, is able to infect a large range of mammalian hosts (Thoen et al., 2006). For this reason it was historically believed that *M.bovis* was a predecessor of *M.tuberculosis* with infected animals transmitting disease to humans (Smith et al.,

2006). However, evolutionary sequence analysis elucidated the existence of two independent lineages originating over 40,000 years ago (Wirth et al., 2008). Both strains were believed to have infected humans but one of which may have been transmitted from humans to their livestock to give rise to the *M.bovis* strain which is rife within the wildlife and domesticated animal populations today (Mostowy et al., 2005).

1.1.2 Human tuberculosis infection

The World Health Organisation (WHO) defined TB as global pandemic within the human population and in 2009 alone reported over 9 million cases of active infection with 1.7 million lives lost as a consequence (WHO, 2010). Strategies such as the DOT initiative (Directly Observed Therapy) introduced in the mid 1990's and the subsequent Stop TB Partnership have contributed to the effective control of TB in the developed world. The majority of disease burden that is still seen today is found in regions of Africa and South East Asia, particularly prevalent as a concurrent infection within immune suppressed individuals (WHO, 2010).

In most cases, TB infection is caused by the virulent strain *M.tuberculosis*, transmitted through inhalation of aerosolised bacilli released from infected individuals. During primary infection, mycobacterial pathogens manifest within the respiratory tract and apical lobes of the lungs and as a rare occurrence may result in active TB infection (Flynn and Chan, 2001). However in most individuals, the immune system possesses the capacity to control bacterial replication and contain the infection within a granulomatous Gohn Focus resulting in a latent infection without clinical symptoms.

It is predicted that one third of the world's population may be latently infected with TB and in the majority, mycobacteria remain dormant for the duration of their lifetime. However, in certain instances where the immune response appears unable to control bacterial replication, re-activation may occur resulting in active TB infection. Despite the focus of substantial research, the trigger which enables re-activation is not known but susceptibility in this manner is strongly associated with periods of immune suppression. This is particularly apparent in the developing world where concomitant

infections of TB commonly afflict HIV sufferers and account for almost 25% of the total TB related deaths (WHO, 2010).

Upon active infection, the manifestation and severity of TB varies substantially between individuals and is influenced by the predisposing disease status as well as the length of time left untreated. If pathogenic bacilli are restricted within the respiratory tract and lungs, infection is defined as pulmonary TB with affected individuals commonly presenting with a persistent cough, haemoptysis, flu-like symptoms, malaise, fever and weight loss. In more severe cases extra-pulmonary infection is defined by local spread of mycobacteria resulting in pleural effusions or spread to local lymph nodes. In rare cases, commonly following re-activation and a long period of undiagnosed infection, patients suffer with severe wide-spread dissemination of mycobacteria defined as miliary TB infection.

Whilst the majority of the cases of TB within humans are considered to be as a result of *M.tuberculosis*, the clinical presentation of zoonotic infection from *M.bovis* is indistinguishable from that of *M.tuberculosis*. Within the UK, *M.bovis* was historically believed to be the major source of extra-pulmonary TB infection of infants due to consumption of unsterilised cow's milk (Thoen et al., 2006, de la Rua-Domenech, 2006). The routine introduction of pasteurisation and strategies to reduce exposure from handling of infected animals reduced the incidence rates substantially and as a result is rare in today's society, accounting for only 1% of cases (de la Rua-Domenech, 2006). However, identification of the TB causing mycobacterial species requires specialised diagnostic facilities often not employed particularly in developing regions, therefore whilst considered a rare occurrence, the true prevalence of TB due to *M.bovis* within these regions is not known but may be as many as 10% of all cases (Cosivi et al., 1998).

1.1.2.1 Diagnostic and treatment strategies

Diagnostic measures to detect active TB infection vary substantially and whilst the protocols employed should depend on individual requirements, often in developing countries financial constraints restrict the use of specialised diagnostic protocols. Upon presentation of symptoms associated with TB, the presence of mycobacteria is

detected by acid fast (Zeihl-Neelson (ZN)) staining of sputum samples followed by chest radiography (Parsons et al., 2011). Detection of mycobacteria by ZN staining is the current 'gold standard' test used world-wide yet there are numerous constraints to this approach if secondary diagnostics are not employed. In particular, accurate diagnosis is heavily reliant on the ability to obtain an adequate sputum sample, may not be sensitive in HIV infected individuals and cannot differentiate between mycobacterial species or establish susceptibility to antimicrobial treatments (Parsons et al., 2011). Second line diagnostics are available within specialised laboratories and include determination of drug susceptibility of isolates by culture on solid or liquid media. Whilst, a long process due to the slow replication rate of mycobacteria, identification of drugs able to restrict mycobacterial growth can enable drug treatment strategies to be tailored to an individual's requirements. More recently, highly specific DNA sequence based techniques have become available and are able to differentiate between strains of mycobacteria by PCR amplification (Reddington et al., 2011).

Tuberculin skin testing regimes such as the Heaf or Mantoux test administer mycobacterial antigens to the skin with the subsequent development of delayed type hypersensitivity (DTH) responses reliant on the presence of antigen specific T cells. It is however important to consider that DTH responses only confirm the presence of mycobacteria specific adaptive immune responses which may be present within vaccinated, infected, treated or latently infected individuals. Furthermore, false negative diagnoses may be made within actively infected individuals that are immune compromised (Michel et al., 2010). More selective diagnostic regimes such as IFN γ release assays (IGRA), currently licensed as QuantiFERON-TB Gold (Cellestis) and T-SPOT[®].TB (Oxford Immunotec) are able to detect *M.tuberculosis* specific release of IFN γ upon re-stimulation with mycobacterial antigens. As such the ELISPOT based assay to diagnose active TB infection improves the specificity and sensitivity (83-97%) over use of the tuberculin skin test. However, no test is able to distinguish between active and latent TB infection (Lalvani, 2007, Menzies et al., 2007).

TB is a treatable infection and the course of treatment is largely determined by the severity of infection. As a standard regime sufferers of pulmonary TB will receive combinations of antibiotic therapy including initially isoniazid, rifampicin, pyrazinamide

and ethambutol for a period of up to six months (NHS, 2011). However, poor patient compliance, amongst other factors, has led to the emergence of multi-drug resistant (MDR) strains of TB which are resistant to the first line antibiotics isoniazid and rifampicin. Yet more serious is the emergence of extremely drug-resistant (XDR) strains of TB which are able to replicate despite treatment with second line antibiotics (Gandhi et al., 2010). For this reason the current treatment regimes are considered antiquated and substantial scientific research is being conducted to aid drug discovery.

Whilst diagnosis of TB by the protocols described identifies infected individuals, it does not circumvent the spread of infection that may have already occurred prior to detection and subsequent treatment. Diagnostic strategies for the future aim to differentiate between the heterogeneous phenotypes resulting following mycobacterial exposure. One of the most significant modern day challenges to the future of TB diagnoses lies in the identification of latently infected individuals susceptible to re-activation. Current strategies to address this are employing cytokine profiling (Sester et al., 2011) or whole genome approaches, seeking to identify signatures evident within the blood which may correlate with latency, vaccination or infection (Parida and Kaufmann, 2010).

1.1.3 Bovine tuberculosis

Several species of mycobacteria within the *M.tb* complex are able to infect wildlife and domesticated animal populations however; *M.bovis* afflicts the widest range of hosts and is most commonly the causative agent for bovine tuberculosis (bTB). Whilst cattle are the primary hosts for infection, *M.bovis* has also been found to be endemic within the wild badger population in the UK (Krebs et al., 1998). Furthermore, bovine TB is an issue of global significance and is rife within many wildlife populations including Brush-Tailed Possums in New Zealand, White-Tail Deer in Michigan and Wild Boar in Spain (Buddle et al., 2011).

1.1.3.1 Pathogenesis of bovine TB

M.bovis is highly virulent in cattle with as little as 1 CFU administered intra-tracheally able to induce a pathological response (Dean et al., 2005). Infection predominantly

occurs by inhalation of bacilli carried within aerosols to the respiratory tract. This is evidenced by the post-mortem examination of naturally infected cattle with caseous and necrotic granulomas in the lymph nodes and tissues within the head, respiratory tract and thorax. Lesions are most commonly found within the respiratory tract alone (approximately 60% of cattle) whereas the remainder may present with additional lesions within tissues of the head (Corner, 1994, Neill et al., 2001, Neill et al., 1994, Whipple et al., 1996). In particular the retropharyngeal, bronchial and mediastinal lymph nodes as well as the lungs are primarily afflicted by infection (Liebana et al., 2008, Neill et al., 2001). Disseminated TB is an extremely rare occurrence largely as a result of active control programs able to identify infected cattle prior to substantial spread of infection.

1.1.3.2 Incidence and control strategies in the UK

In 1934 within the UK, over 40% of dairy cattle were believed to be carriers of bovine TB infection and due to the consumption of contaminated milk, were a significant source of zoonotic TB amongst infants (de la Rua-Domenech, 2006). Subsequent steps were taken by the British government in attempts to eradicate TB within cattle with compulsory testing of herds introduced in the 1950's and still conducted today. Regions with high incidence of bovine TB are tested annually with lower incremental testing in regions with lower incidence. In this manner, up until the 1970's these 'test and slaughter' control strategies contributed to a significant decline with the lowest ever recorded incidences of bovine TB. However, over the past 40 years there has been a substantial increase in the number of herd breakdowns particularly evident in the South West regions of the UK (de la Rua-Domenech, 2006), with spread of infection into areas previously free of bovine TB such as Wales and West Midland regions. In 2009 alone DEFRA (Department for Environment, Food and Rural Affairs) reported over 30,000 cattle reactors with a cost in excess of £100 million to the UK economy (DEFRA, 2009).

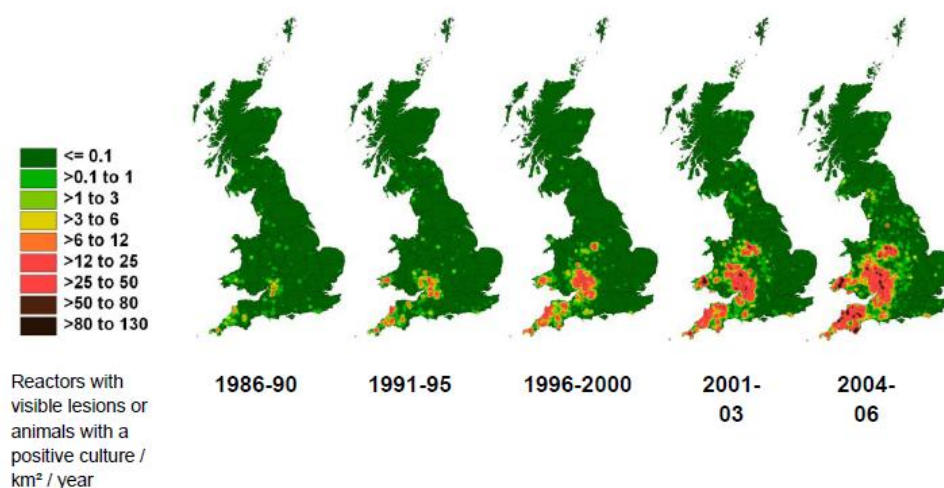


*Illustration of the spread of bovine TB into new areas of the UK from 1985-2006,
Source VLA; January 2009*

The reasons for the dramatic rise in the prevalence of bovine TB are not known however, as an infectious pathogen both cattle-cattle and wildlife-cattle transmission may account for the persistence of infection and the subsequent repeated herd breakdowns that are observed. In particular, within the UK an environmental reservoir of infection exists within the wild badger population and has been implicated as a significant source of transmission to cattle (Krebs et al., 1998, Smith et al., 2006). To determine whether the culling of badgers would benefit the incidence rates of bovine TB, a randomised badger culling trial (RBCT) was implemented in 1998 within the most severely afflicted regions of the UK. Initial analysis suggested that the removal of reservoirs of infection may be beneficial with a reduction in the incidence rates within the areas where active culling was conducted. However, in adjacent regions there was an observed increase in the number of herd breakdowns potentially due to the dissemination of infected badgers (Donnelly et al., 2003). Furthermore, the initial reduction in incidence was not sustained following cessation of culling and would require a repeated long-term initiative to remove infected badgers, not considered as financially viable (Jenkins et al., 2010). In addition, culling strategies employed in Ireland revealed that the removal of infected badgers did not substantially influence disease prevalence within the remaining badger populations (Corner et al., 2008). Despite a large body of evidence suggesting badger culling may be ineffective, the UK

government is likely to approve further widespread trials within England. However, there is also a need for more effective and economical strategies to be employed in the control of bovine TB (1.1.3.4).

1.1.3.3 Diagnostics

In the UK, diagnosis is performed by the single intra-dermal comparative cervical skin test (SICCT) via administration of tuberculin derived from *Mycobacterium avium subspecies avium* (PPDa) and *M.bovis* (PPDb) (Morrison et al., 2000). Measurement of a delayed type hypersensitivity response (DTH) in this manner indicates the presence of antigen specific T cell activity, however sensitivity is often reported as being relatively poor ranging from 68-95% (Monaghan et al., 1994). Further testing strategies include the whole blood assay, BOVIGAM® measuring antigen (PPDb) specific IFN γ production which improves the sensitivity and specificity (96-98%) over use of the tuberculin skin test alone (Coad et al., 2008). Despite this, IFN γ assays are only used in certain circumstances as an ancillary to skin testing in cases where the results may be inconclusive.

The historical incidence rates of bovine TB within different regions of the UK are used as a basis to determine the frequency of testing intervals. Cattle within high incidence areas as is the case within the south west are currently skin tested every 12 months. If the thickening of skin in response to PPDb is greater than 4mm over the response to PPDa, cattle are classified as reactors. A smaller (1-4mm) response results in an inconclusive status with cattle re-tested after a 60 day interval. Reactor cattle will subsequently be slaughtered with restrictions on herd movements placed on the farm in attempts to control the spread of infection. Upon slaughter, all animals undergo post-mortem examinations conducted at the Veterinary Laboratories Agency involving histological examinations of tissues for the presence of lesions, bacteriological culture and molecular typing of isolates to define strain identities.

As is the case with human TB, there is substantial heterogeneity in the phenotypes that result following exposure to mycobacterial pathogens in cattle. For this reason, current testing strategies identifying reactivity to mycobacterial antigens may result in both false positive and false negative diagnoses. In particular, skin testing relies on the

development of an adequate adaptive immune response and therefore, may not be able to detect animals during the early stages of infection, during later stages when the adaptive immune response may wane (de la Rua-Domenech, 2006) or when immune-compromised as may be the case with concurrent infections of bovine viral diarrhoea virus (BVDV) (Charleston et al., 2001). Furthermore, in many instances cattle may respond positively in the tuberculin skin test despite no pathological evidence of infection. For example, environmental exposure to pathogens such as *M. avium* subspecies *Paratuberculosis* may result in non specific reactivity (Olsen and Storset, 2001). A comparative skin test is therefore employed and the relative DTH response to PPDa and PPDb is measured with a stronger response to PPDb considered to be infected with *M.bovis* rather than other mycobacterial species. However, this does not entirely resolve the issue of sensitisation, with elevated responses in very young cattle potentially due to large numbers of NK cells (Olsen et al., 2005) or BCG vaccinated animals which are also able to respond non-specifically. This may also be the case during latent infection, the existence of which however, has not been definitively proven in cattle (Pollock and Neill, 2002, Van Rhijn et al., 2008).

To a certain extent, whole blood IFN γ testing is more sensitive and can circumvent these issues. In particular, use of *M.bovis* specific antigens belonging to the ESAT-6 (early secreted antigen 6KDa) family (Jones et al., 2010a) can differentiate between BCG vaccinated and *M.bovis* infected cattle (Vordermeier et al., 2002) and sensitivity of the assay can be improved by simultaneous testing of IL1 β alongside IFN γ (Jones et al., 2010b). Yet, testing in this manner is not routinely employed due to economic constraints and difficulties with use in the field.

1.1.3.4 Vaccination

As a long-term solution, it is believed that development of a vaccine for cattle will be the most effective strategy in the control of bovine TB infection (Krebs et al., 1998). Ideally, such a prophylactic strategy would prevent the onset of infection and subsequent transmission yet, exposure to mycobacteria results in heterogeneous phenotypes and as such, complete sterilisation is an unlikely occurrence. Within cattle, a protective vaccine is therefore defined by its capacity to significantly reduce or

completely inhibit the pathological signs of TB infection (Hope and Villarreal-Ramos, 2008, Vordermeier et al., 2002). Due to the costly nature of *M.bovis* experimental challenge studies, the identification of correlates of protection to aid selection of vaccine candidates has been a significant research priority.

A Th1 immune response is believed to be imperative for protection against mycobacterial infection (Welsh et al., 2005, Orme, 2004). Therefore, vaccine efficacy has generally been determined by antigen specific IFN γ release following re-stimulation of whole blood, yet post-vaccination levels of IFN γ do not necessarily correlate with protection (Mittrucker et al., 2007, Vordermeier et al., 2002, Waters et al., 2010, Buddle et al., 1995) and post-infection, elevated levels of PPD_b or ESAT6 specific IFN γ are indicative of increased severity of infection (Vordermeier et al., 2002). It has been reported that measurement of IFN γ produced specifically by central memory T cells within a cultured ELISPOT assay may be a more accurate correlate with levels corresponding with vaccine induced protection (Vordermeier et al., 2009b, Hope et al., 2011). However, (Buddle et al., 2005) suggested that measurement of a range of cytokines may be a more accurate indicator of vaccine success or failure and identification of novel correlates is therefore a significant focus of research.

1.1.3.4.1 Bacille Calmette-Geurin (BCG) Vaccine

Following serial passage of *M.bovis*, multiple gene deletions in the RD1 regions resulted in a mycobacterial strain which lacked important virulence factors and appeared to be protective against infection with *M.tuberculosis*. To date, *M.bovis* Bacille Calmette-Geurin (BCG) is the only licensed vaccine for the prevention of TB and is routinely administered to humans world-wide. However, the protective efficacy of BCG varies substantially, thought to be most effective in the prevention of severe disseminated TB infection within infants yet with variable levels of protection against pulmonary infection in both infants and adults (Fine, 1995) and waning protection with increasing age (Weir et al., 2008). In a similar manner, responses in cattle are also variable (Buddle et al., 1995, Buddle et al., 2002) with better protection following neonatal vaccination (Buddle et al., 1995, Hope et al., 2005b, Hope et al., 2011). BCG induced protective responses in cattle are associated with a reduction in the severity

of infection evidenced by inhibition of bacterial colonisation resulting in reduced spread of infection and reduction in shedding (Buddle et al., 1995, Hope et al., 2011, Suazo et al., 2003).

Despite substantial research, the immunological responses that determine BCG efficacy are not fully understood. Several studies have defined a role of prior exposure to environmental mycobacteria as a compromising factor in both humans (Fine, 1995) and cattle (Buddle et al., 2002) resulting in inappropriate priming of the immune response such that subsequent vaccination with BCG is ineffective. However, the type of mycobacteria within the environment appears to be influential as *M.avium* exposure did not interfere with BCG vaccination and conferred a degree of protection to *M.bovis* challenge in cattle, yet did influence diagnostic testing (Hope et al., 2005a, Thom et al., 2008).

Issues relating to environmental mycobacteria may be avoided by immunisation of neonates which has been shown to be protective in humans (Marchant et al., 1999) and cattle (Buddle et al., 1995, Hope et al., 2011, Hope et al., 2005b). In this case, the innate immune system may play a fundamental role in the early development of a Th1 biased memory T cell immune response (Tastan et al., 2005, Marchant et al., 1999, Price et al., 2007, Hope and Villarreal-Ramos, 2008, Buza et al., 2009). BCG is therefore administered to infants world-wide and remains the only effective vaccination strategy for prevention of TB. However, vaccination of cattle with BCG results in non-specific sensitisation to the tuberculin skin test and is therefore not permitted under EU legislation. The use of BCG within cattle will require changes in the law alongside diagnostic regimes which are not compromised by vaccination. Therefore, significant research is being conducted to identify alternative strategies to control the incidence of bovine TB in the UK.

The targeting of wildlife reservoirs directly is an appealing prospect and would circumvent issues related to diagnostic testing in cattle. It has been proposed that the oral delivery of BCG incorporated into baits would be the most cost-effective means of vaccine delivery to wildlife (Buddle et al., 2011). Within experimental challenge studies the oral administration of a lipid formulated BCG vaccine imparted protective

immunity to badgers with a reduced number and severity of lesions (Corner et al., 2010). Despite promising results the implications of faecal shedding resulting in exposure of BCG to cattle suggests that substantial control strategies would need to be implemented prior to vaccine delivery by this route. Recent research has however proposed that subcutaneous BCG vaccination of badgers may also impart a degree of protection (Lesellier et al., 2009) and may be a viable strategy in attempts to control transmission of *M.bovis* to cattle. To this effect the BCG vaccine was licensed for use in badgers and trials are currently being conducted in high burden regions of the UK.

1.1.3.4.2 Current research – vaccines and diagnostics for bovine TB

Numerous strategies have been explored in the development of a vaccine against bovine TB and are often benchmarked against BCG as an indicator of vaccine success. Novel live attenuated strains of *M.bovis* were able to impart protection against subsequent challenge in cattle (Buddle et al., 2002) however; this strategy is unlikely to be successful due to safety concerns in the field (Hope and Villarreal-Ramos, 2008). Alternative strategies include the use of subunit vaccines; DNA vaccines encoding the immuno-dominant antigens MPB70 and MPB83 did not prevent *M.bovis* infection (Wedlock et al., 2003) with protein subunit vaccines showing more promise. Administration of a mixture of mycobacterial proteins in the form of culture filtrate protein (CFP), CpG oligodeoxynucleotides (ODN) and an oil in water adjuvant induced a degree of protection with the adjuvant used of significant importance (Wedlock et al., 2005b). For this reason, identification of novel adjuvants has been a focus of vaccine research; water-in-oil emulsion was found to induce both effector and central memory T cell immune responses (Vordermeier et al., 2009a). Furthermore, the targeting of dendritic cells with adjuvants is considered an important approach to induce innate and adequately prime adaptive immune responses (Hope and Villarreal-Ramos, 2008, Wedlock et al., 2008).

To date, only heterologous prime boosting strategies appear able to improve on the protection observed with BCG alone. DNA vaccines imparted significant protection but only with BCG as a booster vaccine (Skinner et al., 2003). Additionally, the efficacy of the CFP subunit vaccine could be improved when administered with BCG and was

found to be more protective than BCG alone (Wedlock et al., 2005a). The use of replication deficient viral vectors expressing mycobacterial antigens has been a focus of substantial research in the design of prime-boost vaccines in both humans and cattle. In particular, the use of a BCG prime followed by boosting with modified vaccinia virus ankara (MVA) expressing the mycobacterial antigen Ag85a is currently undergoing clinical trials as a protective vaccine against human TB (McShane and Hill, 2005). In cattle, BCG vaccination was followed by administration of either MVA or adenovirus expressing Ag85a. Following infection with *M.bovis*, significant protection was observed over use of BCG alone with a number of cattle displaying signs of complete protection (Vordermeier et al., 2009b). Furthermore, the route of vaccine administration is likely to be a significant factor where strategies employing mucosal immunisation show promise. In mice, only intranasal boosting with adenovirus expressing Ag85a induced antigen specific T cell responses within the airways and subsequent protection against *M.tuberculosis* challenge (Santosuosso et al., 2005). It is not known whether this is the case in cattle, and is currently the focus of research.

Despite significant promise vaccination strategies which employ the use of BCG will not be routinely administered unless there is an appropriate diagnostic testing procedure in place which is not compromised by BCG vaccination as is the case with the current tuberculin skin test. Alongside the development of a vaccine, there is therefore also a need to develop a test for cattle which is able to differentiate between vaccinated and infected animals (DIVA). To this effect, studies have targeted *M.bovis* specific antigens such as ESAT-6 and CFP-10 which are encoded within the RD1 gene regions missing from BCG (Jones et al., 2010a). Stimulation of whole blood with these antigens was able to accurately identify *M.bovis* infected cattle without non-specific reactivity within BCG vaccinated animals (Vordermeier et al., 2002). Furthermore, CD4⁺ T cells were found to up-regulate intracellular IFN γ levels, detectable only within *M.bovis* infected cattle 4 hours following re-stimulation with PPD_b (Sopp et al., 2008, Sopp et al., 2006). In an example of a DIVA skin testing strategy, cattle infected with *M.bovis* produced a detectable DTH response following administration of a cocktail of *M.bovis* specific antigens including ESAT-6, CFP-10, MPB70 and MPB83 with no detectable response from BCG vaccinates (Whelan et al., 2010).

Whilst substantial progress has been made in the development of diagnostic and vaccination strategies to control TB within cattle, it is apparent that the immunological mechanisms that govern the balance between protection or progressive infection are not fully understood. Further research is required to elucidate the fundamental immune responses that occur during mycobacterial infection in order to aid the development of strategies in the future where these may be manipulated to our advantage.

1.2 The immune response to mycobacterial infection

TB causing species; *M.bovis* and *M.tuberculosis* infect via the respiratory route and a multifaceted immune response ensues to control the spread of infection. Pathologically, this can be identified by the formation of widespread bacterially loaded lesions surrounded by infiltrating immune effector cells (Algood et al., 2005) which can develop into necrotic granulomas during chronic infection (Liebana et al., 2008). Despite substantial research, the immune mechanisms that govern protective immunity have not been completely deciphered. It is known that both the innate and adaptive arms of the immune response are involved and Th1 polarisation is critical (Orme, 2004, Welsh et al., 2005). Implicit within this is the fundamental role played by IFN γ , in particular at early stages during the development of the immune response to enable containment of infection and subsequent polarisation of cell mediated immunity. This has been illustrated by gene knock-out studies where deficiencies of IFN γ result in severe dissemination and mortality following *M.tuberculosis* challenge (Flynn et al., 1993, Cooper et al., 1993). In addition, human IFN γ deficiencies correlate with an enhanced susceptibility to mycobacterial infections (Dorman et al., 2004).

1.2.1 The role of antigen presenting cells

Antigen presenting cells such as macrophages and dendritic cells (DCs) residing within the luminal and intra-epithelial regions of the respiratory passages and lungs are largely responsible for the initiation of innate immunity to mycobacterial pathogens and the route of cellular entry can have a substantial impact on the clearance, containment or onset of progressive infection (Hope et al., 2004).

1.2.1.1 Macrophages

During initial immune defence mechanisms, macrophages function to rapidly kill invading pathogens. Expression of an array of cell surface receptors enables recognition of mycobacteria and induction of downstream effector responses. In particular, conserved antigenic regions are recognised by toll like receptors (Means et al., 1999), complement receptors, mannose receptors binding to mannosylated bacterial glycoproteins and also FC receptors able to recognise opsonised molecules (Ernst, 1998). Binding of receptors initiates intracellular signalling pathways leading to activation of transcription factors such as NFκB.

Upon activation, macrophages are able to release reactive oxygen or nitrogen intermediates or can ingest mycobacteria by phagocytosis and target the bacterial phagosome to intracellular lysosomal degradation pathways. Furthermore, macrophages infected with *M.tuberculosis* secrete pro-inflammatory cytokines TNFα, IL-1 and IL-6 (Hickman et al., 2002, Giacomini et al., 2001) and *M.bovis* infected alveolar macrophages up-regulate inflammatory chemokines (Widdison et al., 2011, Widdison et al., 2008) to promote the recruitment and activation of immune effector cells and aid granuloma formation. However, macrophages also release IL-10 following mycobacterial infection which functions to down-modulate the inflammatory immune response (Hickman et al., 2002, Giacomini et al., 2001, Hope et al., 2004, Denis et al., 2005).

Despite this, mycobacteria have evolved to circumvent the anti-microbial activities of macrophages and persist within the host. The route of entry plays a central role with inhibition of cytotoxicity following entry via complement receptor 3 (CR3) or mannose receptor (reviewed by (Hope et al., 2004)). Additionally, mycobacteria can block the maturation of the phagosome and prevent lysosome fusion to subvert degradation by hydrolytic enzymes (Flynn and Chan, 2003, Rohde et al., 2007, Nguyen and Pieters, 2005). Studies have reported that exposure of macrophages to IFNγ may prevent mycobacterial persistence through activation of bactericidal mechanisms and release of pro-inflammatory cytokines (Giacomini et al., 2001, Hickman et al., 2002, Zhang et al., 2010, Flynn et al., 1993). This was only true to a certain extent during *in vitro*

infection with *M.bovis* where treatment with IFN γ did not prevent bacterial replication within bovine macrophages but did promote the production of pro-inflammatory cytokines (Aldwell et al., 1997, Denis et al., 2005, Hope et al., 2004). A consequence of bacterial persistence within the phagosomes of macrophages as well as IL-10 release is likely to be low levels of antigen presentation and priming of T cell responses. For these reasons it is believed that macrophages do not play a significant role in the progression of the adaptive immune response and during chronic infection are the main reservoirs for mycobacterial pathogens.

1.2.1.2 Dendritic cells

Dendritic cells (DCs) are professional antigen presenting cells, specialised in their ability to actively take up antigen and prime naïve or activate memory T cells in the bridge between the innate and adaptive arms of the immune response. Originating in the bone marrow, DCs belong to myeloid or lymphoid lineages that form phenotypically and functionally heterogeneous subsets of cells (Steinman, 2003). Bovine DCs are predominantly of myeloid origin (Howard and Hope, 2000, Miyazawa et al., 2006) and the existence of a small population of plasmacytoid DCs of lymphoid origin, has only recently been identified (Reid et al., 2011).

Only a small percentage of differentiated DCs are found circulating in peripheral blood (Miyazawa et al., 2006), with the vast majority forming resident populations in tissues. For example, DCs are particularly prevalent within the intra-epithelial regions of mucosal surfaces within the gut, respiratory tract and lungs and as such are ideally placed to sample large volumes of antigen pivotal to the induction of tolerance or immunity (Steinman, 2003). Furthermore, DCs and macrophages can be derived from circulating monocytes depending on the stimulus received. *In vitro* human (Sallusto and Lanzavecchia, 1994) and bovine (Werling et al., 1999) monocytes differentiate into immature DCs in the presence of granulocyte monocyte colony stimulating factor (GM-CSF) and interleukin 4 (IL-4).

In tissues, DCs exist in an immature state; highly responsive to inflammatory chemokines enabling accumulation at sites of infection, with increased phagocytic capacity and low expression of cell surface MHC class II and co-stimulatory molecules

(Steinman, 2003). In the murine model of mycobacterial infection, intra-nasal inoculation induced the recruitment of immature DCs to pulmonary regions (Wolf et al., 2007, Pecora et al., 2009), a proportion of which may have been of monocytic origin (Reljic et al., 2005). The expression of a repertoire of antigen recognition receptors by immature DCs enables the efficient capture and recognition of foreign particles. These include the C-type lectins; mannose receptor, dendritic cell specific intracellular adhesion molecule 3 grabbing non-integrin (DC-SIGN), macrophage mannose receptor 1 (DEC-205) and pattern recognition receptors for example toll-like receptors (Demangel and Britton, 2000). In particular, mycobacteria are able to bind to DC-SIGN (Yamakawa et al., 2008, Geijtenbeek and van Kooyk, 2003) and roles of TLR2, TLR4 (Tsuji et al., 2000, Means et al., 1999) and TLR9 (von Meyenn et al., 2006) have also been described in the recognition of conserved molecules expressed by mycobacteria. Upon recognition by endocytic receptors, DCs take up antigen through mechanisms involving formation of caveolae (Werling et al., 1999), clathrin coated pits, macropinocytosis and phagocytosis.

Following antigen uptake DCs migrate from the periphery to draining lymph nodes via afferent lymphatics and undergo a process of maturation involving phenotypic and functional changes rendering the capacity to activate T cell responses. In gene knock-out models, mice deficient in chemokines CCL19 and CCL21 were unable to transport *M.tuberculosis* to local lymph nodes (Wolf et al., 2007) highlighting the fundamental role of the chemokine receptor CCR7 and its ligands in the lymphoid homing capacity of DCs (Jakubzick et al., 2006, Martin-Fontecha et al., 2003, Saeki et al., 1999). As well as during an inflammatory immune response, DCs also constitutively traffic and transport antigen in this manner under steady-state conditions; a process associated with the induction of peripheral tolerance to a multitude of self-antigens (Steinman, 2003).

During the migration and maturation process, previously immature phagocytic DCs are phenotypically transformed into efficient antigen presenting cells. In particular cell surface expression of MHC class II, co-stimulatory molecules and adhesion molecules is augmented. Monocyte derived DCs from cattle up regulated MHC class II, CD40 and CD80 cell surface expression following infection with *M.bovis*; consistent with

pathogen induced maturation (Hope et al., 2004). During classical presentation, MHC class II bearing peptide complexes are presented to CD4⁺ T lymphocytes expressing the cognate T cell receptor along with co-stimulation. Antigen may also be presented by DCs in a non classical manner; in the context of CD1 molecules (Porcelli and Modlin, 1999, Rosat et al., 1999) or during cross-presentation with exogenous antigens presented by MHC class I (Steinman, 2003).

Cytokines produced by maturing DCs are pivotal to the effective polarisation of the adaptive immune response. Infection of DCs with mycobacteria is associated with the production of pro-inflammatory cytokines IL-12, IL-18, TNF α , IL-1, IL-6 and the immune-regulatory cytokine IL-10 (Demangel et al., 2002, Hickman et al., 2002, Hope et al., 2004, Giacomini et al., 2001, Denis and Buddle, 2008). Of fundamental importance is the role of IL-12, implicit to the priming of Th1 immune responses (Hickman et al., 2002). IL-12 released by DCs functions to enhance the IFN γ production and proliferation of T cells (Denis and Buddle, 2008, Hickman et al., 2002, Hope et al., 2000, Khader et al., 2006), NK cells (Hope et al., 2002b) and $\gamma\delta$ T cells (Price, 2009) to potentiate innate and adaptive immune responses. Furthermore in an autocrine fashion, IL-12 was able to maintain CCR7 expression by DCs to enable efficient transport of *M.tuberculosis* to draining lymph nodes and priming of CD4⁺ T cells (Khader et al., 2006).

Within cytokine rich environments, the balance of IL-12 and IL-10 significantly influences the ensuing immune response. Following aerosol infection with BCG, IL-10 inhibited the release of IL-12 and migration of DCs to draining lymph nodes with a subsequent decline in IFN γ production by T cells (Demangel et al., 2002, Giacomini et al., 2001). Abundant release of IL-12 functions to override the down-modulatory effects of IL-10 (Hickman et al., 2002). For these reasons, the role of IL-10 in mycobacterial infection is controversial with elevated levels associated with persistence and progression to chronic infection (Welsh et al., 2005). Importantly, *M.bovis* infected bovine macrophages are a significant source of IL-10 (Denis et al., 2005, Hope et al., 2004) and are therefore implicated as playing a role in the immune evasion by mycobacteria during chronic infection.

As is the case with macrophages, mycobacteria have developed mechanisms enabling persistence within DCs. Uptake via DC-SIGN is associated with interference of intracellular signalling in the subversion of DC derived immune responses (Geijtenbeek and van Kooyk, 2003). However, this was also the case in the absence of DC-SIGN (Gagliardi et al., 2005) suggesting alternative mechanisms are also involved. In particular, mycobacteria can inhibit the differentiation of monocytes to DCs (Gagliardi et al., 2004), prevent migration to draining lymph nodes, interfere with MHC class II presentation of antigen (Wolf et al., 2007) and influence cytokine release (Remoli et al., 2011, Palucka and Banchereau, 2002). As a consequence, mycobacteria induced subversion of DCs may be associated with advanced chronic TB infection. Alternatively, persistence in this manner may provide a constant source of antigen enabling continual stimulation of NK cells, $\gamma\delta$ T cells and the potential priming or activation of T cells (Hope et al., 2004).

1.2.2 Chemokines

Chemokines are proteins that upon binding initiate the directional migration of cells bearing cognate G-protein coupled receptors and are therefore fundamental to almost every aspect of the immune system. Genomic analysis within humans has identified over 40 different chemokines with 18 functional receptors (Zlotnik et al., 2006). Both of these are classified according to the number and position of the cysteine residue into the families; C, CC, CXC, CX3C with ligands expressed as XCL, CCL, CXCL, CX3CL and receptors as XCR, CCR, CXCR, CX3CR (Murphy, 2002, Murphy et al., 2000). In cattle, homologues to all known human chemokine receptors were identified and found to be well conserved with varying homology (60-90%) at nucleotide and amino acid level (Widdison et al., 2010, Blumberman et al., 2007, Boyd et al., 2010).

Functionally, gradients of chemokines and the repertoire of cell-surface chemokine receptor expression determine migratory ability. However, complexity arises within this system as chemokines are able to bind to more than one receptor and any given receptor can bind multiple ligands; a mechanism which enables redundancy. There are two major subclasses of chemokines; homeostatic chemokines which establish and

maintain resident cellular populations and inflammatory chemokines which induce recruitment of cells upon insult (Murphy, 2002, Murphy et al., 2000).

Major roles for chemokines have been defined in the recruitment of immune effector cells in particular; migration to inflammatory sites, to draining lymph nodes and also in the organisation of distinct cellular regions within secondary lymphoid tissue (Cyster, 2005, Luther et al., 2002). Chemokines are secreted by numerous cells and may be present in the extracellular milieu, or loosely tethered to the surface of endothelial cells. In this manner, lymphocytes bearing the appropriate chemokine receptor may be recruited from neighbouring regions or from the periphery in a sequence of adhesion and migratory events. Initially, circulating lymphocytes are arrested by adhesion molecules such as selectins and shear forces induce rolling over endothelial layers (Sallusto et al., 2000). Tethered lymphocytes bearing chemokine receptors may come into contact with chemokines (Sallusto and Baggiolini, 2008), triggering intracellular signalling events to induce integrin mediated firm adhesion and trans-endothelial migration. During this process lymphocytes respond to the gradient of chemokine created between inter-endothelial cell junctions to migrate into tissues (Bromley et al., 2008).

Numerous studies have illustrated the importance of chemokine signalling in the immune response to mycobacterial infection. For example, in the accumulation of effector cells contributing to the formation of granulomatous lesions (Peters and Ernst, 2003) and also in the migration of lymphocytes to secondary lymphoid organs to promote the induction of adaptive immune responses (Orme and Cooper, 1999).

Infection of macrophages and dendritic cells with mycobacteria results in a rapid release of inflammatory chemokines. *In vitro*, macrophages secreted elevated levels of CCL2, CCL3, CCL4, CCL5, CCL20 and CXCL8 (Saukkonen et al., 2002, Scott and Flynn, 2002, Mendez-Samperio et al., 2003). In a similar manner, DCs infected with *M.tuberculosis* up-regulated CCL2, CCL3, CCL4, and CXCL8 followed by increasing levels of CXCL9 and CXCL10 (Gustafsson et al., 2008, Lande et al., 2003). In cattle, inflammatory chemokines were up-regulated as early as 4 hours following *M.bovis* infection of alveolar macrophages (Widdison et al., 2011), supernatants from which

induced the recruitment of granulocytes (Widdison et al., 2008). Furthermore, during inflammatory conditions, epithelial cells within the respiratory tract are believed to respond to macrophage derived cytokines (TNF α and IL1 β) to produce chemokines such as CCL2, CXCL8 and CCL20 which may be important for the recruitment of DCs (Thorley et al., 2007, Thorley et al., 2005). Whether this is also the case with mycobacterial infection is yet to be determined.

Murine gene knock-out models have shed significant light into the roles of specific chemokines and receptors; *M.tuberculosis* infection in CCR2 negative mice resulted in severe and rapid onset of infection as a consequence of deficiencies in lymphocyte migration to the lungs and to mesenteric lymph nodes (Peters et al., 2001, Scott and Flynn, 2002). Furthermore mice lacking CCR1 were unable to recruit NK cells to the site of infection (Shang et al., 2000). Importantly CCR7 appears to be pivotal; expression of which confers the capacity to migrate to lymph nodes. Mice lacking CCR7 succumbed to *M.tuberculosis* infection at faster rates as a consequence of impaired trafficking of DCs resulting in delayed CD4⁺ T cell responses (Olmos et al., 2010, Wolf et al., 2007). Conversely, deficiencies in CXCR3 were found to be beneficial with increased retention of Th1 effector T cells within the lungs of *M.tuberculosis* infected mice. However this was only the case during chronic infection (Chakravarty et al., 2007). Additionally, DCs were retained in lymph nodes of mice lacking CCR5, potentiating adaptive immune responses (Algood and Flynn, 2004).

It is clear that chemokine mediated recruitment during mycobacterial infection is a complex and intricate process which involves the balance of signalling between multiple ligands and receptors. Whilst inflammatory chemokines are implicit to the efficient recruitment of effector lymphocytes, elevated levels may exacerbate immuno-pathological responses. Elevated levels of CCL2, CCL3, CXCL8, CXCL9 and CXCL10 are often found within the pleural fluid of chronically infected TB patients (Hasan et al., 2009, Pokkali et al., 2008). Similarly, elevated expression of CCL3, CCL4, CCL5, CXCL8, CXCL10 within lymph nodes of *M.bovis* infected cattle were found to correlate positively with increased severity of infection (Widdison et al., 2009).

1.2.3 Cell mediated immune responses

1.2.3.1 Conventional $\alpha\beta$ T cells

Antigen specificity in the immune response is conferred by conventional T cells bearing the highly divergent $\alpha\beta$ T cell receptor and MHC restricted co-receptors; CD4 or CD8. In the priming of adaptive immune responses, peptide antigens complexed with MHC molecules are presented to T cells within secondary lymphoid tissue; a process involving binding of a cognate TCR, co-stimulation and cytokine release. Cytokines produced by antigen presenting cells are fundamental to the effective polarisation of T cells such that Th1 priming was found to be reliant on IL-12 production from DCs infected with *M.tuberculosis* (Hickman et al., 2002). In this manner, Th1 polarised T cells secrete IFN γ , IL-2 and TNF α with IFN γ production pivotal to the control of TB infection (Cooper et al., 1993, Flynn et al., 1993). Whilst the role that IFN γ may play in mycobacterial infection is a debated issue (1.1.3.4), within cattle infected with *M.bovis* a Th1 biased cytokine profile was found to correlate with an augmented capacity to control the spread of infection (Welsh et al., 2005).

T cells bearing the CD4 co-receptor are restricted by MHC class II presentation pathways and are able to secrete IFN γ upon activation. For this reason, numerous studies have illustrated the implicit nature of CD4+ T cells in the protective immune response to mycobacterial infection. CD4 knock-out mice infected with *M.tuberculosis* were unable to control bacterial replication within the lungs (Mogues et al., 2001) as a consequence of reduced cellular infiltration and defects in granuloma formation (Saunders et al., 2002). In addition, immune deficiencies of CD4+ T cells as is the case in HIV afflicted individuals' renders sufferers highly susceptible to mycobacterial infections. Within cattle, proliferation and antigen specific recall responses within the CD4 population can be detected following both BCG vaccination (Hope et al., 2000) and *M.bovis* infection (Sopp et al., 2008, Sopp et al., 2006, Walravens et al., 2002). Furthermore, percentages of circulating CD4+ T cells were significantly increased in *M.bovis* infected cattle (Buza et al., 2009, Pollock et al., 1996).

In contrast, CD8+ T cells are restricted by MHC class I presentation pathways and are therefore commonly activated by intracellular peptides. However, in alternative

mechanisms exogenous antigens may be cross-presented via hijacking of MHC class I pathways (Liebana et al., 1999). In addition, CD1 molecules are able to present lipid antigens to non-classically restricted CD8⁺ T cells (Hope et al., 2001, Porcelli and Modlin, 1999, Rosat et al., 1999). Upon activation, CD8 expressing T cells are predominantly cytotoxic but are also able to proliferate and produce IFN γ . It has been reported that BCG vaccination in mice induces antigen specific CD8⁺ T cells which are able to accumulate within the lungs following *M.tuberculosis* infection (Begum et al., 2009). Similarly within cattle, CD8⁺ T cells produced IFN γ in the antigen specific recall response following BCG vaccination (Hope et al., 2000) and *M.bovis* infection (Walravens et al., 2002). Additionally, upon re-stimulation CD8⁺ T cells from BCG vaccinated cattle produced perforin and were able to kill BCG or *M.bovis* infected macrophages (Hogg et al., 2009). However, whilst CD8⁺ T cells appear to be responsive during mycobacterial infection, their relative importance to protection has been challenged. In gene knock-out mice the loss of CD4⁺ T cells or MHC class II was far more detrimental than the loss of CD8⁺ T cells or MHC class I (Mogues et al., 2001). Within cattle, antigen specific IFN γ release was consistently greater in the CD4⁺ T cell population (Liebana et al., 1999). Importantly, cattle depleted of CD8⁺ T cells presented with lower lesion scores in head lymph nodes following infection with *M.bovis* (Villarreal-Ramos et al., 2003). Therefore, whilst CD8⁺ T cell specific release of IFN γ and cytotoxicity may contribute to the initial control of infection, it is not considered essential for long-lived protective immunity (Liebana et al., 1999) and may contribute to the immuno-pathology associated with chronic TB infection (Villarreal-Ramos et al., 2003)

1.2.3.2 B cell responses and humoral immunity

Th2 polarisation of the immune response results in B cell derived antibody production, with prominent roles of cytokines such as IL-4 and IL-10. It has long been regarded that this response is driven by extracellular pathogens and therefore, antibody responses are generally believed not to be associated with protection in the context of intracellular mycobacterial infection (Johnson et al., 1997). In cattle, mycobacteria specific IgG and IgM were detected within the sera as early as 4 weeks post-*M.bovis* infection (Waters et al., 2006). However within humans and cattle, Th2 polarisation

and elevated antibody titres are often indicative of chronic progressive infection and high bacterial burden (Waters et al., 2010). For example, IgG levels within the sera of *M.bovis* infected cattle positively correlated with disease induced pathology (Welsh et al., 2005). Furthermore, BCG vaccination induced antibody responses were not associated with protection (Buddle et al., 2005).

Despite this, B cells appear to participate in the immune response to mycobacterial infection and are particularly prevalent within pulmonary granulomas (Maglione et al., 2007). Following *M.tuberculosis* infection, B cell knock-out mice were unable to contain bacteria with deficiencies in granuloma formation (Maglione et al., 2007) and reduced cellular infiltration to the lungs (Bosio et al., 2000). In addition, immune responses associated with Th1 and Th2 polarisation are unlikely to be mutually exclusive with a balance of signalling likely to determine the outcome of infection (Abebe and Bjune, 2009). De Valliere et al., (2005) reported that antibody mediated opsonisation and phagocytosis of mycobacteria potentiated the expansion of IFN γ producing CD4 $^{+}$ and CD8 $^{+}$ T cells. For these reasons, it has been proposed that immunotherapeutic approaches may be beneficial in the treatment of TB infection and development of novel monoclonal antibodies has shown promise (Balu et al., 2011).

1.2.3.3 Unconventional T Cells

1.2.3.3.1 $\gamma\delta$ T cells

Lymphocytes expressing the $\gamma\delta$ T cell receptor (TCR) represent a small population in humans and mice however, in comparison may constitute up to 60% of all circulating lymphocytes within young calves (Davis et al., 1996, Kampen et al., 2006) and are also particularly prevalent within tissues such as the intra-epithelial regions of the gut and respiratory tract. The expression of multiple receptors enables recognition of a diverse array of antigens such that $\gamma\delta$ T cells participate in both innate and adaptive immune responses. As is the case with conventional T cells, recombination events of the V, D and J genomic regions confers specificity however, $\gamma\delta$ T cells also express invariant TCRs or pattern recognition receptors and are therefore not always restricted by classical presentation pathways. In particular, lipid antigens may be presented in the

context of CD1 molecules (Porcelli and Modlin, 1999, Van Rhijn et al., 2006) or $\gamma\delta$ T cells may respond directly to pathogen associated molecular patterns.

Substantial evidence exists to suggest that $\gamma\delta$ T cells play a prominent role in the immune response to mycobacterial infection. Following BCG infection in mice, $\gamma\delta$ T cells were recruited to the lungs and were an early innate source of IFN γ , potentiating CD8 $^{+}$ T cell effector responses (Caccamo et al., 2006). In cattle and other ruminants, a significant proportion of $\gamma\delta$ T cells uniquely express workshop cluster 1 (WC1) and expression of isoforms of this molecule define functionally distinct subsets (Rogers et al., 2005). Percentages of WC1 $^{+}$ $\gamma\delta$ T cells increased in response to BCG, with levels peaking at 2 weeks post-vaccination. Importantly, a corresponding increase in IFN γ was also observed (Buza et al., 2009). In contrast, as early as 5 days post-*M.bovis* infection, WC1 $^{+}$ $\gamma\delta$ T cells decreased in numbers from the periphery (Kennedy et al., 2002, Pollock et al., 1996) and following BCG inoculation, percentages of IFN γ producing WC1 $^{+}$ $\gamma\delta$ T cells increased within the tissues of the lungs and upper respiratory tract (Price et al., 2010). Furthermore, WC1 $^{+}$ $\gamma\delta$ T cells reciprocally interacted with *M.bovis* infected DCs to produce IFN γ and enhanced IL-12 release from DCs (Price and Hope, 2009). In depletion studies, the loss of WC1 $^{+}$ $\gamma\delta$ T cells influenced the early innate levels of IFN γ observed however, did not substantially alter the progression of *M.bovis* infection highlighting the existence of redundancy within the immune system (Kennedy et al., 2002).

1.2.3.3.2 NKT cells

Natural Killer T cells exist as a small distinct sub-population of lymphocytes within humans and mice, phenotypically and functionally related to T cells and NK cells. The most widely studied of which express an invariant TCR, restricted to antigen recognition via CD1d molecules presenting glycolipids such as α -galactosylceramide (α -GalCer) (Bendelac et al., 2007, Lantz and Bendelac, 1994). NKT cells can be phenotypically distinguished from NK cells by the expression of conserved TCRs and lack of expression of natural cytotoxicity receptor NKp46 (Walzer et al., 2007a). In the murine model of *M.tuberculosis* infection invariant NKT cells were able to augment the bactericidal activity of macrophages to reduce bacterial burden within the lungs; a

process which involved recognition of antigen in the context of CD1d (Sada-Ovalle et al., 2008). Furthermore, NKT cells may restrict mycobacterial growth through release of cytolytic proteins such as granulysin (Gansert et al., 2003) and promote pro-inflammatory immune responses through cytokine and chemokine production (Kim and Chung, 2011).

Activation of NKT cells in this manner requires the presentation of antigen in the context of CD1d. However cattle do not express functional CD1d molecules, with genomic homologues identified as pseudogenes. Furthermore, bovine lymphocytes did not respond to tetramers of human and murine CD1d molecules loaded with α -GalCer antigen (Van Rhijn et al., 2006). It has therefore been suggested that cattle may lack functional CD1d restricted NKT cells (Boysen and Storset, 2009, Van Rhijn et al., 2006). It is yet to be determined whether NKT cells exist within cattle and recognise antigen through novel mechanisms distinct from humans and mice.

1.2.3.4 Natural Killer Cells

Natural Killer (NK) cells are multi-functional lymphocytes that have been widely studied with respect to their diverse roles and interplay within the immune system. Expression of germ-line encoded receptors confers responsiveness without prior stimulation enabling innate cytolytic effector responses towards transformed or infected cells. Upon stimulation NK cells are able to produce immuno-modulatory cytokines, interact with antigen presenting cells and may also be capable of memory-like responses. It is therefore hypothesised that NK cells function as a bridge between the innate and adaptive immune systems.

As innate effectors, NK cells contain preformed granules of lytic proteins (Reefman et al., 2010), released upon recognition of target cells. During this process membranous protrusions extend to form immunological synapses to enable polarised delivery of perforin and granulysin (Chauveau et al., 2010, Culley et al., 2009, Davis and Dustin, 2004, Reefman et al., 2010). In addition, NK cells express cytotoxic effector molecules to induce apoptotic events within target cells. The functional responses of NK cells are however far more diverse. Upon activation, NK cells produce abundant cytokines such as IFN γ , TNF α and GM-CSF (Cooper et al., 2001, Boysen et al., 2006b, Fehniger et al.,

1999b) and have also been reported as source of inflammatory mediators IL-10 (Fehniger et al., 1999b, Cooper et al., 2001) and IL-22 (Cella et al., 2009, Dhiman et al., 2009). Furthermore, cytokine stimulated NK cells are able to secrete inflammatory chemokines (Dorner et al., 2002, Fehniger et al., 1999b, Nieto et al., 1998), to aid accumulation of effector cells during early innate immune responses.

In further complexity, the role of NK cells within adaptive immune responses is the focus of intense research. Following vaccination and re-infection, NK cells respond indirectly to CD4⁺ T cell derived IL-2 (Bihl et al., 2010, Horowitz et al., 2010a). However, (O'Leary et al., 2006) demonstrated direct antigen specific recall responses of NK cells in the murine model of contact hypersensitivity. This has subsequently been demonstrated in viral infections (Sun et al., 2009, Paust et al., 2010) with memory-like responses restricted to a small sub-population of CXCR6 expressing hepatic NK cells (Paust et al., 2010). In addition, discrete subsets of NK cells have been reported to express MHC class II molecules (Burt et al., 2008, Evans et al., 2011) and may possess the capacity to present antigen to CD4⁺ T cells (Burt et al., 2008, Hanna et al., 2004, Roncarolo et al., 1991). However the mechanisms that enable acquisition, internalisation and presentation of antigen are not understood.

NK cell derived IFN γ is believed to be implicit to the Th1 polarisation of the immune response (Martin-Fontecha et al., 2004). Considerable evidence exists to suggest that NK cells play a role in the immune response to mycobacterial infection. In addition, abundant numbers of NK cells within neonates may participate in the enhanced efficacy of BCG vaccination (Endsley et al., 2009). Following *M.tuberculosis* or BCG infection in mice, numbers of activated NK cells within the lungs increased with augmented IFN γ release (Junqueira-Kipnis et al., 2003, Feng et al., 2006, Saxena et al., 2002). Importantly, the IFN γ functioned to regulate macrophage activation and control bacterial replication and dissemination (Feng et al., 2006). *In vitro*, *M.tuberculosis* infected antigen presenting cells were able to recruit NK cells (Lande et al., 2003) and augment cytolytic activity and IFN γ release (Vankayalapati et al., 2002). Furthermore, BCG may bind directly to NK cells to induce proliferation, IFN γ production and cytolytic activity (Esin et al., 2004, Esin et al., 2008, Batoni et al., 2005, Marcenaro et al., 2008). In cattle, NK cells are also present in large numbers in neonates (Graham et al., 2009)

and were a source of IFN γ following stimulation with mycobacterial antigens (Olsen et al., 2005) or BCG (Hope et al., 2002b). In addition, NK cells were able to produce IFN γ and kill *M.bovis* (Denis et al., 2007) and BCG (Endsley et al., 2006) infected macrophages.

1.2.3.4.1 Human NK cells

NK cells originate in the bone marrow as CD34+ hematopoietic progenitor cells, responsive to growth factors and cytokine stimulation (Mrozek et al., 1996). The precise pathways that govern commitment to an NK lineage have not been fully defined however; the identification of developmental intermediates suggests that precursor cells may undergo a process of differentiation and maturation within secondary lymphoid tissue (Freud et al., 2006, Juelke et al., 2010). This process involves acquisition of an NK cell specific repertoire and induction of tolerance to prevent self-reactivity (Colucci et al., 2003).

Mature NK cells represent a heterogeneous population of cells that are widely distributed but found in large numbers within tissues underlying mucosal surfaces, within lymph nodes (Ferlazzo et al., 2004b, Fehniger et al., 2003) and as a constituent of the peripheral blood lymphocyte population. Human NK cells can be primarily defined by lack of expression of CD3 and varying levels of expression of CD56 and CD16 (Cooper et al., 2001) to form functionally distinct subsets. CD56^{dim} CD16+ NK cells are more commonly found within the blood and are predominantly cytotoxic in nature (Jacobs et al., 2001). In comparison CD56^{bright} CD16- NK cells found within lymph nodes are competent cytokine producers (Ferlazzo et al., 2004a, Ferlazzo et al., 2004b, Fehniger et al., 2003, Fehniger et al., 1999b, Jacobs et al., 2001, Cooper et al., 2001). Studies have proposed that the CD56^{bright} NK cells may be precursors to the CD56^{dim} subset (Chan et al., 2007), with expression of CD94 identifying a functional intermediary (Yu et al., 2010), yet the precise mechanisms that may enable differentiation *in vivo* have not been determined. In addition, human NK cell subsets may not be as functionally distinct as previously believed with CD56^{dim} NK cells also able to produce cytokines such as IFN γ at early time-points following activation (De Maria et al., 2011).

1.2.3.4.2 Murine NK cells

Murine NK cells also exist as a heterogeneous population of cells existing within tissues at differing stages of maturation (Gregoire et al., 2007). Due to the lack of a murine homologue for CD56, NK cells are identified by unique phenotypic characteristics for example; expression of NK1.1 or CD49b (DX5) (Arase et al., 2001). Subsets can further be divided by expression of CD11b and CD27 with blood derived CD27^{low} CD11b^{high} NK cells representing the most mature population (Chiossone et al., 2009, Hayakawa and Smyth, 2006). As is the case within humans, murine NK cells are also widely distributed however importantly during resting conditions have been shown to be largely excluded from lymph nodes (Bajenoff et al., 2006), potentially as a consequence of the lack of expression of the lymphoid homing receptor CCR7 (Gregoire et al., 2007).

1.2.3.4.3 Bovine NK cells

In cattle, NK cells constitute 0.5-10% of the total leukocyte population, with calves possessing higher numbers than adults in order to compensate for an immature adaptive immune response (Graham et al., 2009, Kampen et al., 2006, Kulberg et al., 2004). Bovine NK cells do not express CD56 (Endsley et al., 2006), but can be accurately identified and purified by expression of the natural cytotoxicity receptor NKp46 (Storset et al., 2004) which has also been shown to be constitutively expressed and highly conserved by NK cells within humans, mice (Walzer et al., 2007a) and other ruminant species (Connelley et al., 2011, Elh mouzi-Younes et al., 2010). Phenotypic analysis has revealed that the vast majority of NKp46+ NK cells are CD3 negative, express variable levels of CD8, CD16, CD44 and CD25 and have been reported to exist as distinct sub-populations, most effectively differentiated by expression of CD2 (Boysen et al., 2006b). Whilst the majority of peripheral blood derived NK cells within cattle express CD2, the opposite is true within lymphoid tissue, with larger populations of CD2-/low NK cells (Boysen et al., 2008, Boysen et al., 2006b).

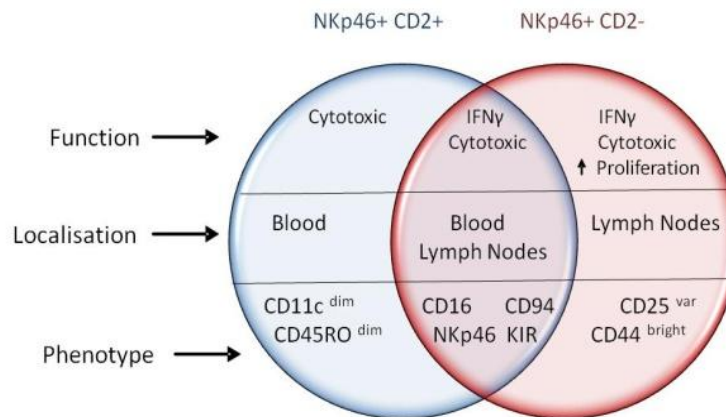


Illustration of the phenotypic and functional differences between NK cell subsets in cattle – Adapted from (Boysen, 2006)

Whilst the CD2 molecule is widely expressed within lymphocyte populations, in particular by T cells and NK cells, its functional role is largely unknown. Studies have reported involvement as an adhesion receptor or co-receptor for activation (McNerney and Kumar, 2006, Tangye et al., 2000). However, blocking of CD2 had little effect on bovine NK cells and therefore, expression appears to be redundant for effective NK cell derived functional responses. Furthermore, the CD2-/low population has been shown to be the more functionally activated population following IL-2 stimulation with faster proliferation, elevated IFN γ production and cytolytic activity (Boysen et al., 2006b).

1.2.3.4.4 The control of NK cell derived effector responses

NK cells express a diverse array of receptors which function to control cytolytic and cytokine producing effector responses through an intricate balance between activatory and inhibitory signalling mechanisms. During the process of 'education' NK cells acquire germ-line encoded receptors, such that mature NK cells are tolerant to self antigens whilst competent to respond as effector cells. Under steady-state conditions, NK cell responses are dampened by inhibitory receptors bound to MHC class I molecules. The precise mechanisms which enable peripheral tolerance are not known; theories suggest inhibitory receptors may engage with MHC class I molecules to 'license' NK cells and enable functional competence of activatory receptors (Kim et al., 2005); a process that may be aided by cytokines (Juelke et al., 2009). Alternative

hypotheses suggest that NK cells lacking inhibitory receptors exist in an auto-reactive state and are 'disarmed' to become hyporesponsive as a consequence of chronic stimulation without inhibition (Bolanos and Tripathy, 2011).

During an immune response, the 'missing self hypothesis' proposes that down-modulation of MHC class I by transformed or infected cells may initiate NK cell effector responses resulting from the lack of an inhibitory signal, an event commonly associated with viral infection or tumour transformation (Lanier, 2005). However, it is increasingly apparent that co-stimulatory signals are required and may be provided via activatory receptors and cytokines (Ortaldo and Young, 2003). Furthermore, the existence of a threshold has been proposed such that activatory signals must outweigh inhibition prior to induction of NK cell effector responses (Ortaldo and Young, 2003, Holmes et al., 2011) and it is therefore, a complex integration of signals that determines the response.

1.2.3.4.4.1 Activatory and inhibitory receptors of NK cells

The MHC restricted killer immunoglobulin-like receptors (KIRs) are encoded by polymorphic genes to give both activatory and inhibitory receptors, distinguishable by the presence of differing intracellular tyrosine based motifs. The variable extracellular region of these receptors confer diversity in the discrimination of allelic variants of MHC class I (Lanier, 2005). Multiple KIR genes have been identified in humans and primates (Vilches and Parham, 2002, Wilson et al., 2000) whereas rodents express distinct Ly49 c-type lectin receptors, encoded by novel genes yet functionally related to KIRs (Lanier, 2005). It has been reported that cattle express both KIR and Ly49 genes conferring a high degree of diversity in the recognition of MHC class I ligands and a possible reflection of the complexity of cattle MHC (Storset et al., 2003, Dobromylskyj and Ellis, 2007, Dobromylskyj et al., 2009, McQueen et al., 2002). Additional inhibitory receptors include the heterodimeric CD94/NKG2 complexes which recognise non-classical MHC molecules and are widely expressed by NK cells in numerous species (Braud et al., 1998, Lanier, 2005). In particular CD94/NKG2A was found to be highly expressed by CD56^{bright} NK cells (Jacobs et al., 2001, Cooper et al., 2001) and multiple genes have also been identified in cattle (Birch and Ellis, 2007, Storset et al., 2003).

In contrast, NKG2D activatory receptors derived from a single gene, function in the recognition of stress related molecules encoded by MHC class I (MIC) related genes (Lanier, 2005). NKG2D receptors are widely expressed by NK cells within numerous species and may be highly expressed by NKp46+ cells in cattle, activation through which was shown to induce IFN γ release (Guzman et al., 2010). Roles within mycobacterial infection have also been reported with NK cell mediated lysis of *M.tuberculosis* infected alveolar macrophages via NKG2D recognition of stress induced ULBP1 ligand (Vankayalapati et al., 2005). In addition CD16 is variably expressed by human NK cells (Cooper et al., 2001) and highly expressed in cattle (Boysen et al., 2008, Boysen et al., 2006b). As a low affinity IgG receptor, CD16 functions in the recognition of opsonised molecules to induce antibody dependent cell mediated cytotoxicity responses of NK cells (Lanier, 2005).

Further activatory receptors of NK cells include NKp46, NKp30 and NKp44 belonging to the natural cytotoxicity complex. In humans, both NKp46 and NKp30 are highly expressed by NK cells whereas expression of NKp44 is augmented following activation (Pessino et al., 1998, Walzer et al., 2007a). Triggering via these receptors induces cytotoxic responses, yet the ligands are largely unknown. However, viral haemagglutinin has been shown to bind to NKp46 (Mandelboim and Porgador, 2001) with the position of binding found to be implicit in determining whether a cytolytic effector response is induced (Mandelboim et al., 2001, Achdout et al., 2010). Furthermore, NKp46 was reported to play a role in the induction of NK cell mediated lysis of *M.tuberculosis* infected monocytes (Garg et al., 2006, Vankayalapati et al., 2005, Vankayalapati et al., 2002). In addition, NKp44 expressed by CD56^{bright} NK cells was able to bind directly to BCG to induce proliferation, IFN γ production and augmented cytolytic activity (Esin et al., 2008, Batoni et al., 2005, Esin et al., 2004). Bovine NK cells have also been reported to possess the capacity to interact directly with *Neospora caninum* to produce IFN γ (Boysen et al., 2006a). This may be through recognition via natural cytotoxicity receptors or toll-like receptors which are known to be expressed by NK cells (Lauzon et al., 2006), with BCG reported to interact directly with human NK cells via TLR2 to trigger NK cell mediated cytolytic activity (Marcenaro et al., 2008).

1.2.3.4.4.2 Cytokine control

NK cells are responsive to a multitude of cytokines during both homeostatic and inflammatory immune conditions. For example IL-15 has been shown to be fundamental to the effective differentiation, maturation and maintenance of peripheral NK cells (Cooper et al., 2002, Mrozek et al., 1996). During an immune response cytokines are potent stimulants for NK cells, with the capacity to override inhibitory signals (Orr et al., 2010, Ortaldo and Young, 2006) with the cytokine milieu influencing the resulting NK cell phenotype and response. IL-12 functions to induce IFN γ release, in particular with a co-stimulus such as IL-18 (Cooper et al., 2001, Fehniger et al., 1999b). Bovine NK cells produced abundant IFN γ in response to IL-12 released following stimulation with mycobacterial antigens (Olsen et al., 2005). However, the combination of IL-12 and IL-15 has been reported to induce IL-10 and TNF α release from NK cells (Cooper et al., 2001, Fehniger et al., 1999b) and augment the cytolytic activity of bovine NK cells (Endsley et al., 2006).

Several reports suggest that subsets of NK cells may respond differentially to cytokine stimulation with CD56^{bright} NK cells found to produce abundant levels of IFN γ following IL-12 and IL-18 stimulation (Fehniger et al., 1999b). In a similar manner, IL-2 augmented the proliferative, cytolytic and IFN γ responses of both human CD56^{bright} NK cells (Ferlazzo et al., 2004b, Fehniger et al., 2003) and CD2- NK cells in cattle (Boysen et al., 2006b). This may be particularly relevant within lymph nodes where abundant T-cell derived IL-2 may contribute to the presence of resident populations of activated and cytolytic NK cells (Boysen et al., 2008).

1.3 Aims and Objectives

The innate immune system is important in the early control of mycobacterial infection but has also been demonstrated to play a prominent role in the shaping of adaptive immunity. Th1 biased cell mediated immune responses are required for protective immunity during TB infection and early innate production of IFN γ is implicit within this. NK cells function as cytolytic effectors but are also a significant source of immunomodulatory cytokines such as IFN γ . Human and murine studies have demonstrated the prevalence of NK cells within tissues afflicted by mycobacterial infection. However, fully functional effector responses of NK cells are reliant upon signals from accessory cells. In particular, DC derived chemokines function to recruit NK cells to within a close proximity enabling interactions to ensue. Importantly, the interactions are reciprocal in nature influencing the effector functions of NK cells and the maturation status of DCs to promote subsequent Th1 polarisation.

Bovine NK cells exist as distinct heterogeneous subsets with differential functions and anatomical localisations. Given that within human and murine studies, the interactions between NK cells and DCs have been shown to substantially influence the immune responses during mycobacterial infection; this thesis sought to explore these interactions further within the bovine system. In particular, whether functionally distinct subsets of bovine NK cells played differing roles in *M.bovis* infection.

This thesis therefore had four main aims; (1) to comprehensively analyse the patterns of chemokine receptor expression with a view that this would provide insight into the migratory behaviour of NK cells, (2) to determine the capacity of *M.bovis* infected DCs to recruit and (3) reciprocally interact with subsets of bovine NK cells. Finally (4), to determine whether models of mycobacterial infection and vaccination could be utilised to demonstrate the *in vivo* responsiveness of bovine NK cells.

2.1 Media and Suppliers

Media used is specified in (9.1) and all suppliers of reagents and equipment in (9.9)

2.2 Animals

Experiments were carried out on British Holstein-Friesian calves housed at the Institute for Animal Health (IAH), Compton according to Home Office guidelines and with full ethical approval. The herd at the IAH has been certified free of bovine TB for over 10 years. The majority of animals were below 6 months of age and calves were age-matched within experimental groups. For isolation of blood derived leukocytes, blood was collected by jugular venepuncture into sodium heparin (10u/ml).

2.2.1 Subcutaneous BCG vaccination

In vaccination experiments, calves received 1×10^6 cfu BCG Pasteur (2.7.1) in 2ml phosphate buffer saline (PBS) delivered subcutaneously into the left shoulder.

For BCG time course experiments, blood was sampled at regular time points up until 12 weeks post-vaccination and NK cell number and phenotype were assessed as described (2.12.1). Whole blood stimulation was employed to confirm the antigen specific response to BCG vaccination at week 7 post-vaccination; 5ml of blood was either left unstimulated or supplemented with purified protein derivative from *M.bovis* (PPD-b) 10µg/ml and incubated overnight at 37°C with 5% CO₂. Following stimulation, cells were pelleted and the collected supernatant was assessed for IFNγ levels by ELISA (2.8)

To assess recruitment of NK cells *in vivo*, BCG Pasteur was administered subcutaneously as described above and prescapular lymph nodes (PSLN) were obtained at post-mortem at days 1, 3, 7 and 14 post-vaccination for experimental analysis of NK cell number and phenotype (2.12) within the draining LN. PSLN from the right shoulder served as a control.

2.2.2 Intranasal BCG inoculation

For intranasal inoculation, age matched calves were challenged with 1.5×10^7 cfu (Price et al., 2010) of BCG Pasteur suspended in 2ml PBS through a catheter inserted into the left nostril. Age matched naive calves were used as controls. After 7 days the head associated lymph nodes, lung tissue, lung associated lymph nodes and peripheral lymph nodes as specified in Table 2.1 were recovered at post-mortem.

	Tissue	Location
RPLN R	Retropharyngeal right lymph node	Head associated lymph nodes
RPLN L	Retropharyngeal left lymph node	
Pall R	Pallatine right tonsil	
Pall L	Pallatine left tonsil	
Ph Ton	Pharyngeal tonsil	
Apical R	Apical right lung	Lung tissue
Apical L	Apical left lung	
Int	Intermediate lung	
Medial R	Medial cardiac right lung	
Medial L	Medial cardiac left lung	
Diaph R	Diaphragmatic right lung	
Diaph L	Diaphragmatic left lung	
CLN	Cervical lymph node	Lung associated lymph nodes
BLN	Bronchus associated lymph node	
MLN	Mediastinal lymph node	
PS R	Prescapular right lymph node	Peripheral lymph nodes
PS L	Prescapular left lymph node	

Table 2.1 Tissues collected at post-mortem

2.2.3 *M.bovis* infection

Samples were obtained from *M.bovis* infected cattle which were a part of a larger duration of BCG induced immunity trial (*unpublished*) conducted by the Institute for Animal Health in collaboration with the Veterinary Laboratories Agency. Within this experiment, cattle were challenged at 24 months of age with 1.9×10^2 cfu *M.bovis* strain AF 2122/97 delivered via bronchoscope to the bifurcation of the trachea to

mimic the natural route of infection (Vordermeier et al., 2002). For evaluation of NK cells, blood was received at days 2, 5, 7, 14 and 21 days post-infection in line with the samples which were taken as a part of the trial. All handling and experimentation with samples from *M.bovis* infected animals was conducted at ACDP containment level III. Animals were slaughtered at 12 weeks post-infection followed by post-mortem examination to determine the number and size of lesions in the head and respiratory tract as an indicator of the severity of infection (Vordermeier et al., 2002). Pathology scores are specified in appendices table (9.8).

2.3 Isolation of cells

2.3.1 Peripheral blood leukocytes (PBLs)

Erythrocytes from bovine blood were lysed using 0.1M Ammonium Chloride lysis buffer (9.1) followed by three washes (first wash at 400g for 10 minutes at 4°C followed by 300g for 8 minutes at 4°C and all subsequent washes at 400g for 5 minutes at 4°C) with PBS to obtain a leukocyte population. Viable cell numbers were established using a haemocytometer and 0.1% Trypan Blue exclusion. The PBL population was resuspended to approximately 1×10^8 cells/ml in tissue culture medium (TCM) (9.1) prior to further experimentation.

2.3.2 Peripheral blood mononuclear cells (PBMCs)

15ml of heparinised blood was diluted with 15ml PBS, underlayered with 15ml Histopaque 1083 followed by centrifugation at 1200g, 30-40 minutes, 20°C without brake, to obtain a buffy coat. Peripheral blood mononuclear cells (PBMCs) were retrieved from the interface, washed three times with PBS and viable cells assessed as described (2.3.1). The PBMC population was resuspended to approximately 1×10^8 cells/ml in TCM.

2.3.3 Mononuclear cells (MNCs) from tissues and lymph nodes

Tissue samples from the head and respiratory tract were cut into small pieces and completely homogenised into PBS using a pestle and mortar. Lymph nodes were sliced in half and scored to expose the inner regions of the tissue. Using a blunt scalpel, cells

were gently teased away from the capsule and into PBS. The resulting cell suspensions were filtered through 40µm cell strainers and washed once with PBS. Cells were then diluted to approximately 2×10^7 cells/ml in PBS and placed over a Histopaque density gradient (2.3.2). Live cells were removed from the interface, washed three times and resuspended to approximately $1-2 \times 10^8$ cells/ml in PBS prior to further analysis.

2.4 Isolation of bovine NK cells

It has previously been established that NKp46 (CD335) is a highly restricted NK cell marker and can be used to accurately identify and isolate bovine NK cells (Storset et al., 2004). Mononuclear cells (MNCs) obtained from peripheral blood or lymph nodes as described (2.3), were resuspended in PBS at approximately $1-2 \times 10^8$ cells/ml and labelled for bovine NKp46 using mouse anti-bovine CD335 (3µg/ml). Cells were rolled gently for 10 mins followed by three washes (400g for 5 minutes at 4°C) in PBS to remove unbound antibody. Bovine NK cells were isolated using the MACS cell separation system via indirect labelling. MNCs stained for NKp46, were further incubated for 10 minutes with Rat Anti-Mouse IgG1 MicroBeads at $2.5 \mu\text{l}/10^7$ cells, washed once and resuspended in approximately 5ml solution of FACSFlow/Bovine Serum Albumin (FF/BSA, 0.5%). NK cells were positively selected by passing the labelled MNCs through MACS LS columns and were eluted into TCM. Viable cell numbers were established by trypan blue exclusion.

Purity of the MACS sorted NK cell population was assessed by staining the positively selected cells using goat anti-mouse IgG-RPE (9.4) and analysing the percentage of positively stained cells by flow cytometry. Only cell populations with over 95% purity were selected for further experimentation (3.2.1)

2.4.1 Bovine NK cell subsets

Blood or lymph node derived NKp46+ cells isolated by MACS sorting, were further labelled for CD2 to enable the isolation of NK cell subsets. NKp46+ cells were incubated with the CD2 specific monoclonal antibody IL-A43 (Howard and Naessens, 1993) which was transiently coupled to IgG2a-FITC (Zenon® labelling kit) prior to labelling. Cells were rolled gently for 10 minutes followed by two washes in FF/BSA

(0.5%). Labelled cells were filtered and purified using the FACS Aria™ to obtain NKp46+ CD2+ and NKp46+ CD2- subsets. Following sorting cells were washed 4-5 times in TCM to remove contaminating sheath fluid. This resulted in NK cell subset populations with over 98% purity which were resuspended in TCM at 2×10^6 cells/ml.

2.5 NK cell stimulation

Previous work within the group has established that bovine NK cells are optimally stimulated by IL-12 + IL-18 (M. Thom, unpublished data). Within experiments utilising stimulated cells, NKp46+ cells were cultured with COS cell derived recombinant bovine (rbo) IL-12 (20U/ml) (Hope et al., 2002a) and recombinant human (rhu) IL-18 (20ng/ml) in 24-well tissue culture plates at 1×10^6 cells/ml in TCM for 24 hours at 37°C and 5% CO₂.

2.6 Isolation and culture of monocyte derived dendritic cells (DC)

CD14+ monocytes were isolated using the MACS cell separation system. PBMCs were labelled with anti-human CD14 coupled magnetic beads at $2.5 \mu\text{l}/10^7$ cells, incubated for 10 minutes followed by positive selection using MACS LS columns. Monocytes isolated were washed twice in PBS (400g for 5 minutes at 4°C) and viable cell number was determined by trypan blue exclusion. Cells were cultured at 37°C + 5% CO₂ in media supplemented with COS cell derived recombinant bovine GM-CSF (0.2u/ml) and IL-4 (200u/ml) (9.1) for 3 days to obtain immature DCs (Sallusto and Lanzavecchia, 1994, Werling et al., 1999).

2.7 *In vitro* mycobacterial infection

2.7.1 Mycobacterial cultures

All bacterial cultures were handled at appropriate bio-security levels; ACDP containment level II (BCG) and level III (*M.bovis*). BCG Pasteur (P3) and *M.bovis* AF2122 (P3) cultures were obtained by inoculating 1ml bacteria (1×10^6 to 1×10^7 cfu) into 9ml 7H9 medium (9.1) which was cultured for one week at 37°C with shaking at 100rpm. This was further bulked up to 100ml in 7H9 medium and grown for an additional 3-4

days at 37°C, 100rpm. After sufficient growth was visually observed, mycobacterial samples were aliquoted and stored at -80°C.

Bacterial titre was determined after a single freeze-thaw cycle using the droplet enumeration technique where bacteria were serially diluted (10-fold) in 7H9 medium (9.1). 50µl droplets of bacteria were spotted onto 7H10 (BCG) or 7H11 (*M.bovis*) agar plates (9.1) in triplicate and incubated at 37°C for 3-4 weeks. After growth was observed and seen not to be increasing, colonies were counted to determine the titre of the bacterial culture.

2.7.2 Infecting DCs and macrophages

Antigen presenting cells (APCs), cultured as described (2.6) were removed from plates by incubating adherent cells with 1ml cell dissociation fluid at 37°C for 30 minutes. Cells were washed in PBS and resuspended to 1×10^6 cells/ml in TCM without antibiotics, prior to infection with either *M.bovis* at a multiplicity of infection (MOI) of 1:1 or BCG Pasteur at a MOI of 10:1. It has previously been established that these ratios give an equivalent rate of infection as determined by infecting DCs with GFP labelled bacteria (Thom, 2005). Cells were infected overnight at 37°C + 5% CO₂.

2.8 Enzyme Linked Immunosorbent Assay (ELISA)

ELISAs were conducted to detect the presence of bovine IFN γ , IL-12 (Hope et al., 2002a), IL-10 (Kwong et al., 2002), TNF α (Kwong et al., 2010), IL-6 and IL-2. Reagents used are specified in (Table 2.2). Supernatants from various experiments were retrieved and stored at 4°C prior to analysis. 96-well transparent plates (IFN γ , IL-6, IL-2) or black plates (TNF α , IL-12, IL-10) were coated with the monoclonal capture antibody diluted in carbonate/bicarbonate buffer at previously established optimal concentrations. Plates were sealed and incubated overnight at room temperature. Non-specific binding of the antibody was blocked using Casein at 1mg/ml in PBS for 1 hour at room temperature and plates were washed with PBS/Tween-20 (0.05%) after every incubation step. Following washing, supernatants (100µl) and recombinant cytokine standards (dilution series) were added in triplicate and left for 1 hour. Plates were washed again, and incubated with the biotinylated secondary antibody for a

further hour. After washing, Streptavidin Horseradish peroxidase; 1:500 with Tween-20 (0.05%) was added to the wells and left for 45 minutes. For IFN γ , IL-6 and IL-2 assays, plates were developed using 0.1mg/ml 3,3'-5,5' tetramethylbenzidine (TMB) diluted in sodium acetate buffer (pH 6.0). The reaction was stopped using 50 μ l/well of 1M Sulphuric Acid after the appearance of a blue colour in the wells containing the standards. For all other luminescent assays, plates were washed three times with 5 minute soaks in PBS/Tween-20 and were developed using supersignal ELISA Femto Maximum Sensitivity luminescent substrate at 100 μ l per well. Absorbance was measured at 450nm subtracted from the non specific absorbance at 690nm using the FLUOstar Optima Luminometer. Concentrations of cytokines within supernatants were extrapolated after plotting a curve from the standards. Where precise concentrations could not be calculated (IL-12 and IL-10), protein levels were expressed as biological units (bu) of activity as has previously been described (Hope et al., 2002a, Kwong et al., 2002).

	Capture monoclonal antibody	Secondary biotinylated antibody	Cytokine standards
IFNγ	Mouse anti-bovine CC330 (IgG1)	Mouse anti-bovine CC302 (IgG1)	COS cell derived recombinant bovine IFN γ (IAH, Compton)
TNFα	Mouse anti-bovine CC327 (IgG2b)	Mouse anti-bovine CC328 (IgG2a)	COS cell derived recombinant bovine TNF α (IAH, Compton)
IL-10	Mouse anti-bovine CC318 (IgG2b)	Mouse anti-bovine CC320 (IgG1)	CHO cell derived recombinant bovine IL-10 (Moredun Research Institute)
IL-12	Mouse anti-bovine CC301 (IgG2a)	Mouse anti-bovine CC326 (IgG2b)	COS cell derived recombinant bovine IL-12 (IAH, Compton)
IL-6	Mouse anti-bovine CC310 (IgG2b)	Anti-ovine IL-6 polyclonal rabbit serum, sheep anti-rabbit IgG biotin	COS cell derived recombinant bovine IL-6 (IAH, Compton)
IL-2	Bovine IL-2 ELISA detection kit		(R&D systems)

Table 2.2 ELISA reagents

2.9 Chemotaxis Assays

Chemotaxis assays were carried out using Costar tissue culture treated transwells (5 µm pore size, 6.5 mm membrane diameter) in duplicate or triplicate. Input migrating cells (2.9.1) were placed on the transwell membrane at 2×10^5 cells per well suspended above the chemotactic agent. Plates were incubated at 37°C + 5% CO₂ for 4 hours. Recombinant human CXCL12 (200ng/ml) was used as a positive control (3.2.6). Following incubation, the migrated and input cells were diluted; 80µl of input cells were diluted in 520µl PBS to match the 600µl migrated cell suspension. Cells were counted for one minute using a FACSCalibur flow cytometer. Live cells were gated and identified by their FSC and SSC properties and migration was expressed as a percentage of the input population.

2.9.1 Input cells

Migration of PBLs or PBMCs was tested in response to recombinant chemokines (Table 3.3). Prior to testing within chemotaxis assays, cells isolated as described were stimulated for 24 hours with Concanavlin A (ConA) at 5µg/ml and cultured at 2×10^6 cells/ml in TCM for 3 days at 37°C + 5% CO₂. Migrated populations of granulocytes, monocytes and lymphocytes were identified by their FSC and SSC properties.

For all other migration experiments, input cells were NKp46+ cells, NKp46+ CD2+ or NKp46+ CD2- subsets isolated as described (2.4.1). All NK cell populations were resuspended in TCM at 2×10^6 cells/ml and left to rest overnight before use in chemotaxis assays to prevent interference from MACS beads bound to the cell. Migrated cell numbers were analysed and cell populations were also assessed for expression of NK cell surface receptors where replicate wells were pooled and labelled as described (2.12.1). Cell death within migrated populations was determined by labelling with Propidium Iodide (PI - 25µg/ml) and analysis by flow cytometry.

2.9.2 Attractants

Recombinant human and murine chemokines were used in chemotaxis assays to establish cross-reactivity (Table 3.3) at concentrations ranging from 50ng/ml to 1µg/ml diluted in TCM. A positive result was a significant increase in cell migration above

background. Of those chemokines found to cross react; dilution curves were used to establish optimal concentrations for cell migration of purified NK cells.

Supernatants from *M.bovis* infected DCs (2.7.2) were also used within chemotaxis assays. DCs were cultured and infected as described and supernatants retrieved and filtered (0.1µm) before being used at several dilutions as chemoattractants.

Within co-cultured chemotaxis experiments, DCs were cultured and infected with either BCG or *M.bovis* (2.7.2) within 24 well Costar chemotaxis plates. After overnight infection, purified NK cells or NK cell subsets were assessed for migratory responses towards infected antigen presenting cells left *in situ*. Cells within wells containing bacteria, were fixed with 2% paraformaldehyde (PFA) prior to analysis.

2.10 Co-culture of NK cells with antigen presenting cells

Monocyte derived DCs cultured as described (2.6), were diluted to 4×10^5 cells/ml in TCM without antibiotics and placed in 96 'U' well plates at 50µl cells per well. Cells were infected with either BCG or *M.bovis* at 10:1 or 1:1 respectively (2.7.2). Total NK cells or NK cell subsets were diluted to 2×10^6 cells/ml and added to the co-culture at 100µl per well. Each well was made up to a 200µl volume with TCM to give a final NK:DC ratio of 5:1 which was previously determined to result in optimal activation of NK cells.

For blocking experiments, the mAb CC326 was also supplemented into the co-culture at concentrations determined to be optimal (10µg/ml) to diminish the IL-12 produced (5.2.2.2). Within stimulated co-cultures, rbo IL-12 and rhu IL-18 were added into the co-culture at concentrations known to be optimal for NK cell activation (2.5). Each culture condition was replicated in triplicate and cells were incubated for approximately 18 hours (overnight) at 37°C and 5% CO₂. After an overnight co-culture period, cells were pelleted and supernatants retrieved for analysis of cytokine production by ELISA (2.8). Triplicate wells were analysed independently.

To determine phenotypical changes after co-culture; expression levels of CD16, CD27, CD8, CD62L, CD25 and Perforin by NK cells and MHC class II, CD40, CD80 and CD86 by DCs (2.12.1) were analysed by flow cytometry.

In contact dependent assays, co-cultures were set up in 96 well flat bottomed plates and NK cells were separated from infected antigen presenting cells using a transwell insert (0.2µm).

2.11 Killing Assays

Murine tumour (P815) cells are known *in vitro* target for NK cells (Boysen et al., 2006b) and were used to assess NK cell killing activity. P815 cells were cultured in T75 tissue culture flasks in TCM at 37°C for approximately 1 week and were sub-cultured every 48 hours and also one day prior to use. For killing assays, the P815 target cells were washed and labelled with Vybrant® CFDA-SE cell tracer kit (1µm final) for ease of identification. Effector cells used included IL-12 and IL-18 stimulated NK cells (2.5) or NK cells from co-cultures (2.10). Stimulated NK cells were cultured with the labelled target cells in triplicates either at a range of effector target ratios or at 12.5:1 which was determined as optimal to assess killing activity of NK cells from co-cultures (5.2.2.2). Cells were incubated for 3 hours at 37°C and 5% CO₂ and NK cell specific killing was analysed by labelling with PI (25µg/ml) followed by analysis of the proportions of CFDA-SE and PI positive populations by flow cytometry.

2.12 Multi-colour immunofluorescent staining

All antibodies and fluorochromes are specified in table (9.2) and were used at previously determined optimal concentrations. Where possible, single stained cell populations, isotype controls (IC) or unstained cells were used as compensation and negative controls. Each 10 minute incubation step was followed by two washes (100µl/well) with PBS/BSA/Azide (9.1). Blocking steps were performed with 2% Normal Mouse Serum (NMS).

For intracellular staining of perforin (Hogg et al., 2009) and cytokines, cells were pre-incubated with TCM supplemented with Brefeldin A (BFA) 10µg/ml and left overnight at 37°C with 5% CO₂ prior to further labelling. Fixation was performed using 50µl 2% PFA and permeabilisation with 50µl BD FACSPerm solution (1X). Labelled cells were analysed by flow cytometry using the BD FACSCalibur.

2.12.1 Staining protocols

2.12.1.1 Intracellular labelling of PBMCs

	Step 1	Step 2	Step 3	Step 4
1	Unstained			
2	NKp46 (CD335-FITC) + CD2 (CC42-647)	PFA		
3	IgG1-FITC + IgG1-647 (IC)			
4	NKp46 (CD335-FITC) + CD2 (CC42-647)			
5			Permeabilise	IFN γ (CC330-PE)
6				Perforin-PE
				IgG2b-PE (IC)

2.12.1.2 NK cell phenotyping

	Step 1	Step 2	Step 3	Step 4	Step 5	Step 6
1	NMS	-	-	-	-	PFA
2			IgG-RPE	NMS	NKp46 (CD335-FITC) + CD2 (CC42-647)	
3		CD3 (MM1A)				
4		CD4 (CC30)				
5		CD8αα (CC63)				
6		CD8αβ (CC58)				
7		γδ TCR (GB21A)				
8		WC1 (CC39)				
9		CD16 (KD1)				
10		CD27 (MT-271)				
11		CD14 (CCG33)				
12		CD25 (IL-AIII)				
13		MHC II (CC158)				
14		CD11b (CC94)				
15		CD21 (CC21)				
16		CD62L (CC32)				
17		SIRPα (CC149)				
18		AV20 (IC)				
19		AV29 (IC)				
20		AV37 (IC)				

2.12.1.3 Labelling NK cells for CD25 and intracellular Perforin

	Step 1	Step 2	Step 3	Step 4	Step 5
1	NMS		NMS	NKp46 (CD335-PE) + CD2 (CC42-647)	PFA
2		CD25 (CACT108A) - FITC*			
3		AV37(IC) - FITC*			

**Purified mAbs CACT108A (CD25) and AV37 (IC) were transiently coupled to IgG2a Alexa Fluor - 488 using a Zenon® labelling kit prior to use.*

	Step 1	Step 2	Step 3	Step 4	Step 5
1	NMS	NKp46 (CD335-PE) + CD2 (CC42-647)	PFA		
2				Permeabilise	Perforin-FITC
3					IgG2b-FITC (IC)

2.12.1.4 DC phenotype

	Step 1	Step 2	Step 3	Step 4	Step 5
1	-	-	NMS	SIRPα (CD172a 647/PE)	PFA
2	MHC II (CC158)	IgG2a-FITC			
3	AV37 (IC)				
4	CD40 (IL-A156)	IgG1-FITC			
5	CD80 (N32/52-3)				
6	CD86 (IL-A190)				
7	AV20 (IC)				

**All wash steps were performed with ice cold PBS/BSA/Azide to promote the release of adherent cells.*

2.13 Isolation of RNA and synthesis of cDNA

NK cells (2.4) or DCs (2.6); isolated, cultured and infected (2.7.2) by the methods described were lysed and stored at -80°C in 1ml GTC buffer (9.1) with 2-mercaptoethanol (0.1M) prior to RNA extraction.

Lysed cells were thawed and homogenised using Qia shredders and RNA was extracted using RNeasy mini or micro kits according to the manufacturer's instructions. RNA samples were eluted into nuclease free water and DNase treated (DNAfree kit) to remove any genomic DNA contaminants. The quality and concentration of the RNA was assessed using the NanoDrop 1000 spectrophotometer. When necessary, RNA was further cleaned using RNeasy mini columns and re-eluted into RNase free water and stored at -80°C.

100ng RNA was reverse transcribed using the SuperScript® III Reverse Transcriptase system and concentrations were analysed using the NanoDrop spectrophotometer. The cDNA was then further diluted to 50ng/μl with nuclease free water prior to further analysis.

2.14 Identification and cloning of chemokine receptors and toll like receptors

As a collaborative effort with the bovine genomics research group at the Institute for Animal Health, the bovine chemokine receptors and toll like receptors were identified, cloned and sequenced. Briefly, primers were designed to span full length regions of the coding sequence and PCR amplified from a variety of cDNA sources using GoTaq DNA polymerase and the Expand High Fidelity^{plus} PCR system. PCR products were cleaned using the Qiaquick PCR purification kit and cloned into TA vectors; pCR2.1-TOPO, pGEM-T Easy or pDrive. Plasmid DNA was generated by transforming competent E-coli and purification using the Qiaprep spin miniprep kit. Inserted sequences were confirmed as described (Widdison et al., 2010).

2.15 Quantitative (TaqMan) polymerase chain reaction (PCR)

Primers and probes were designed based on already established sequences or those predicted from the bovine genome (Sequences - 9.5), designed to span at least one

intron-exon boundary. Probes were labelled with the FAM (6-carboxyfluorescein) dye at the 5' end and the quencher dye TAMRA (6-carboxytetramethyl-rhodamine) at the 3' end. Quantitative PCR analysis was carried out using TaqMan FAST Universal PCR Master Mix on an ABI Prism 7500 Fast Real-Time PCR System. Each sample was tested in triplicate using 100ng cDNA. All results were quantified against a standard curve created through use of known copy number plasmid DNA containing the gene of interest. Data was normalised to the housekeeping gene BoPo (Bovine homologue of the large fragment of RNA polymerase) to establish the efficiency of conversion of RNA to cDNA.

In addition quantitative PCR assays for the detection of Ly49 and NKp30 were designed as described (2.15) based on published sequences and are specified in section 9.5. Plasmid DNA standards were provided as a gift from Melanie Dobromylskyj, IAH (Ly49) and Tim Connelly, Edinburgh (NKp30).

2.16 Data Analysis

Data analysis was carried out using the software programs; Microsoft Excel 2007, FCS Express 3, Primer3 v0.40, 7500 Fast System SDS Software, Sequencher™ v4.14. Statistical analysis was carried out using Minitab v16 or GraphPad Prism v5. Data was transformed appropriately prior to assessment for normality and variance and statistical methods employed included paired T-tests, 1-Way ANOVA and general linear models (GLM), where appropriate with Tukey post-hoc comparisons at 95% confidence intervals.

3 The chemokine receptor repertoire of bovine NK cells

3.1 Introduction

The discovery that NK cells and their subsets reside in many different compartments has given rise to the theory that complex trafficking mechanisms may be involved both under steady state conditions as well as during an inflammatory immune response. NK cells express receptors for the lipids lysophosphatidic acid (LPA) (Jin et al., 2003) and sphingosine-1-phosphate (Kveberg et al., 2002) both of which have been implicated in the induction of migratory responses of NK cells. In particular, binding to sphingosine-1-phosphate enables egress from bone marrow and lymph nodes and deficiency within this system resulted in aberrant trafficking of NK cells (Walzer et al., 2007b). Furthermore, NK cells migrate in response to Chemerin binding to ChemR23 receptors (Parolini et al., 2007) as well as IL-15 (Allavena et al., 1997). However, it is believed that chemokine signalling is fundamental to the retention of NK cells within tissues to form resident populations as well as the effective recruitment of NK cells to the site of infection and it is the repertoire of chemokine receptors that confers migratory ability.

Under steady-state conditions, the homeostatic control of NK cell migration is influenced by a number of chemokine receptors. Immature NK cells express high levels of CXCR4 contributing to retention within the bone marrow (Bernardini et al., 2008, Luther et al., 2002) and CX3CR1 enables adhesion of NK cells to endothelial cells bearing the CX3CL1 ligand (Barlic et al., 2003). In addition, functionally distinct populations of NK cells are differentially distributed as a consequence of their chemokine receptor repertoire. For example, murine NK cells with a memory phenotype expressed CXCR6 and were selectively located within the liver (Paust et al., 2010) and a discrete sub-population of IL-22 producing NK cells expressing CCR6 were identified within mucosal associated lymphoid tissue (Cella et al., 2009).

Chemokine receptor expression patterns also vary substantially between subsets of human NK cells resulting in altered migratory responses with implications for selective recruitment and tissue localisation (Robertson, 2002). Most significantly, the lymphoid homing receptor CCR7 is highly expressed by CD56^{bright} CD16⁻ NK cells (Berahovich et al., 2006) conferring responsiveness to high endothelial venule (HEV) cell derived

CCL21 and stromal cell derived CCL19 (Luther et al., 2002, Campbell et al., 2001, Kim et al., 1999, Martin-Fontecha et al., 2008) such that this population may selectively migrate towards and be retained within secondary lymphoid tissue (Fehniger et al., 2003). Furthermore, CD56^{bright} CD16⁻ NK cells express high levels of CCR5 and CXCR3 and are able to migrate in response to the ligands CCL4, CCL5, CCL7, CXCL10 and CXCL11 (Berahovich et al., 2006, Campbell et al., 2001, Taub et al., 1995). In comparison the CD56^{dim} CD16⁺ subset preferentially express CCR4, CCR9, CXCR1 and CX3CR1 and migrate in response to CXCL8 and CX3CL1 (Berahovich et al., 2006, Campbell et al., 2001).

Phenotypically and functionally heterogeneous subsets of NK cells, defined by expression of NKp46 and CD2 (Storset et al., 2004, Boysen et al., 2006b) have also been identified in cattle. As is the case in humans (Sundstrom et al., 2007) percentages of NK cells in the periphery are highest within neonates with a rapid decline with increasing age (Graham et al., 2009, Kulberg et al., 2004). This may be as a consequence of re-distribution to tissues with bovine NK cells found as resident populations in numerous compartments. In particular, large numbers are located within the lungs, spleen, liver and lymph nodes as well as a constituent of the circulating lymphocyte population (Boysen et al., 2008, Storset et al., 2004). Importantly, the patterns of NK cell subset distribution vary between the different compartments such that CD2⁺ NK cells are predominantly localised within peripheral blood whereas CD2⁻ NK cells constitute the major NK cell population within lymph nodes. It has been proposed that this may be as a result of selective expansion of CD2⁻ NK cells within lymph nodes (Boysen et al., 2008, Boysen et al., 2006b) however; the chemokine receptor expression patterns or migratory characteristics of bovine NK cells may also account for these observed differences, but have previously not been investigated.

In recent years, there have been significant advances in the discovery of bovine chemokines and their corresponding receptors. Genomic analysis identified bovine homologues for all known human chemokine receptors with varying similarities (on average between 60-90%) to their human counterparts (Widdison et al., 2010, Blumberman et al., 2007). For example, both bovine CCR7 and CXCR3 are over 80%

homologous at nucleotide and amino acid level to their corresponding human receptors (Blumerman et al., 2007) and cross reactivity of human and murine chemokines has been reported in the induction of bovine lymphocyte migration (Wilson et al., 2002).

Initial investigations therefore sought to confirm the reported phenotypic characteristics of bovine NK cells and to further extend these studies by defining the chemokine receptor expression patterns of NK cell subsets derived from peripheral blood and prescapular lymph nodes as an insight into their potential migratory characteristics.

3.2 Results

3.2.1 Identifying and isolating bovine NK cells and subsets

Expression of the natural cytotoxicity receptor NKp46 is highly restricted to NK cells and lymphocytes from cattle which express this receptor display NK-like characteristics. NKp46 has therefore been shown as an accurate marker by which bovine NK cells can be identified and isolated (Storset et al., 2004). Blood or lymph node derived MNCs were labelled with the mAb mouse anti-bovine CD335-FITC, identifying a small population (1-5%) of NKp46+ lymphocytes (Figure 3.1.2). As previously observed, the percentages of NK cells in peripheral blood varied substantially; in particular with the age of the animal (Graham et al., 2009) however, the intensity of NKp46 expression within animals remained stable over time.

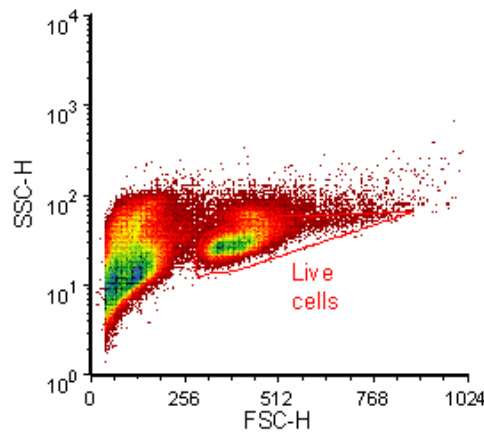
Total NK cells were isolated by positively selecting NKp46 labelled cells with IgG1 coated magnetic beads (2.4) to purities >95% (Figure 3.2.1). Whilst negative selection was investigated as an option to isolate bovine NK cells, a variety of depletion techniques did not result in adequate enough purities to continue with this method (data not shown).

NKp46+ NK cells can be divided into phenotypically and functionally distinct subsets based on expression of CD2 (Boysen et al., 2006b). MNCs were further labelled using mAb mouse anti-bovine CD2 (CC42-647) to identify sub-populations of NK cells. This confirmed that blood derived NK cells formed two distinct populations with 70-80% of NK cells found to express high levels of CD2, referred to as NKp46+ CD2+ (CD2+ NK cells) whereas 20-30% of NK cells expressed low levels of CD2 (Figure 3.1.3). Whilst CD2 was not completely absent from this population, in line with the published literature this population will be referred to as NKp46+ CD2- (CD2- NK cells).

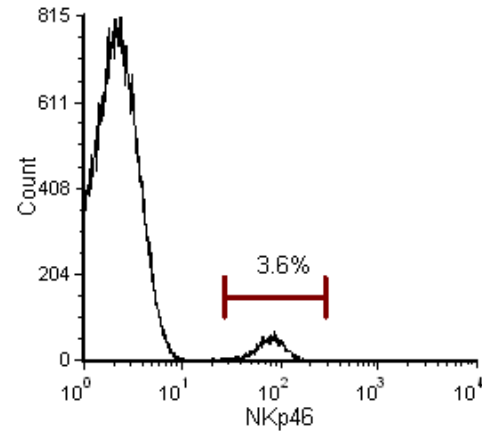
In comparison to the distinct sub-populations of NK cells found in peripheral blood, the CD2 expression profile of NK cells derived from lymph nodes was less well defined with varying degrees of expression. In this case, CD2- NK cells were the predominant population (40-60%), with a smaller population of CD2+ NK cells (Figure 3.1.4).

To purify subsets, positively selected NK cells from peripheral blood and lymph nodes were labelled for CD2 using mAb mouse anti-bovine IL-A43 transiently coupled to IgG2a-FITC (2.4.1) and analysed by flow cytometry. By gating on the relevant population (Figure 3.1.3 and 3.1.4), labelled cells could be sorted into CD2⁺ and CD2⁻ sub-populations using the FACS Aria™ cell sorter (BD) to purities >98% (Figure 3.2.2 and 3.2.3).

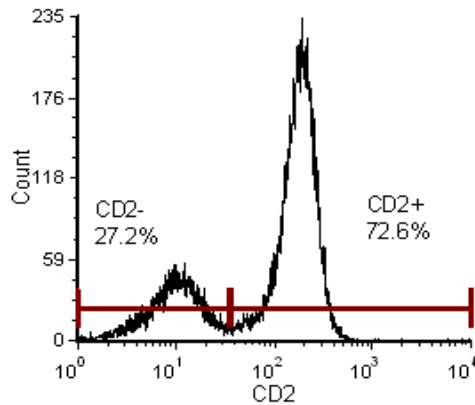
3.1.1



3.1.2



3.1.3



3.1.4

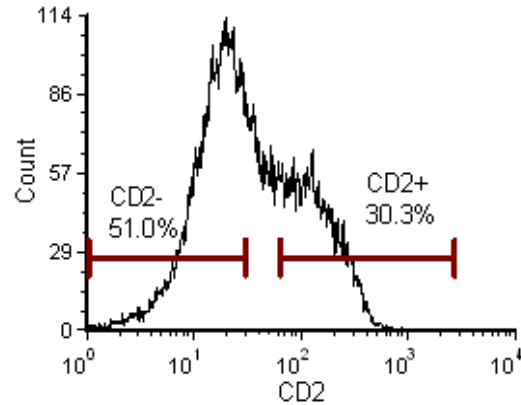
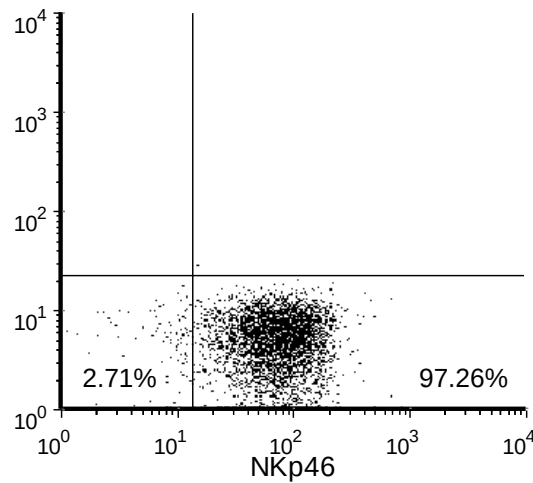


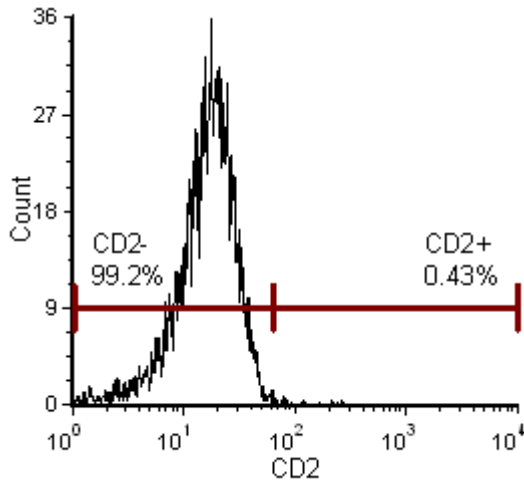
Figure 3.1 Identification of bovine NK cells

PBMCs were isolated by density gradient centrifugation and live lymphocytes identified by their FSC and SSC properties (Fig 3.1.1). Cells were labelled with the mAb CD335-FITC to define the NKp46+ population (Fig 3.1.2) assessed by flow cytometry. To identify bovine NK cell subsets, MNCs were further labelled with mAb CC42-647 with plots representing the CD2+ and CD2- populations of NK cells within the NKp46+ population from peripheral blood (Fig 3.1.3) or prescapular lymph nodes (Fig 3.1.4). Representative plots from 2 animals are shown.

3.2.1



3.2.2



3.2.3

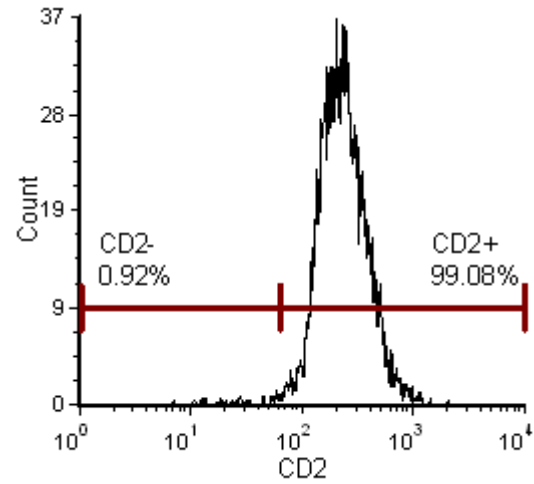


Figure 3.2 Isolating bovine NK cells

PBMCs were isolated by density gradient centrifugation and labelled for NKp46 with the mAb, mouse anti- bovine CD335 at 3 μ g/ml. Total NK cells were isolated by positive MACS selection (2.4) and cells were assessed for purity by labelling the sorted population with IgG-RPE (Fig 3.2.1) and comparing with unstained cells by flow cytometry. Populations with purities >95% were selected for further experimentation. To obtain subsets, NK cells isolated by positive MACS selection were further labelled for CD2 using the mAb mouse anti-bovine IL-A43 transiently coupled to IgG2a-FITC (Zenon[®] labelling kit). NK cells could be purified into CD2+ (Fig 3.2.2) and CD2- (Fig 3.2.3) subsets by gating on the relevant sub-populations (Fig 3.1.3 and 3.1.4) and sorting using the FACSaria[™] (BD) cell sorter to purities >98%. Representative plots from one animal are shown.

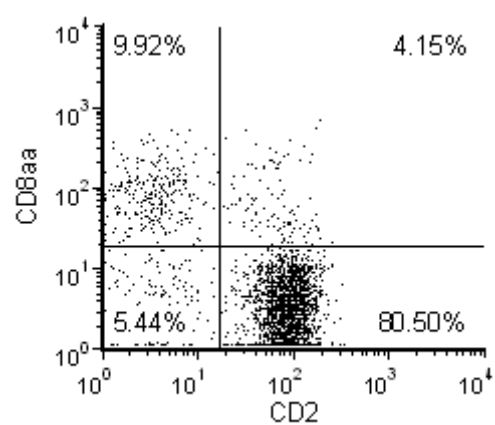
3.2.2 Phenotyping bovine NK cells

The lymphocyte population identified by expression of NKp46 and CD2 was further characterised to confirm an NK cell phenotype. PBMCs from four animals were labelled with monoclonal antibodies for T cell, B cell, monocyte and DC related surface molecules and expression within the NKp46⁺ CD2⁺ and NKp46⁺ CD2⁻ subsets was analysed.

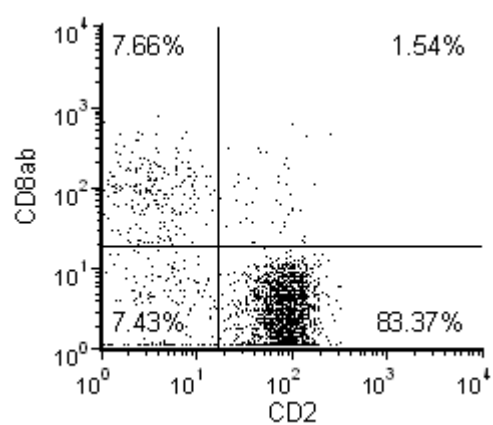
As has been previously reported (Boysen et al., 2006b, Storset et al., 2004) the vast majority of NK cells did not express the T cell specific receptors; CD3 (MM1A), CD4 (CC30), $\gamma\delta$ TCR (GB21A) or WC1 (CC39). In addition NK cells were negative for monocyte receptor; CD14 (CCG33), B cell receptor; CD21 (CC21) and further myeloid cell related molecules; SIRP α (CC149) and MHC class II (CC158) (data not shown).

However, both CD8 $\alpha\alpha$ (CC63) (Figure 3.3.1) and CD8 $\alpha\beta$ (CC58) (Figure 3.3.2) receptors were expressed by bovine NK cells and predominantly found on the NKp46⁺ CD2⁻ subset. The low affinity FC receptor; CD16 (KD1) has been reported to define subsets of human NK cells (Cooper et al., 2001) and was found to be expressed by both the CD2⁺ and CD2⁻ NK cell subsets in cattle (Figure 3.3.3). In comparison, only a small percentage of NK cells were found to express CD27 (MT-271) (Figure 3.3.4) and were largely negative for CD11b (CC94) (Figure 3.3.5); receptors which commonly differentiate subsets of human and murine NK cells (Chiossone et al., 2009, Hayakawa and Smyth, 2006).

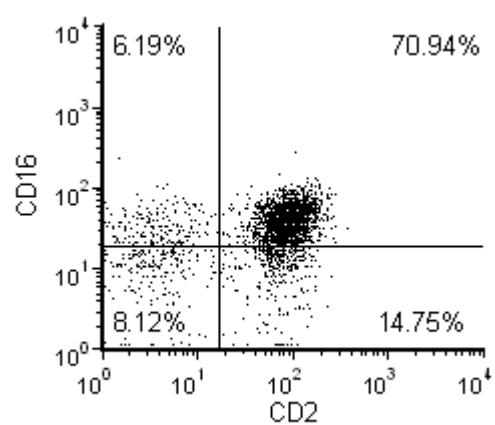
3.3.1



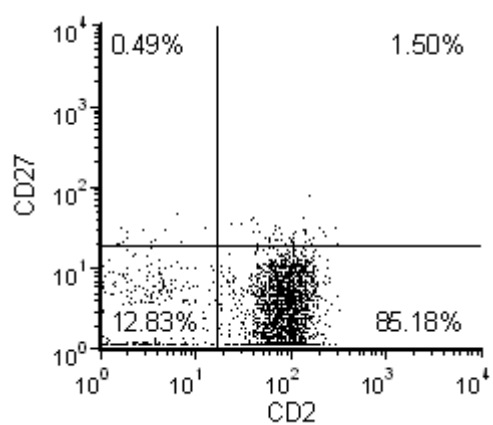
3.3.2



3.3.3



3.3.4



3.3.5

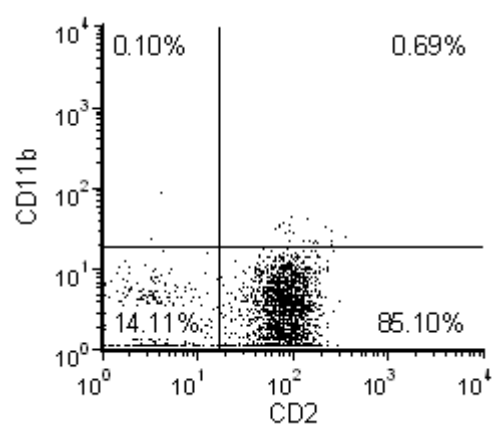


Figure 3.3 Phenotyping bovine NK cells

PBMCs were isolated by density gradient centrifugation and labelled with the mAb, mouse anti-bovine CD335-FITC (NKp46) and CC42-647 (CD2) to identify the NKp46⁺ CD2⁺ and NKp46⁺ CD2⁻ populations by flow cytometry. Cells were further labelled with mAbs for T cell, B cell, NK cell, monocyte and DC related surface molecules to phenotype NK cells (2.12.1.2). FACS plots demonstrate the expression of CD8 α α (mAb; CC63), CD8 α β (CC58), CD16 (KD1), CD27 (MT-271) and CD11b (CC94); (monoclonal antibodies are specified in section 9.2) within the NKp46⁺ population of lymphocytes. Isotype controls and cells labelled with fluorochrome alone were used as controls to set the quadrants. Representative plots from one animal are shown.

3.2.3 Designing quantitative PCR assays for chemokine receptor analysis

In collaboration with the bovine genomics group at the Institute for Animal Health, chemokine receptor genes were identified using published sequences (Blumberman et al., 2007) or those predicted from the bovine genome. Homologues to all known human chemokine receptors were identified in cattle and appeared to share a high degree of similarity with the corresponding human receptors. The genes for CCR1-10, CXCR1-CXCR6, CX3CR1 and XCR1 were cloned and sequenced and gene specific plasmid DNA standards were developed (Widdison et al., 2010).

To detect transcript levels of chemokine receptors, primers and probes were designed (9.5) using Primer3 software; in order to amplify gene specific regions between 50 and 150 base pairs in length and spanning over at least one exon boundary to prevent amplification of contaminating genomic DNA. Regions to be amplified were analysed by a basic nucleotide local alignment search tool (BLASTn) to confirm specificity and primers and probes were optimised using the ABI Prism 7500 Fast Real-Time PCR System and gene specific plasmid DNA standards (2.14).

3.2.4 The chemokine receptor repertoire of bovine NK cells

Bovine NK cells display varied tissue distribution with phenotypically distinct subsets of cells found within different anatomical locations. It is thought that the chemokine and chemokine receptor axis plays a significant role within a homeostatic context; enabling NK cells to migrate and exist within tissues, as well as during an inflammatory immune response; initiating re-distribution of NK cells to effector sites. Therefore, elucidating the chemokine receptor profiles of bovine NK cell subsets will enable insight into their potential migratory characteristics.

NK cell subsets were isolated from peripheral blood of eight animals and prescapular lymph nodes from seven animals (2.4.1). RNA was isolated and reverse transcribed into cDNA prior to quantitative PCR analysis for the bovine chemokine receptors CCR1-CCR10, CXCR1-6, CX3CR1 and XCR1 (2.15).

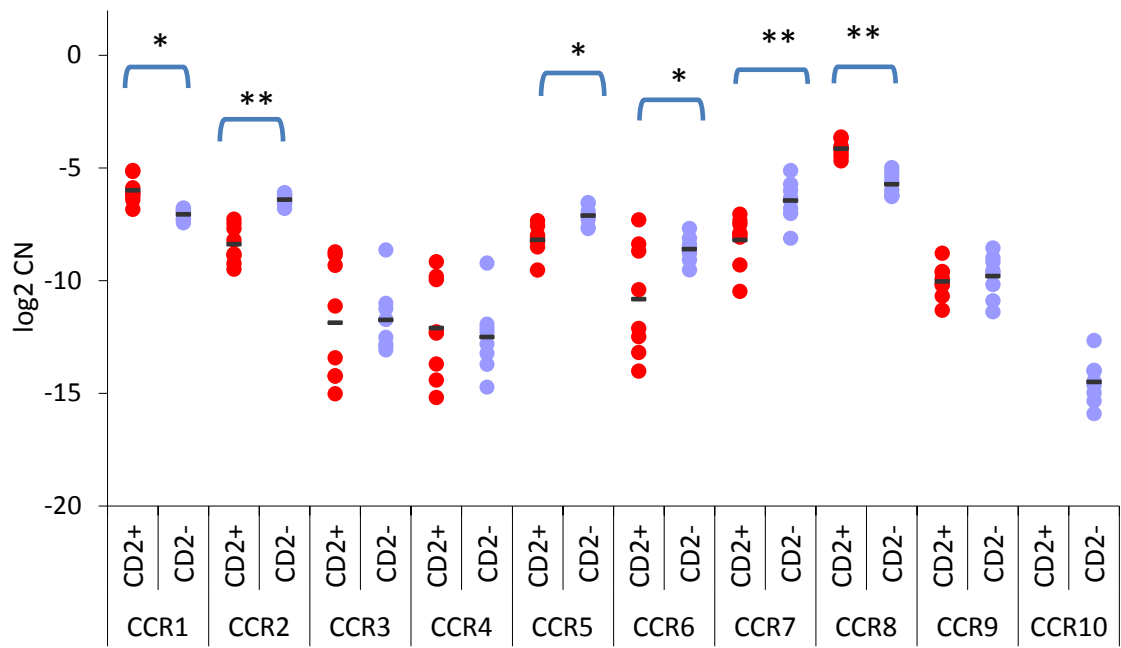
Transcripts for all of the measured chemokine receptors were detected in bovine NK cells. Within the blood derived NK cell population CCR8, CXCR6 and CX3CR1 were found to be transcribed at high levels (log2 copy number (CN) within the range of 0 to -6). CCR1, CCR2, CCR5, CCR6, CCR7, CCR9, CXCR2, CXCR3, CXCR4 and XCR1 at intermediate levels (log2 CN within the range of -6 to -12) and CCR3, CCR4, CCR10 and CXCR1 at comparatively lower levels (log2 CN below -12) (Figure 3.4). The pattern of expression was considerably different in NK cells derived from prescapular lymph nodes with the majority of receptors being expressed at high levels; CCR1, CCR2, CCR5, CCR6, CCR7, CCR8, CXCR3 CXCR4, CXCR5, CXCR6 and CX3CR1 or intermediate levels; CCR3, CCR4, CXCR1, CXCR2 and relatively few at low levels; CCR9, CCR10 and XCR1 (Figure 3.4). Despite these observations, statistical analysis conducted using a general linear model (GLM) with Tukey post-hoc comparisons (95% CI) revealed that within both peripheral blood and prescapular lymph node NK cells populations, no one receptor was transcribed at significantly higher or lower levels than any other receptor.

However, comparison between the CD2+ and CD2- subsets of NK cells, assessed using pair-wise T-Tests (95% CI), indicated significant differences in the transcript levels of a large number of chemokine receptors (Table 3.1). NKp46+ CD2- NK cells were found to transcribe significantly higher levels of CCR2, CCR5, CCR6, CCR7, CCR10, CXCR3, CXCR4 and CXCR5 when compared with their CD2+ counterparts. However, higher levels of CCR1, CCR8, CXCR6 and CX3CR1 were found on CD2+ NK cells. The differences between the subsets of NK cells were far less apparent within prescapular lymph nodes. In a similar statistical comparison, CD2+ NK cells were found to transcribe significantly higher levels of CCR1, CCR7, CCR8 and CX3CR1 when compared with the expression levels of CD2- NK cells (Table 3.1).

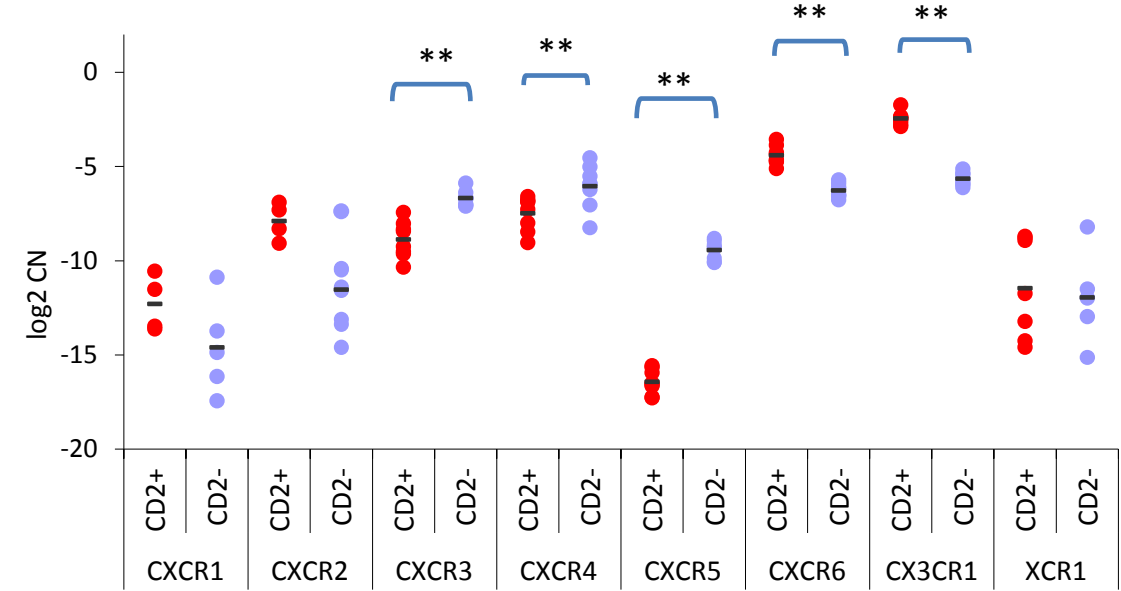
Having established that subsets of NK cells expressed unique chemokine receptor transcript profiles, further comparisons were made to determine whether the anatomical location may also have an influence. T-Tests (95% CI) were conducted for each chemokine receptor to compare the observed transcript levels between peripheral blood and prescapular lymph nodes (Table 3.2). Generally, transcript levels for most chemokine receptors were significantly higher in NK cells derived from lymph

nodes including; CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CXCR3, CXCR4, CXCR5, CXCR6 and CX3CR1 on CD2+ NK cells and CCR3, CCR5, CCR6, CXCR1, CXCR3, CXCR4, CXCR5, CXCR6 and CX3CR1 on CD2- NK cells.

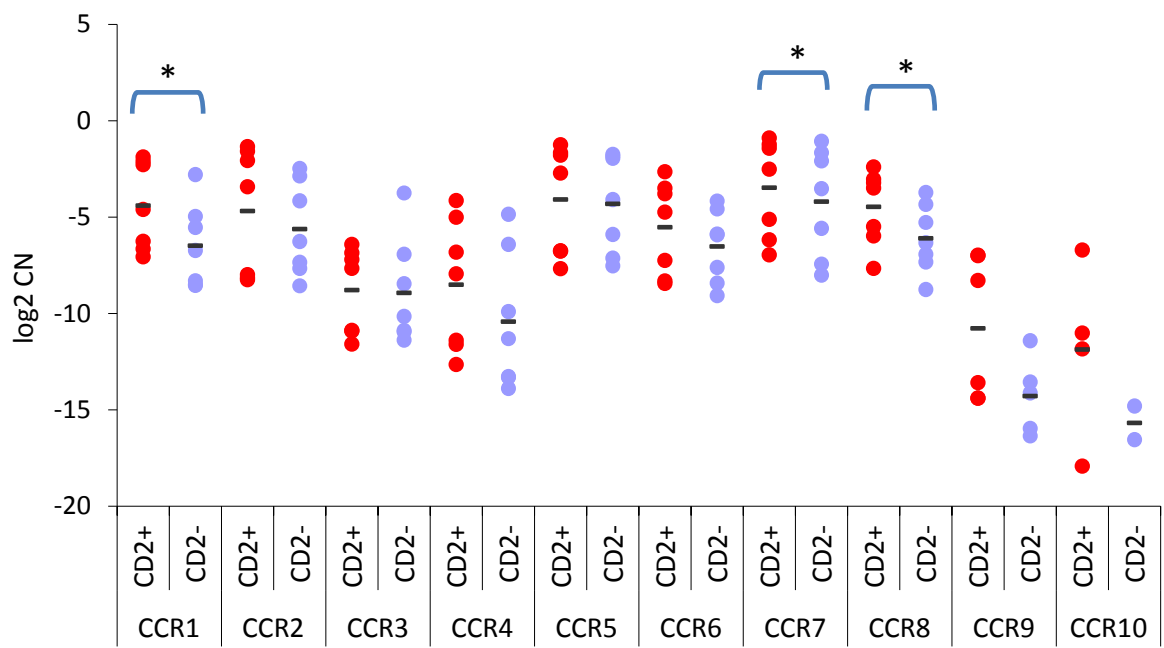
3.4.1



3.4.2



3.4.3



3.4.4

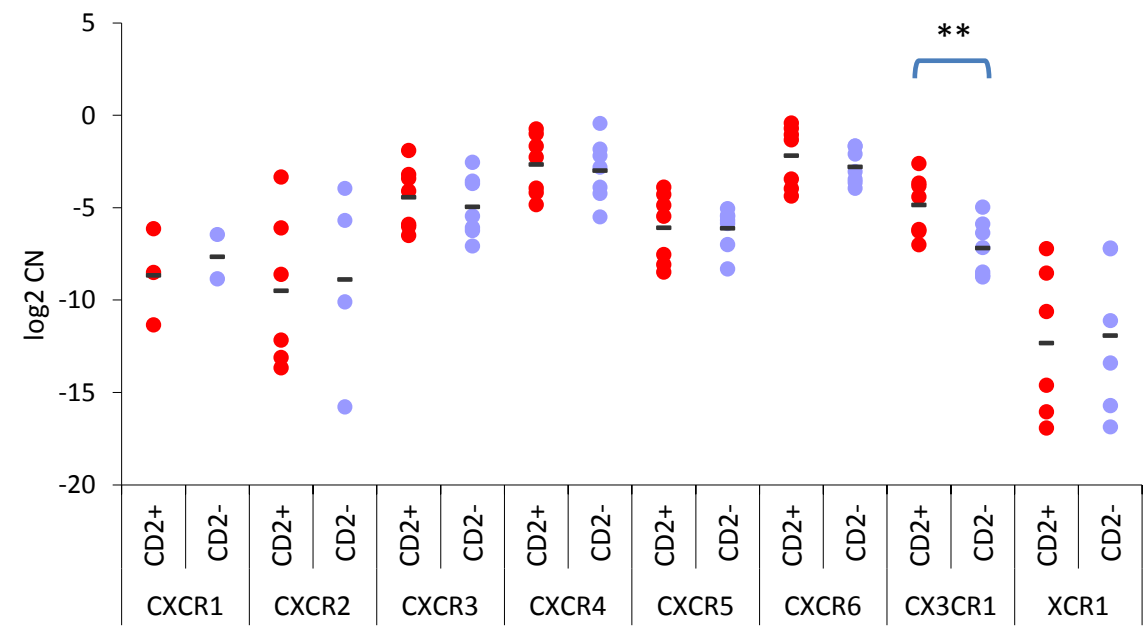


Figure 3.4 The chemokine receptor repertoire of NK cell subsets

*NK cell subsets (NKp46+ CD2+ and NKp46+ CD2-) were isolated from peripheral blood from eight animals and prescapular lymph nodes from seven animals using the FACSARIA™ cell sorter to purities >98%. RNA was reverse transcribed and subjected to quantitative PCR analysis for the cloned and sequenced bovine chemokine receptors. Transcription levels from blood derived NK cells CCR1-10 (Fig 3.4.1), CXCR1-XCR1 (Fig 3.4.2) and from prescapular lymph nodes CCR1-10 (Fig 3.4.3) and CXCR1-XCR1 (Fig 3.4.4) are shown. Each sample was tested in triplicate, quantified against a plasmid DNA standard curve and normalised to the housekeeping gene BoPo. Data represents the Log2 normalised copy number (CN), CD2+ NK cells are shown in red and CD2- in blue with each point denoting the transcriptional value obtained from one animal. Copy numbers between -6 and 0 were considered high, -6 to -12 intermediate and below -12 as low. Significance between receptors was assessed using a GLM and between subsets using the paired T-test $p < 0.05$ *, $p < 0.01$ **.*

	Blood		Prescapular lymph nodes	
	CD2+	CD2-	CD2+	CD2-
CCR1	+		+	
CCR2		++		
CCR3				
CCR4				
CCR5		+		
CCR6		+		
CCR7		++	+	
CCR8	++		+	
CCR9				
CCR10		++		
CXCR1				
CXCR2				
CXCR3		++		
CXCR4		++		
CXCR5		++		
CXCR6	++			
CX3CR1	++		++	
XCR1				

Table 3.1 Comparison of chemokine receptor expression by NK cell subsets

NK cell subsets from peripheral blood and prescapular lymph nodes were analysed for chemokine receptor expression levels by quantitative PCR. Significance between the CD2+ and CD2- subsets was assessed at each location using the pair-wise T-Test with $p < 0.05$ considered significant (+) and $p < 0.01$ considered highly significant (++). P values are specified in Table 9.7. Pooled data from all animals is summarised in the table above.

	CD2+		CD2-	
	Blood	PSLN	Blood	PSLN
CCR1				
CCR2		++		
CCR3		+		+
CCR4		+		
CCR5		++		++
CCR6		++		+
CCR7		++		
CCR8				
CCR9				
CCR10				
CXCR1				+
CXCR2				
CXCR3		++		+
CXCR4		++		++
CXCR5		++		++
CXCR6		++		++
CX3CR1				+
XCR1				

Table 3.2 Comparison of chemokine receptor expression by location

NK cell subsets from peripheral blood and prescapular lymph nodes were analysed for chemokine receptor expression levels by quantitative PCR. Significance between the values obtained for peripheral blood and prescapular lymph node (PSLN) was assessed for each subset using the Students T-Test with $p < 0.05$ considered significant (+) and $p < 0.01$ considered highly significant (++). P values are specified in Table 9.7. Pooled data from all animals is summarised in the table above.

3.2.5 Testing recombinant chemokines

Whilst the transcript levels of chemokine receptors on NK cells varied substantially, the cell surface expression could not be confirmed due to lack of commercially available bovine antibodies. However, phylogenetic analysis has suggested that both chemokines and chemokine receptors are highly conserved and bovine sequences cluster well with both human and other related species (Widdison et al., 2010). In addition, recombinant murine (mu) CCL19, CCL21 and human (hu) CXCL12 were reported to induce the migration of bovine lymphocytes (Wilson et al., 2002).

To determine whether the observed transcriptional differences between subsets of NK cells could influence their capacity to migrate, human and murine chemokines were tested for cross-reactivity in chemotaxis assays. Chemokines to be tested were selected based on previously published evidence suggesting that they may be influential in the induction of NK cell migration or chemokines with reported cross-reactivity within the bovine system. Whilst recombinant bovine chemokines became available (Kingfisher Biotech) at later stages during these studies, their biological activity could not be confirmed and were therefore not used for chemotaxis experiments. The tested human and murine recombinant chemokines are shown in Table 3.3.

<i>Human</i>	<i>Murine</i>
CCL1 (I-309)	CCL3 (MIP-1 α)
CCL2 (MCP-1)	CCL5 (RANTES)
CCL3 (MIP-1 α)	CCL19 (ELC)
CCL17 (TARC)	CCL21 (SLC)
CCL19 (ELC)	CXCL10 (IP-10)
CCL22 (MDC)	CXCL13 (BCA-1)
CXCL8 (IL-8)	CXCL16
CXCL9 (MIG)	
CXCL10 (IP-10)	
CXCL11 (I-TAC)	
CXCL12 (SDF-1 α)	
CXCL13 (BCA-1)	
CXCL16	
CX3CL1 (Fractalkine)	

Table 3.3 **Recombinant chemokines tested for cross-reactivity**

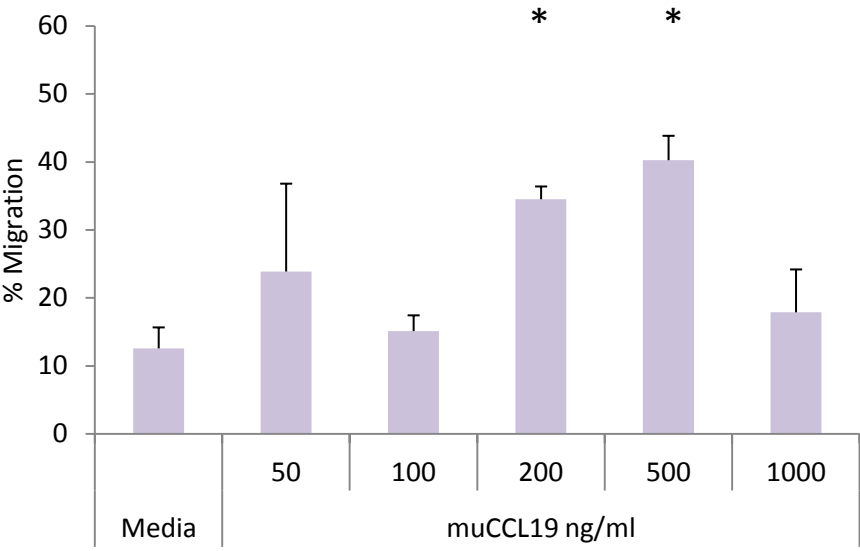
Initial experiments to establish cross-reactivity of chemokines, used bovine PBMCs stimulated for three days with ConA (5µg/ml) as input cells within chemotaxis assays (2.9.1). Migration was assessed in response to recombinant chemokines at a range of dilutions (50ng/ml-1µg/ml) and media (TCM) alone (9.1) was used as a negative control. A positive result was considered as a significant induction of migration above background assessed by a 1-Way ANOVA with Tukey post-hoc comparisons (95% CI).

Of the chemokines tested, only recombinant huCXCL9, CXCL10, CXCL12 and muCCL19 (Figure 3.5) appeared to induce detectable levels of migration above the background levels observed with media alone. As has previously been shown (Wilson et al., 2002), muCCL19 was chemotactic for bovine PBMCs and the migration followed a typical 'bell-shaped' dose-response. At concentrations of 500ng/ml, CCL19 specific recruitment peaked at about 30% PBMCs migrating and at concentrations above this, migration was significantly reduced indicating receptor saturation may have been reached (Figure 3.5.1). Despite previous observations suggesting muCCL21 may also cross-react with bovine receptors and induce the migration of lymphocytes (Wilson et al., 2002), no migration was observed in chemotaxis assays with this chemokine.

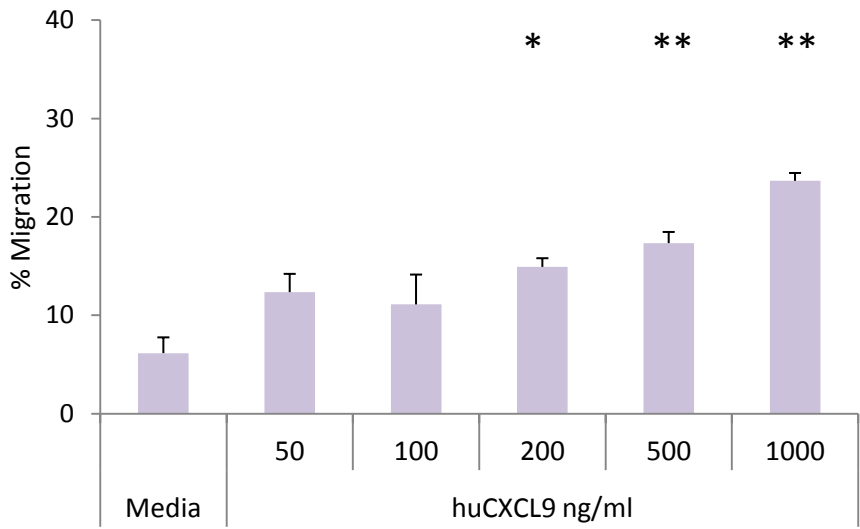
In comparison, PBMCs migrated towards huCXCL9 but only in significant numbers (5-15%) at concentrations above 200ng/ml (Figure 3.5.2). The migratory response was still elevated at 1µg/ml and higher concentrations were not tested to determine if the saturation point had been reached. Whilst low levels of migration towards huCXCL10 (500ng/ml) were observed, this was not found to be significant at any of the concentrations analysed (Figure 3.5.3).

The highest level of chemokine induced recruitment was in response to recombinant huCXCL12 (Figure 3.5.4). In a typical dose-response, migration of stimulated PBMCs was significantly elevated at all concentrations tested (50ng-1µg/ml). At the peak of the response (500ng/ml), huCXCL12 was able to induce migration of over 50% of the PBMC population.

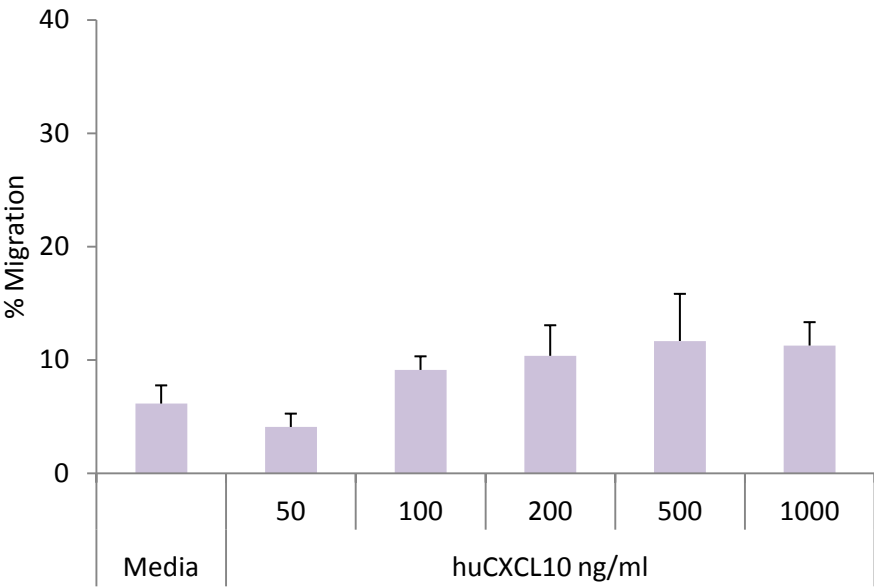
3.5.1



3.5.2



3.5.3



3.5.4

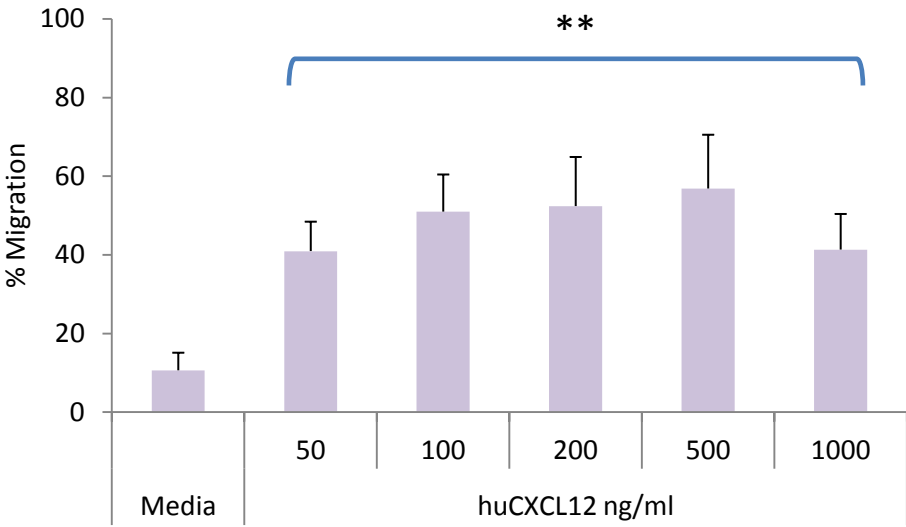


Figure 3.5 Migration of stimulated PBMCs towards recombinant chemokines

*PBMCs were stimulated with ConA (5µg/ml) for three days before being assessed for migration in a transwell chemotaxis assay. Recombinant chemokines; murine (mu) CCL19 (Fig 3.5.1), human (hu) CXCL9 (Fig 3.5.2), CXCL10 (Fig 3.5.3) and CXCL12 (Fig 3.5.4) were used as attractants at concentrations ranging from 50ng/ml to 1µg/ml with media (TCM) alone as a negative control. Each concentration was assayed in duplicate and data represents the average migration, expressed as a percentage of the input population from three animals, with error bars denoting the standard deviation between animals. Significance was assessed using a 1-Way ANOVA with Tukey post-hoc comparisons (95% CI) to determine significantly increased migration above the media control; $p < 0.05$ *, $p < 0.01$ **.*

3.2.6 NK cell migration to recombinant chemokines

Initial migration assays suggested that recombinant muCCL19, huCXCL9 and huCXCL12 may be cross-reactive with bovine MNCs derived from peripheral blood. To determine whether NK cells were migrating in response to these chemokines, the migrated cells from these experiments were pooled and analysed by immuno-fluorescent labelling. Input and migrated cells were labelled with the mAbs (9.2) CD335-PE (NKp46) and CC42-647 (CD2) to identify the percentage of NK cells and proportions of subsets within each population.

NK cells were not detected within migrated populations of PBMCs responding to muCCL19 and huCXCL9, however were found to migrate in response to huCXCL12. A representative plot from one animal is shown in Figure 3.6. Comparison of the percentage of NK cells in the input (3.36%) and migrated (1.43%) populations suggested that in this instance, 40% of the NK cells within the total PBMC population migrated in response to huCXCL12.

To determine whether the observed migration was optimal, further chemotaxis assays were conducted utilising purified NK cells from peripheral blood and a dilution range (50ng/ml to 1µg/ml) of the recombinant chemokines. As was observed in previous assays, NK cells did not migrate in response to muCCL19 or huCXCL9. This was also the case when IL-12 and IL-18 stimulated NK cells (2.5) were used as input cells in chemotaxis assays (data not shown). In comparison, significant percentages of purified NK cells migrated in response to huCXCL12 at all concentrations tested (Figure 3.7). The response was optimal at 200ng/ml with an average 35-45% of the NK cells migrating. For this reason, huCXCL12 was employed as positive control for NK cell migration in all subsequent chemotaxis assays.

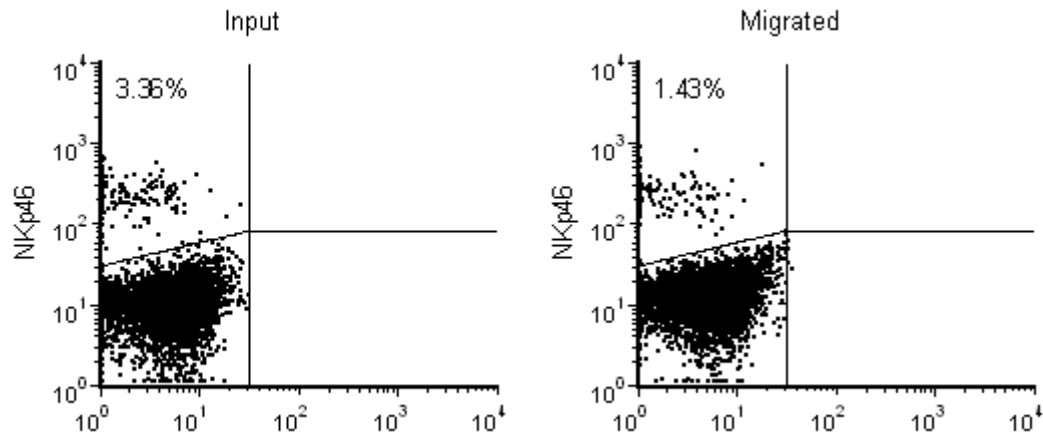


Figure 3.6 Identification of recombinant huCXCL12 induced NK cell migration

PBMCs from three animals were stimulated with ConA 5 μ g/ml for three days before being assessed for migration in transwell chemotaxis assays in response to recombinant huCXCL12. Duplicate wells for both the input and migrated cell populations were pooled separately, labelled with the mAb CD335-PE and analysed by flow cytometry. NKp46+ cells were identified by gating against unstained PBMCs and the percentage of NK cells in the input and migrated populations were compared. Representative plots from one animal are shown.

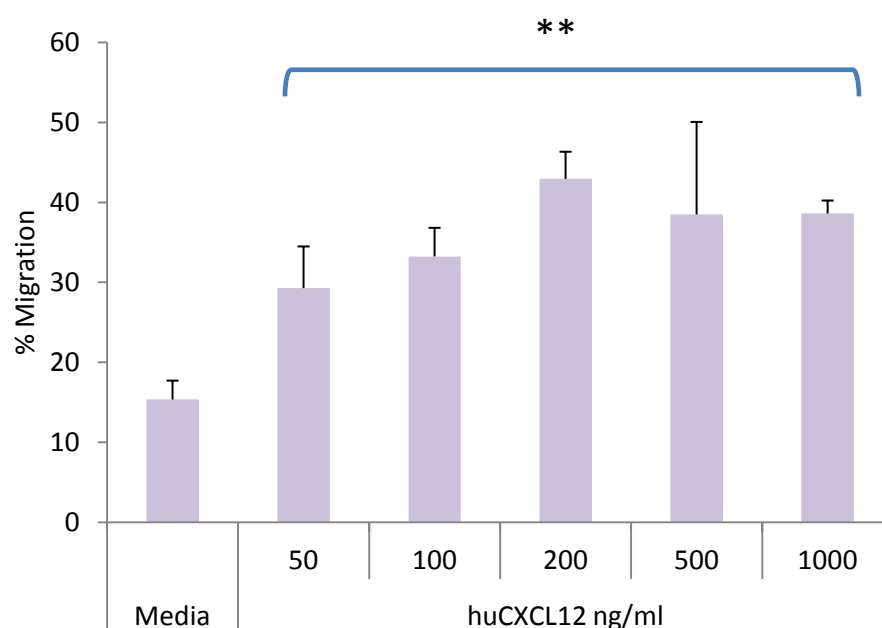


Figure 3.7 Migration of NK cells in response to recombinant huCXCL12

*NKp46+ cells were isolated from peripheral blood of three animals by positive MACS selection and left to rest overnight in TCM at 37°C. Chemotaxis assays were used to assess the migratory responses of NK cells towards recombinant huCXCL12 at concentrations ranging from 50ng/ml to 1µg/ml. Media alone was used as negative control and each concentration was assayed in duplicate. Data represents the average migration, expressed as a percentage of the input population from three animals, with error bars denoting the standard deviation between animals. Significance was assessed using the 1-Way ANOVA with Tukey post-hoc comparisons (95% CI) $p < 0.05$ *, $p < 0.01$ **.*

3.3 Discussion

NK cells are a small population of lymphocytes with multifunctional responses in the innate and adaptive immune systems. Both human and murine NK cells are commonly defined by unique phenotypic characteristics (1.2.3.4) however, the natural cytotoxicity receptor NKp46 is highly conserved and has been reported to represent a unifying marker by which NK cells can be identified across many mammalian species (Walzer et al., 2007a). This is also the case within cattle, with expression restricted to a small percentage of granular lymphocytes with cytolytic and cytokine producing properties. Storset et al., (2004) reported that NKp46 was highly and stably expressed and therefore isolation of lymphocytes bearing this receptor was an accurate method by which bovine NK cells could be identified and purified. Despite this, recent studies have proposed that NKp46 may also be expressed by a minor sub-population of bovine splenic $\gamma\delta$ T cells. However, this was only the case following long term stimulation with IL-15 (Johnson et al., 2008) or very transient expression on a small population of WC1+ $\gamma\delta$ T cells in Foot and Mouth Disease Virus (FMDV) infected cattle (Toka et al., 2009). As such, NKp46 bearing lymphocytes are primarily defined as NK cells with phenotypic and functional characteristics corroborating this definition.

Within initial studies, we confirmed published reports that bovine NK cells from peripheral blood positively selected by NKp46, were phenotypically distinct from T cells, B cells, monocytes and $\gamma\delta$ T cells. This technique has therefore been employed in all subsequent experiments as a method to isolate bovine NK cells to a high degree of purity. It should be noted however, that positive selection of NK cells in this manner may exclude discrete NK-like populations which do not express NKp46. These have been reported to exist within humans (Ferlazzo and Munz, 2004) and may also be found in the peripheral blood of cattle; predicted to be an immature population of NK cells (Graham et al., 2009). In addition, it is important to consider that NKp46 functions as an activatory receptor, signalling through which induces NK cell effector responses. In cytotoxicity studies, cross-linking of NKp46 augmented the killing responses against target cell populations; yet cells within this study were also pre-stimulated with IL-2 (Storset et al., 2004). Additionally, the binding of haemagglutinin to NKp46 has been reported to initiate cytolytic activity against virally infected cells (Mandelboim et al.,

2001). However (Achdout et al., 2010) further elucidated that binding did not necessarily lead to activation with an NK cell response determined by the distinct site at which the ligand bound to the NKp46 molecule. Furthermore, it has also been reported that monoclonal antibodies to NKp46 may mask interactions with natural ligands and function to block cytolytic activity (Pessino et al., 1998, Sivori et al., 1999). It is therefore not known the degree to which the use of monoclonal antibodies against NKp46 may influence NK cell effector responses. However, despite these factors, positive selection utilising NKp46 was found to be the only definitive technique by which bovine NK cells could be isolated with numerous attempts at negative selection failing to provide sufficient purities.

In further phenotypic analysis, we confirmed that significant numbers of bovine NK cells expressed CD16 and in contrast to human NK cells, the levels of expression were not variable (Boysen et al., 2008). Most significantly, blood derived NK cells could be divided into sub-populations with a larger proportion (70-80%) of cells expressing CD2. CD2 is primarily defined as an adhesion molecule but studies have also suggested roles as a co-receptor for activation (McNerney and Kumar, 2006, Tangye et al., 2000). Despite this, CD2 expression has been reported to be redundant for effective NK cell derived effector responses with blocking mAbs having little effect. In addition it is the NKp46⁺ CD2⁻/low subset of bovine NK cells that is the more functionally responsive with augmented cytolytic activity, cytokine production and faster proliferation following IL-2 stimulation (Boysen et al., 2006b).

As such, parallels have been drawn between subsets of bovine and human NK cells with CD56^{bright} CD16⁻ human NK cells demonstrating similar functional responses and localities to bovine NKp46⁺ CD2⁻ NK cells. However, it is not possible to draw direct comparisons between populations of cells defined by differing phenotypic characteristics. Therefore, whilst emerging reports in the human field suggest that human NK cell subsets may represent populations of cells at differing stages of maturation (Marquez et al., 2010, Juelke et al., 2010, Yu et al., 2010); it is not known whether this may also be the case in the bovine field. Both CD2⁺ and CD2⁻ NK cells possess the capacity for mature NK cell derived effector responses (Boysen et al., 2006b). It is possible that specific stimulatory signals may be required as was found to

be the case for human NK cells in contact with fibroblasts (Chan et al., 2007) or through cross-linking of CD16 (Marquez et al., 2010) and is an area where further research is required.

In this thesis significant numbers of NK cells were observed within resting prescapular lymph nodes with varying levels of CD2 expression such that the distinction between subsets was less clear. Nevertheless, NKp46⁺ CD2⁻/low NK cells constituted the predominant population within lymph nodes (Figure 3.1.4). It has been proposed that this may be as consequence of selective expansion of CD2⁻ NK cells which are known to be highly proliferative within IL-2 rich environments (Boysen et al., 2006b), as would be the case within lymph nodes. Yet it is not known whether the observed distribution may also be as a result of selective recruitment of CD2⁻ NK cells from the periphery. Bovine NK cells are known to express L-Selectin (CD62L) (Boysen et al., 2008), an adhesion receptor which confers the ability to home to secondary lymphoid tissue via HEVs. However, the migratory responses of leukocytes are more commonly determined by expression of an array of chemokine receptors which function in synergy to initiate migration in response to gradients of locally produced chemokines. This study therefore sought to gain an insight into the migratory characteristics of bovine NK cells with initial investigations conducted to determine the patterns of chemokine receptor expression between subsets of NK cells in the blood and prescapular lymph nodes.

Transcripts for both homeostatic and inflammatory chemokine receptors could be detected in bovine NK cells. Whether the observed levels equate to translation is not known as at present there are no antibodies to detect their surface expression. Despite this, it has been suggested that the functional levels of chemokine receptors are well reflected at mRNA level (reviewed by (Widdison et al., 2010)). Results herein demonstrated considerable variations in the patterns of chemokine receptor expression between subsets of bovine NK cells. NKp46⁺ CD2⁺ NK cells which constitute the larger population within peripheral blood, expressed significantly higher levels of homeostatic receptors CXCR6 and CX3CR1 in comparison to their CD2⁻ counterparts (Table 3.1). The expression of homeostatic chemokine receptors is associated with the formation of resident populations with NK cells bearing CXCR6 preferentially retained

within murine hepatic regions (Paust et al., 2010) and CX3CR1 functioning as an adhesion receptor to potentiate the retention of lymphocytes to endothelial cells bearing CX3CL1 ligands (Barlic et al., 2003, Fraticelli et al., 2001). In addition, during *in vivo* studies we have observed significant numbers of CD2⁺ NK cells within the lung tissue of cattle (6.2.1). Functional expression of homeostatic chemokine receptors may therefore contribute to the selective localisation or retention of CD2⁺ NK cells within non-lymphoid compartments.

In comparison, CD2⁻ NK cells derived from peripheral blood transcribed significantly higher levels of a number of inflammatory and lymphoid homing chemokine receptors (Table 3.1). In particular, elevated expression of CCR2, CCR5 and CXCR3 would confer responsiveness to multiple inflammatory chemokines produced rapidly at the site of infection. Both virally (Piqueras et al., 2006) and bacterially (Lande et al., 2003) infected antigen presenting cells have been reported to up-regulate expression of inflammatory chemokines CCL2, CCL3, CCL4, CCL5 and CXCL10 to aid the accumulation of effector cells, with NK cells migrating in a CXCR3 dependent manner. In addition, the significantly increased expression of CXCR3 and CCR7 by CD2⁻ bovine NK cells may influence the lymphoid homing capacity of this subset. This has previously been demonstrated within human CD56^{bright} CD16⁻ NK cells expressing CCR7 enabling migration in response to CCL19 and CCL21 and preferential accumulation within secondary lymphoid tissue (Berahovich et al., 2006, Campbell et al., 2001, Robertson, 2002, Kim et al., 1999, Fehniger et al., 2003). Furthermore, murine NK cells migrated to draining lymph nodes in response to CXCR3 ligands produced by infected DCs (Martin-Fontecha et al., 2004). The observed chemokine receptor repertoire of CD2⁻ NK cells may therefore contribute to the preferential accumulation of this subset within lymphoid tissue, as well as recruitment to the site of infection during an inflammatory immune response. These responses were further investigated as a part of this thesis within the context of mycobacterial infection.

In further phenotypic analysis, the patterns of chemokine receptor expression within prescapular lymph nodes was far more uniform, with fewer receptors being preferentially expressed by either subset of bovine NK cells (Table 3.1). This is also the case with CD2 expression levels and may be as a consequence of a more

heterogeneous population of NK cells. Despite this, CD2⁺ NK cells within lymphoid tissue preferentially expressed CCR7 and CX3CR1. Local conditions within lymph nodes favour CD2⁻ NK cells enabling proliferation and persistence (Boysen et al., 2008) thus; elevated expression of these receptors within lymph nodes may aid the retention of the minor population of CD2⁺ NK cells. Overall, the transcript levels for the majority of chemokine receptors were significantly higher in NK cells derived from prescapular lymph nodes when compared with the levels seen within the blood (Table 3.2). It has previously been reported that bovine NK cells within lymph nodes possess a more activated phenotype with elevated expression of CD25 and this may be as a consequence of exposure to T cell derived IL-2 which is found in abundant levels within lymph nodes (Boysen et al., 2008). Human NK cells stimulated with IL-2 expressed enhanced levels of inflammatory chemokine receptors; CCR2, CCR5 (Nieto et al., 1998) and CXCR3 (Beider et al., 2003) and in further studies IL-2 augmented the migratory response to multiple inflammatory and lymphoid homing chemokines (Inngjerdigen et al., 2000, Maghazachi et al., 1997, Berahovich et al., 2006, Beider et al., 2003, Allavena et al., 1996). It should be noted however, that the comparison between chemokine receptor expression levels of NK cells derived from blood and prescapular lymph nodes was made between different animals. Whilst this factor was taken into consideration within the statistical analysis, a more accurate comparison would be to directly compare NK cells from multiple compartments within the same animal.

To corroborate the findings by quantitative PCR, further experimentation involved the development of chemotaxis assays to evaluate the migratory responses of bovine NK cells. When these studies were conducted, functional bovine chemokines were not commercially available therefore; we sought to identify cross-reactive human and murine recombinants. The chemokine family of receptors and ligands is well conserved between species and genomic analysis demonstrated a high degree of homology between certain human, murine and bovine sequences. In general, similarities at both nucleotide and amino acid level range from 60-90%, with the most conserved receptors CCR7 (91%), CXCR3 (87%) and CXCR4 (92%) found to be highly homologous between humans and cattle (Boyd et al., 2010, Blumberman et al., 2007, Widdison et

al., 2010). Additionally, muCCL19, muCCL21 and huCXCL12 were reported to mediate the migration of bovine lymphocytes within chemotaxis assays (Wilson et al., 2002).

Following testing of multiple chemokines (Table 3.3), bovine lymphocytes migrated in response to the CCR7 ligand; muCCL19 and the CXCR3 ligand; huCXCL9. Despite the use of a range of concentrations, percentages of migrating cells were considerably low and NK cells did not appear to be recruited. It is likely that the relative affinity and avidity of receptor-ligand interactions also plays a significant role in determining activity. For example, it has been reported that human CXCL10 binds CXCR3 with lower affinity than CXCL9, through occupation of alternative binding sites (Cox et al., 2001) and may therefore explain the different responses between these two chemokines observed herein. Overall, cross-reactivity was deemed to be relatively low and no further experimentation was conducted with these recombinants. In contrast, at optimal concentrations the CXCR4 ligand; huCXCL12 recruited 50-70% of bovine lymphocytes. Both CXCL12 and its receptor CXCR4 are highly conserved across many species (Basu et al., 2002) and human and bovine sequences are 92% similar at amino acid level. Therefore, the highly homologous receptor/ligand pair induced substantial migratory responses of bovine lymphocytes.

Resting human NK cells constitutively express CXCR4 (Inngjerdingen et al., 2001) and murine studies have demonstrated homeostatic roles for CXCR4/CXCL12 associated with the retention or release of NK cells from the bone marrow (Beider et al., 2003, Bernardini et al., 2008). Herein, peripheral blood derived bovine NK cells expressed CXCR4 and migrated in significant numbers in response to huCXCL12. Thus, in all further chemotaxis experiments, huCXCL12 was utilised as a positive control to evaluate efficient migratory responses of NK cells. Given the substantial heterogeneity in the levels of chemokine receptor transcript between subsets of bovine NK cells, further experimentation within this thesis was conducted to determine whether this may influence migratory capacity in particular within models of mycobacterial infection.

4 The migratory responses of NK cells during *M.bovis* infection

4.1 Introduction

During mycobacterial infection, the recruitment of leukocytes to the site of infection as well as re-distribution to draining lymph nodes are pivotal events in the induction of innate and adaptive immune responses. Within the respiratory tract, infiltrating lymphocytes enable the formation of lesions and granulomas to restrict the replication and spread of mycobacteria (Peters and Ernst, 2003, Algood et al., 2003). Whilst this process contributes to the immuno-pathology associated with chronic TB infection, it is also essential to the formation of an appropriate early immune bias for the induction of protective immunity.

NK cells are prevalent at the site of an inflammatory immune response as well as within the draining lymph nodes of humans and cattle (Boysen et al., 2008, Fehniger et al., 2003, Ferlazzo et al., 2004b). Murine models of Cytomegalovirus (Salazar-Mather and Hokeness, 2003), *Leishmania major* (Vester et al., 1999), *Toxoplasma gondii* (Khan et al., 2006) and *Klebsiella pneumonia* (Zeng et al., 2003) have demonstrated accumulation of NK cells upon antigenic insult. Furthermore, intranasal challenge with BCG or *M.tuberculosis* induced increases in the absolute numbers of activated, IFN γ producing NK cells within the lungs (Feng et al., 2006, Junqueira-Kipnis et al., 2003, Saxena et al., 2002). A similar response was also seen within the localised lymphoid tissue following administration of mycobacterial antigens; Ag85 and ESAT6 (Hall et al., 2010). In particular, CD56^{bright} NK cells are often reported as abundant within inflammatory sites (Maghazachi, 2005, Dalbeth et al., 2004). This has also been reported to be the case during TB infection, with selective enrichment of CD56^{bright} NK cells within the pleural fluid (Schierloh et al., 2005).

Whilst the observed increase in the proportions of NK cells may at least in part be attributed to localised expansion, NK cells are highly responsive to chemokines and have been reported to possess a higher migratory capacity during TB infection (Pokkali et al., 2009). Numerous studies have demonstrated the fundamental role that chemokines play in the recruitment of NK cells. For example, NK cell accumulation following *Leishmania major* infection in mice was dependent on CXCL10 release

(Vester et al., 1999) and a role of CCL3 during *Klebsiella pneumonia* infection has been shown (Zeng et al., 2003). Prlic et al., (2005) reported that upon antigenic challenge, NK cells were expanded within the spleen and recruited from the periphery to the site of infection in a process reliant on G-protein coupled receptor signalling. Within gene knock-out mice, lack of CCR1 (Shang et al., 2000) or CCR5 (Khan et al., 2006) had a substantial impact on the migratory responses of NK cells with impaired trafficking to the site of infection. Additionally, expression of CXCR3 has been demonstrated as implicit for NK cell migration to draining lymph nodes (Martin-Fontecha et al., 2004, Watt et al., 2008).

The recruitment of lymphocytes such as NK cells is controlled by inflammatory chemokines rapidly secreted by alveolar macrophages, DCs and also epithelial cells. However, it has been reported that DCs in particular play a fundamental role in the effective recruitment of both NK cells and also CD4+ T cells (Uchida et al., 2007). The release of chemokines by DCs has been reported to be a dynamic process in order to orchestrate the differential recruitment of effector cells in a time-dependent fashion (Piqueras et al., 2006, Sallusto et al., 1999). DCs infected with *M.tuberculosis* rapidly up-regulated the inflammatory chemokines CCL2, CCL3, CCL4, and CXCL8 followed by increasing levels of CXCL9 and CXCL10 (Gustafsson et al., 2008, Lande et al., 2003) over the course of the infection period.

Of particular importance is the co-localisation of NK cells with DCs (5.1), a process which enables reciprocal interactions to ensue such that DC maturation and NK cell effector responses are potentiated both at the inflammatory site and within draining lymph nodes to influence the shaping of the adaptive immune response. In this manner, DCs exposed to influenza virus recruited NK cells in significant numbers by chemokine mediated mechanisms (Piqueras et al., 2006). Furthermore, following *M.tuberculosis* infection the migration of NK cells towards DCs was reliant on CXCR3 expression and CXCL10 secretion (Lande et al., 2003) with an important role of CCR5 also described in the DC derived recruitment of NK cells (Van Elssen et al., 2010). *In vivo*, LPS matured DCs mediated the rapid recruitment of NK cells from the periphery to draining lymph nodes, mediated by CXCR3 expression by NK cells (Martin-Fontecha et al., 2004), ligands for which are readily produced by DCs (Gustafsson et al., 2008).

Herein, we have demonstrated significant variations in the chemokine receptor expression patterns between subsets of bovine NK cells (3.2.4) however their migratory responses, in particular during mycobacterial infection have previously not been investigated. This study therefore sought to elucidate whether *M.bovis* infected DCs could recruit NK cells and whether the previously observed patterns of chemokine receptor expression could be reflected within the migratory responses of NK cells subsets.

4.2 Results

4.2.1 Chemotactic activity of supernatants from *M.bovis* infected DCs

DCs were cultured from peripheral blood derived monocytes for three days and infected with *M.bovis* overnight at a MOI of 1:1 (2.7.2). Supernatants were retrieved and their migratory capacity was evaluated using chemotaxis transwell assays with PBLs as input cells. Specific migration at each dilution could be ascertained by flow cytometry and FSC/SSC differentiation of granulocytes, monocytes and lymphocytes.

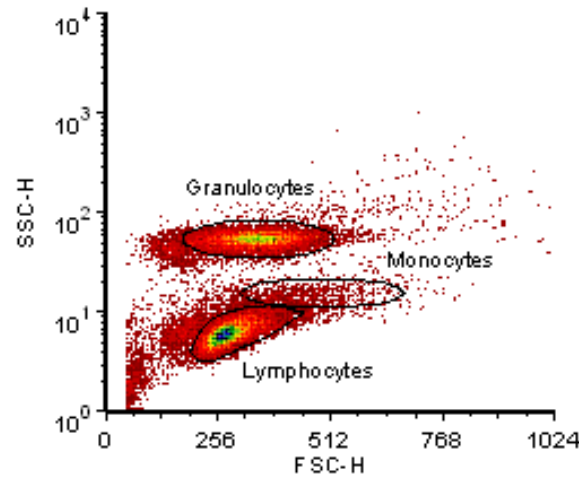
Within total PBLs, gating of a high SSC population of cells (Figure 4.1.1) identified granulocytes, which demonstrated an inherent capacity to migrate with over 40% of cells non-specifically responding to the media (TCM) control (Figure 4.1.2). Significant migration over background (media-control) was assessed using log transformed data within a GLM (95% CI) with $p < 0.05$ considered significant. The highest level of migration was observed in the granulocyte population of cells (vs monocytes $p = 0.004$ and vs lymphocytes $p < 0.001$) with supernatants from *M.bovis* infected DCs able to specifically recruit an additional 30-40% of cells at optimum (1:2) (peak) supernatant dilutions (vs media $p = 0.021$). Monocytes were identified by their high FSC and SSC properties and 20-30% of cells migrated ($p = 0.012$) at the peak of the response (1:2). In contrast, no significant migration of lymphocytes was observed at any of the tested dilutions.

To clarify optimal conditions for lymphocyte migration, supernatants from *M.bovis* infected DCs were obtained at 24, 36 and 72 hours post-infection and assessed in further chemotaxis assays with either ConA stimulated PBMCs or NK cells (unstimulated or IL-12 + IL-18 stimulated) as input cells. As previously observed, PBMCs or purified NK cells did not migrate in response to supernatants from *M.bovis* infected DCs and extending the period of infection appeared to have no effect (data not shown).

It has previously been suggested that, *M.bovis* infected DCs could induce the activation and migration of bovine $\gamma\delta$ T cells, however only when infected DCs were in direct culture with the $\gamma\delta$ T cells (Price, 2009). In light of this, chemotaxis assays were

adapted to include the *M.bovis* infected DC population and the resultant supernatant *in situ* and subsequent lymphocyte migration was assessed. Initial studies utilising this co-culture format indicated an induction of NK cell migration. Further experiments to optimise the time period for the chemotaxis assay suggested that the peak of the NK cell migratory response could be observed after 4 hours and that time periods longer than this (up to 24 hours) did not significantly alter the percentages of migrating cells (data not shown). Therefore, these conditions were used in all subsequent migration assays.

4.1.1



4.1.2

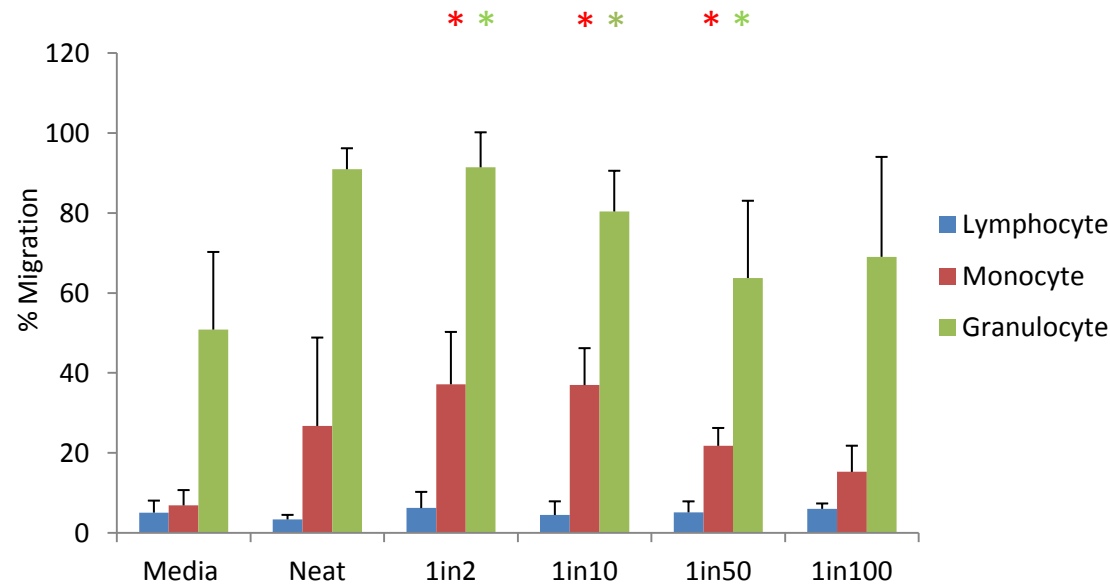


Figure 4.1 Migration of PBLs towards supernatants from *M.bovis* infected DCs

*Monocytes were isolated from peripheral blood and cultured for three days to obtain DCs. Supernatants from DCs were retrieved after overnight infection with M.bovis and migration of PBLs was assessed in transwell chemotaxis assays (Fig 4.1.2). Granulocytes, monocytes and lymphocytes could be differentiated within the total PBL population, by flow cytometry based on FSC and SSC properties (Fig 4.1.1). Supernatants were used as attractants at a variety of concentrations ranging from neat to 1:100 and media (TCM) alone was used as a negative control. Each dilution was assayed in duplicate and data represents the average migration, expressed as a percentage of the input population from three animals, with error bars denoting the standard deviation between animals. Significance was assessed using a GLM with Tukey post-hoc comparisons (95% CI) to determine significantly increased migration above the media control; $p < 0.05$ **

4.2.2 Co-cultured chemotaxis assays

4.2.2.1 Migration of peripheral blood derived NK cells

Having established optimum conditions for NK cell migration, *in vitro* co-cultured chemotaxis assays were used to determine to what extent mycobacterial infection of DCs induced the recruitment of NK cells. Monocytes were isolated from peripheral blood and cultured for three days to obtain DCs (2.6). Within initial chemotaxis assays, DCs were infected with the attenuated mycobacterial strain BCG overnight (2.7.2) and peripheral blood derived NK cells (2.4), were assayed for migratory responses. Statistical significance was assessed using the 1-Way ANOVA with Tukey post-hoc comparisons (95% CI).

The control conditions; media (TCM) or BCG alone ((MOI 10:1) (2.7.2)) were not able to induce the migration of NK cells (Figure 4.2.1). In contrast, a small percentage of the total NK cell population (5-20%) migrated towards uninfected DCs, significantly above the migratory responses observed with media ($p=0.001$) or BCG alone ($p=0.004$). Whilst NK cells (15-25%) were recruited by BCG infected DCs, for the majority of animals no significant increase was seen in comparison to that observed with uninfected DCs. NK cells from all animals responded to the rhu CXCL12 positive control (data not shown).

In further chemotaxis assays, NK cells did not respond to the media or *M.bovis* alone (MOI 1:1) (2.7.2)) control conditions (Figure 4.3.1). As previously shown, a small induction of migration was observed in response to uninfected DCs; however in this case this was found not to be significantly different to media or *M.bovis* alone. Upon infection of DCs with *M.bovis*, NK cells from three out of six animals were recruited with an increase in the percentage of migrated NK cells (15-25%). However, analysis of data across all six animals revealed no significant response towards *M.bovis* infected DCs despite NK cells from each of the animals responding to the rhu CXCL12 positive control (data not shown).

To further clarify how subsets of NK cells may be responding within these assays, migrated fractions from each chemotactic condition were labelled with the mAb CC42-

647 and analysed by flow cytometry to determine the proportions of CD2+ and CD2- NK cells. Statistical significance between different chemotactic conditions was assessed using a GLM with Tukey post-hoc comparisons (95% CI).

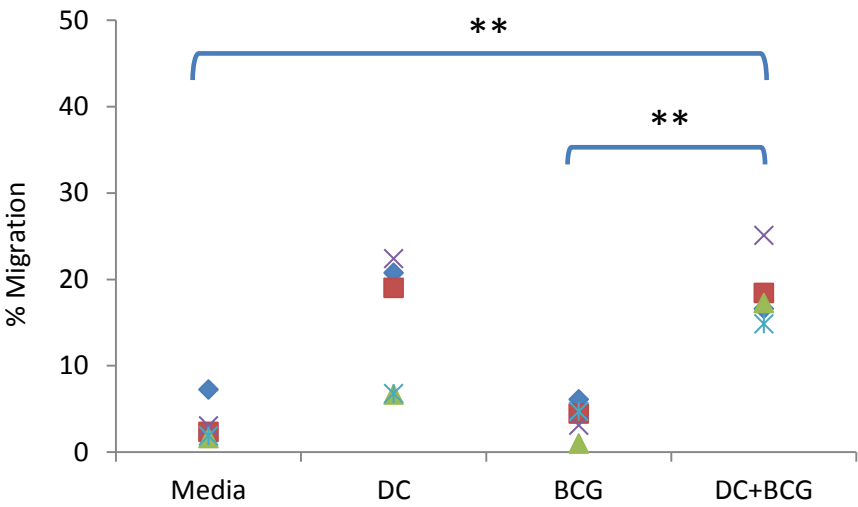
As has been described previously, bovine NK cells consist of subsets defined by their expression of CD2. The majority (70-80%) of blood derived NK cells are CD2+ and a comparatively smaller population (20-30%) have lower levels of CD2 expression (3.2.2). NK cells used as input populations within chemotaxis assays were consistent with this distribution and proportions of CD2+ and CD2- subsets did not change within migrated fractions of cells responding to media alone (Figure 4.2.2). Whilst a minor increase in the proportion of CD2- NK cells within the migrated fractions responding to BCG was often detected, this was found not to be a significant increase compared to the media control. In contrast, analysis of NK cells recruited by DCs revealed considerable alterations in the proportions of NK cell subsets; with significantly increased percentages of CD2- NK cells compared with the input NK cell population ($p=0.001$). Infection of DCs with BCG induced a similar effect, with increased CD2- NK cells ($p=0.002$) compared to media however, the response was similar to that observed with DCs alone and was therefore, not significantly altered upon infection with BCG.

Further chemotaxis assays with *M.bovis* infected DCs showed a similar redistribution of subsets within migrated fractions of NK cells (Figure 4.3.2); with significantly increased proportions of CD2- NK cells in response to DCs ($p<0.001$) or DCs infected with *M.bovis* ($p<0.001$). Importantly, the percentage of CD2- NK cells in migrated fractions recruited by *M.bovis* infected DCs was significantly elevated compared with *M.bovis* ($p<0.001$) or DCs alone ($p=0.006$) suggesting *M.bovis* specific preferential recruitment of CD2- NK cells.

Chemotaxis data from blood derived NK cells suggested that *M.bovis* infected DCs may preferentially recruit CD2- NK cells. However, labelling of total NK cells in this manner was not sufficient to determine whether the CD2- population was selectively migrating, whether the CD2+ NK cells were down-regulating expression of CD2 or selectively dying in culture; all of which could potentially result in the observed increase in proportions of CD2- NK cells. Labelling of migrated fractions with Propidium

Iodide (2.9.1) indicated no significant cell death within either population of NK cells. In further analysis, no significant alterations in the mean fluorescence intensity of CD2 could be detected, therefore suggesting that CD2- NK cells are likely to be selectively recruited.

4.2.1



4.2.2

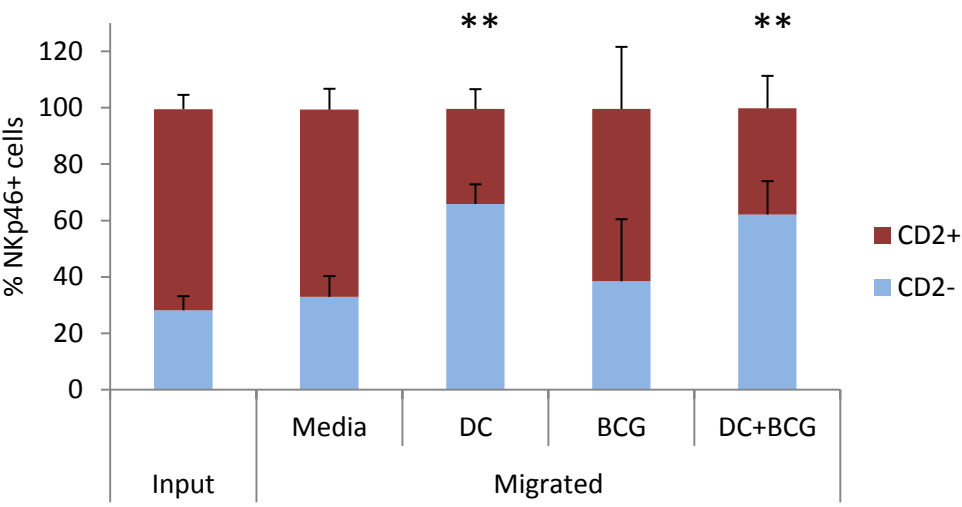
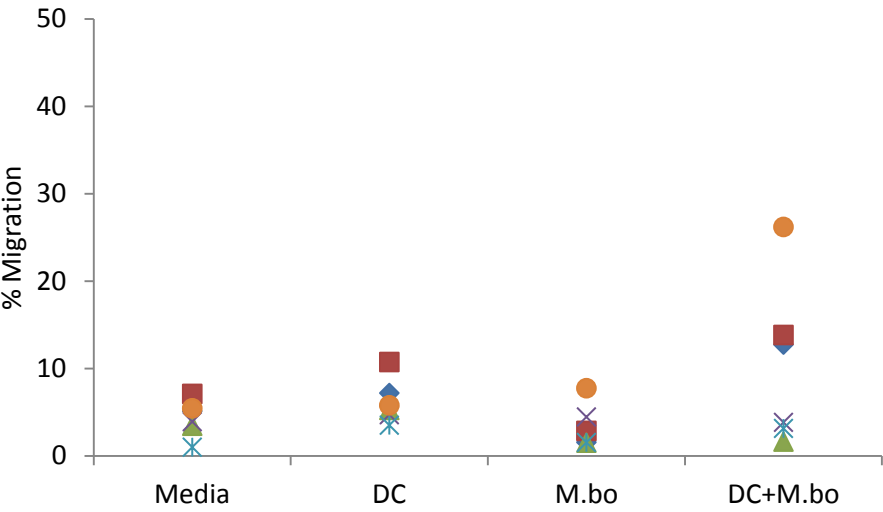


Figure 4.2 Migration of NK cells in response to BCG infected DCs

Monocyte derived DCs were infected with BCG (MOI 10:1) and after overnight infection were used as attractants in chemotaxis transwell assays. Input NK cells were isolated from peripheral blood by positive MACS selection and migration was assessed in response to BCG infected DCs (Fig 4.2.1) with Media (TCM), DCs or BCG alone as controls. Each condition was assayed in triplicate and data represents the average migration, expressed as a percentage of the input population from a group of five animals with each data point denoting the individual responses of animals. Rhu CXCL12 was used as a positive control (data not shown).

To determine proportions of migratory NK cells subsets, triplicates of input and migrated fractions from each condition were pooled independently and labelled for CD2 (CC42-647) and analysed by flow cytometry (Fig 4.2.2). Data represents the proportions of NKp46+ cells that were CD2+ (red) or CD2- (blue) in migrated populations with error bars demonstrating the standard deviation between animals. Statistical significance between conditions was assessed using the 1-Way ANOVA (Fig 4.2.1) or a GLM (Fig 4.2.2) with Tukey post-hoc tests with 95% CI $p<0.05^*$, $p<0.01^{**}$

4.3.1



4.3.2

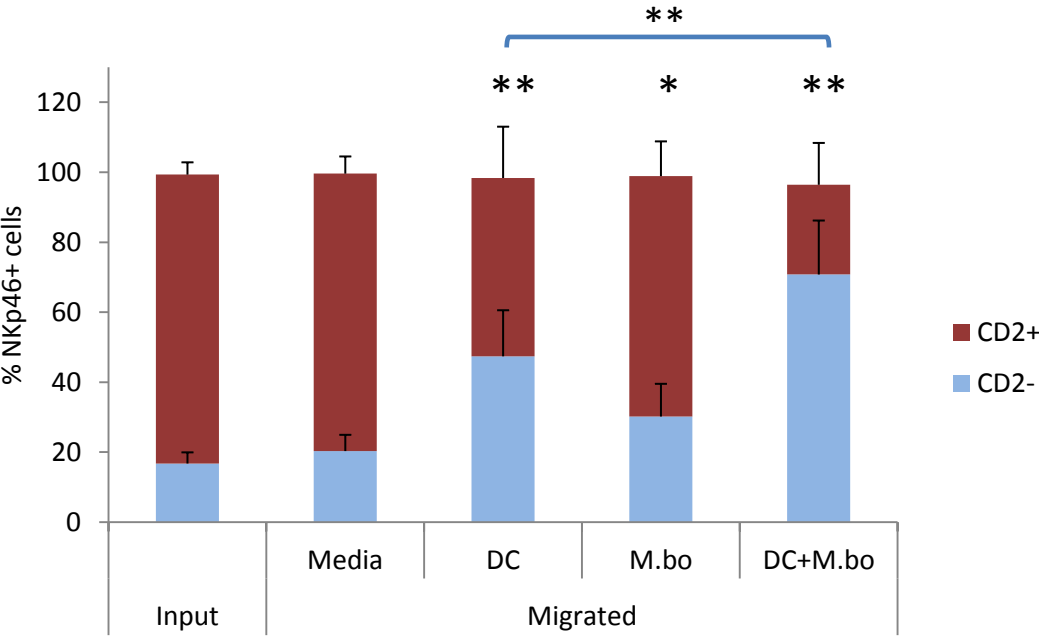


Figure 4.3 Migration of NK cells in response to *M.bovis* infected DCs

Monocyte derived DCs were infected with M.bovis (MOI 1:1) and after overnight infection were used as attractants in chemotaxis transwell assays. Input NK cells were isolated from peripheral blood by positive MACS selection and migration was assessed in response to M.bovis infected DCs (Fig 4.3.1) with Media (TCM), DCs or BCG alone as controls. Each condition was assayed in triplicate and data represents the average migration, expressed as a percentage of the input population from a group of six animals with each data point denoting the individual responses of animals. Rhu CXCL12 was used as a positive control (data not shown).

To determine proportions of migratory NK cells subsets, triplicates of input and migrated fractions from each condition were pooled independently and labelled for CD2 (CC42-647) and analysed by flow cytometry (Fig 4.3.2). Data represents the proportions of NKp46+ cells that were CD2+ (red) or CD2- (blue) in migrated populations with error bars demonstrating the standard deviation between animals. Statistical significance between conditions was assessed using the 1-Way ANOVA (Fig 4.3.1) or a GLM (Fig 4.3.2) with Tukey post-hoc tests with 95% CI $p<0.05^*$, $p<0.01^{**}$

4.2.2.2 Migration of NK cell subsets

Previous assays with total NK cell populations derived from peripheral blood implied migratory responses may be subset specific however, did not fully clarify the individual responses of sub-populations of NK cells (4.2.2.1). For this reason, CD2⁺ and CD2⁻ NK cell subsets were purified and analysed for migratory response to DCs infected with *M.bovis* using conditions which had previously demonstrated a specific induction of NK cell migration (4.2.2.1).

Small percentages of NK cells expressing CD2 migrated in response to *M.bovis* infected DCs, (Figure 4.4) but observed levels were not significantly above those found in response to the control conditions of media, DCs or *M.bovis* alone. In comparison, *M.bovis* infected DCs specifically recruited CD2⁻ NK cells with significantly higher percentages of migrating cells compared with media ($p < 0.001$), DCs ($p = 0.004$) or *M.bovis* ($p = 0.042$). Furthermore, significantly higher percentages of CD2⁻ NK cells migrated in response to *M.bovis* ($p < 0.001$) or *M.bovis* infected DCs ($p < 0.001$) when compared with their CD2⁺ counterparts. Further analysis of migrated fractions of NK cells indicated no alterations in the intensity of CD2 expression prior to and after migration and no significant cell death within either population.

Whilst *M.bovis* infected DCs were able to specifically recruit CD2⁻ NK cells, only a proportion of the overall NK cell population (25-35%) responded in this manner (Figure 4.4). In order to further phenotype this population, migrated fractions (DC+M.bo) were labelled for NK cell associated molecules; CD16, CD27, CD8 $\alpha\alpha$, CD8 $\alpha\beta$ and CD62L (2.12). No significant differences were found in either the percentage of cells, or the intensity of expression for any of the NK cell surface expressed molecules (data not shown). Further analysis will be required to determine the specific functional or phenotypic attributes of the small proportion of NKp46⁺ CD2⁻ cells that preferentially migrate towards *M.bovis* infected DCs.

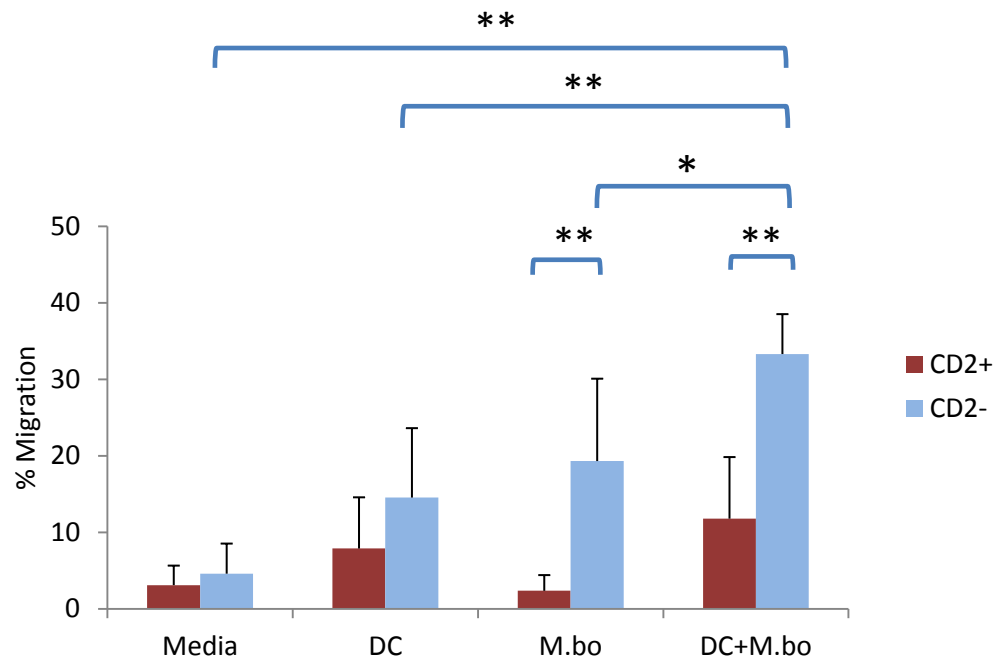


Figure 4.4 Migration of NK cell subsets in response to *M.bovis* infected DCs

Monocyte derived DCs were infected with *M.bovis* and after overnight infection were used as attractants in chemotaxis transwell assays. Input NK cell subsets were purified from peripheral blood and migration was assessed in response to *M.bovis* infected DCs (Fig 4.4) with Media (TCM), DCs or *M.bovis* alone as controls. Each condition was assayed in triplicate and data represents the average migration of CD2+ (red) and CD2- (blue) NK cells, expressed as a percentage of the input population, from a group of six animals - error bars denote standard deviation between animals. Rhu CXCL12 was used as a positive control (data not shown). Statistical significance between subsets and conditions was assessed using the GLM with Tukey post-hoc comparisons (95% CI) $p < 0.05^*$, $p < 0.01^{**}$

4.2.3 Chemokine expression by BCG or *M.bovis* infected DCs

Within migratory assays, infected DCs were able to selectively recruit sub-populations of NK cells. Recruitment of cells in this manner is commonly associated with gradients of chemokines able to induce the migration of neighbouring cells expressing cognate receptors. In the absence of assays to detect protein levels within the supernatants of infected DCs, chemokine expression was assessed by quantitative PCR. Monocyte derived DCs were infected with either BCG or *M.bovis* and chemokine transcripts were analysed at 3, 6, 12 and 24 hours post-infection. Chemokines to be analysed were selected based on known involvement in NK cell migration during an inflammatory immune response or during lymphoid homing.

Uninfected DCs transcribed high basal levels of the inflammatory chemokines CCL2, CCL3, CCL4, CCL5 and CXCL8 (Figure 4.5). In comparison, CCL8, CCL20, CXCL9 and CXCL10 were present at comparatively lower levels. Upon infection with *M.bovis*, DCs underwent significant transcriptional changes with the inflammatory chemokines; CCL3, CCL4, CCL5, CCL20 and CXCL8 augmented within 3-6 hours post-infection (Figure 4.6). After the initial increase in transcription at 3 hours post-infection, levels of CCL4 (Figure 4.6.2) and CCL5 (Figure 4.6.3) remained elevated over the 24 hour period, with no further increase in expression (Table 4.2). In comparison transcription of CCL3 (Figure 4.6.1), CCL20 (Figure 4.6.4) and CXCL8 (Figure 4.6.5) continued to increase over time, with levels of CCL3 and CXCL8 at 24 hours post-infection significantly augmented compared with levels at 3 and 6 hours (Table 4.2). Following *M.bovis* infection, CCL20 was the most highly induced chemokine with transcripts peaking at 12 hours post-infection (Table 4.2). In contrast, *M.bovis* infection of DCs did not affect the expression levels of CCL2, CCL8, CXCL9 or CXCL10 (Table 4.1), with no detectable levels of CCL19 at any time points.

Infection with BCG did not induce large transcriptional changes of chemokines with only CCL5 (Figure 4.6.6) and CCL20 (Figure 4.6.7) up-regulated after 6-12 hours of infection. However, despite up-regulation, the levels of CCL5 ($p=0.035$) and CCL20 ($p=0.001$) induced with BCG were significantly lower than those transcribed after

M.bovis infection suggesting the virulent mycobacterial strain resulted in greater transcriptional induction of chemokines by DCs.

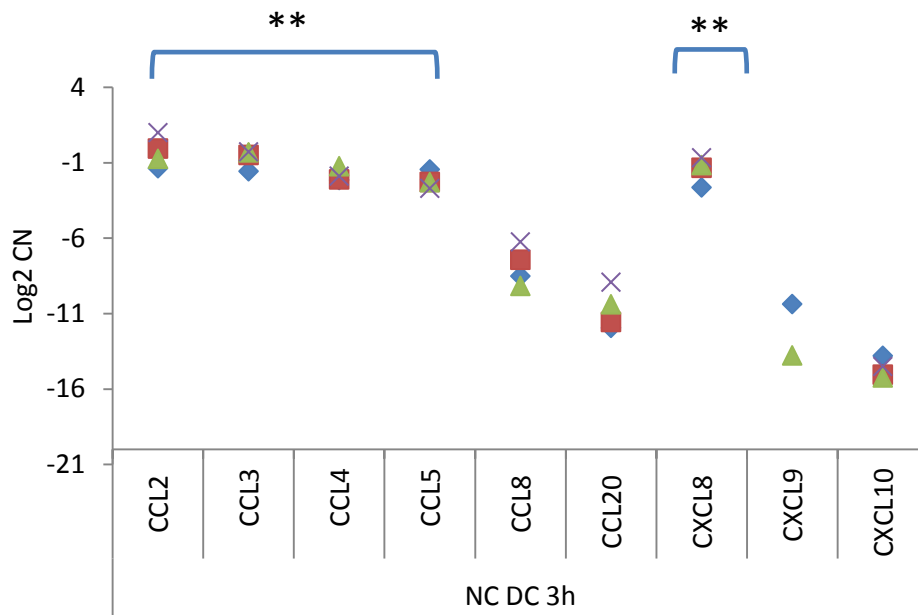
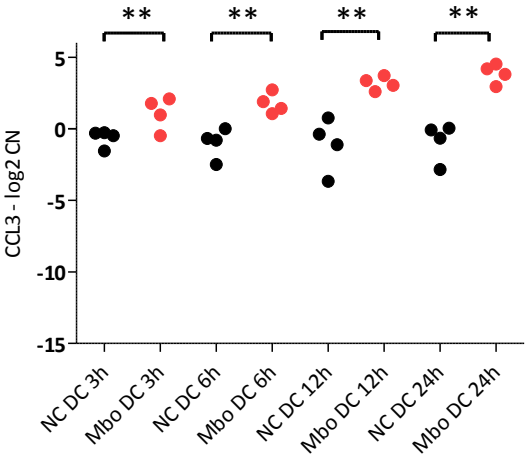


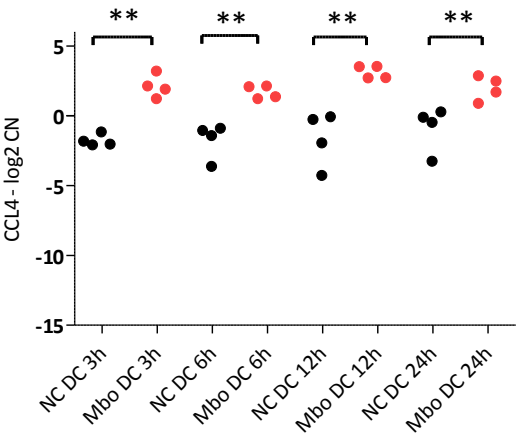
Figure 4.5 Chemokine expression by uninfected DCs

Peripheral blood derived monocytes from four animals were cultured for 3 days to obtain DCs. RNA was obtained from uninfected samples at 3hr and was reverse transcribed to cDNA for analysis of chemokine transcripts by quantitative PCR. Data represents the Log2 copy number (CN) of CCL2, CCL3, CCL4, CCL5, CCL8, CCL20, CXCL8, CXCL9 and CXCL10. Significance between chemokines was assessed using a GLM with Tukey post-hoc comparisons (95%) CI $p < 0.05^*$ $P < 0.01^{**}$

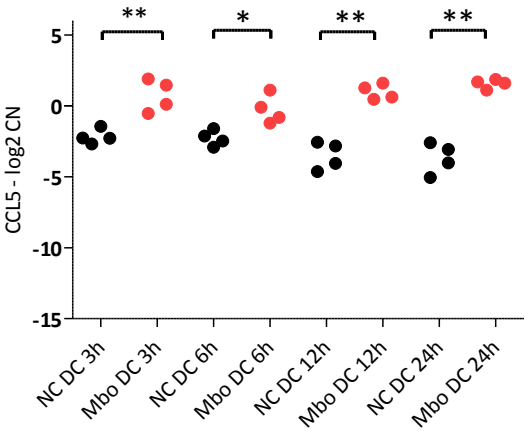
4.6.1



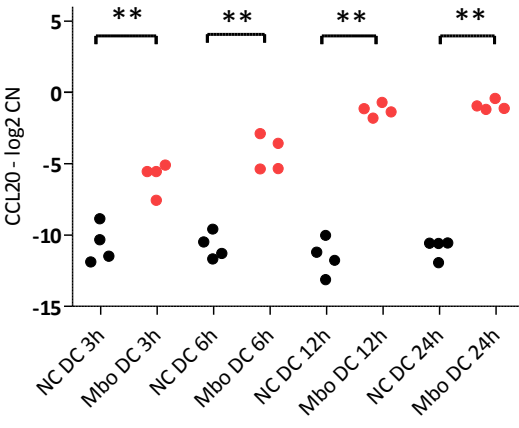
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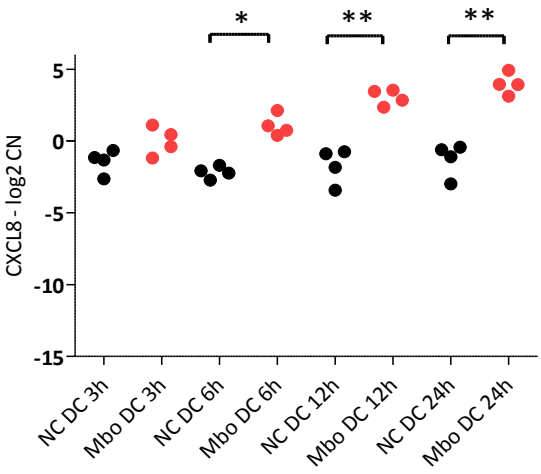
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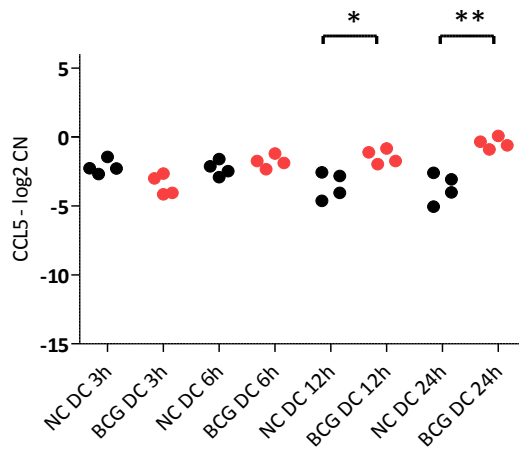
4.6.4



4.6.5



4.6.6



4.6.7

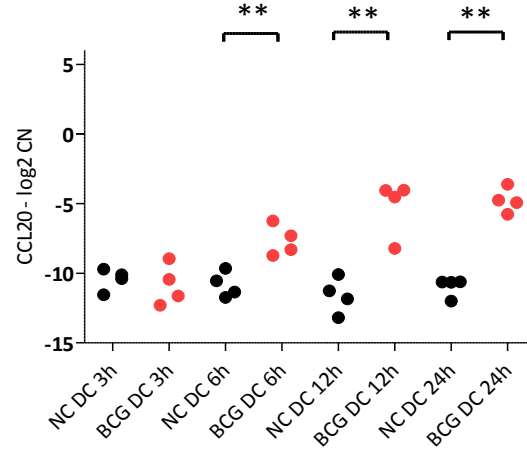


Figure 4.6 Chemokine expression by BCG or *M.bovis* infected DCs

Peripheral blood derived monocytes from four animals were cultured for 3 days to obtain DCs which were infected with BCG (MOI 10:1) or *M.bovis* (MOI 1:1). RNA was obtained from samples at 3, 6, 12 and 24 hours post-infection with uninfected DCs at each time point serving as a negative control. The RNA was reverse transcribed to cDNA and analysed for chemokine transcripts by quantitative PCR. Data represents the Log2 copy number (CN) of CCL3 (Fig 4.6.1), CCL4 (Fig 4.6.2), CCL5 (Fig 4.6.3), CCL20 (Fig 4.6.4), CXCL8 (Fig 4.6.5) from *M.bovis* infected DCs and CCL5 (Fig 4.6.6) and CCL20 (Fig 4.6.7) from BCG infected DCs. Significance between chemokines, infections or between uninfected (black) and infected DCs (red) was assessed using a GLM with Tukey post-hoc comparisons (95%) CI $p < 0.05$ * $P < 0.01$ **

		CCL2 (MCP-1)	CCL3 (MIP1 α)	CCL4 (MIP1 β)	CCL5 (RANTES)	CCL8 (MCP-2)	CCL20 (MIP3 α)	CXCL8 (IL-8)	CXCL9 (MIG)	CXCL10 (IP10)
DC + M.bo	3hr	NS	++	++	++	NS	++	NS	NS	NS
	6hr	NS	++	++	+	NS	++	+	NS	NS
	12hr	NS	++	++	++	NS	++	++	NS	NS
	24hr	NS	++	++	++	NS	++	++	NS	NS
DC + BCG	3hr	NS	NS	NS	NS	NS	NS	NS	NS	NS
	6hr	NS	NS	NS	NS	NS	++	NS	NS	NS
	12hr	NS	NS	NS	+	NS	++	NS	NS	NS
	24hr	NS	NS	NS	++	NS	++	NS	NS	NS

Table 4.1 Summary of chemokines up-regulated by infected DCs

DCs infected with BCG or M.bovis were analysed for chemokine expression levels by quantitative PCR. Significance between uninfected and infected samples was assessed at each time point using a GLM with Tukey post-hoc comparisons; $p < 0.05$ (+), $p < 0.01$ (++) or no significance (NS). Pooled data from four animals is summarised in the table above.

DC + M.bo	CCL3	3hr	6hr	12hr	24hr	CCL4	3hr	6hr	12hr	24hr
	3hr		NS	++	++	3hr		NS	NS	NS
	6hr			NS	++	6hr			NS	NS
	12hr				NS	12hr				NS
	24hr					24hr				
	CCL5	3hr	6hr	12hr	24hr	CCL20	3hr	6hr	12hr	24hr
	3hr		NS	NS	NS	3hr		+	++	++
	6hr			NS	+	6hr			++	++
	12hr				NS	12hr				NS
	24hr					24hr				
DC + BCG	CXCL8	3hr	6hr	12hr	24hr	CCL5	3hr	6hr	12hr	24hr
	3hr		NS	+	++	3hr		NS	NS	+
	6hr			NS	+	6hr			NS	NS
	12hr				NS	12hr				NS
	24hr					24hr				
	CCL20	3hr	6hr	12hr	24hr	CCL20	3hr	6hr	12hr	24hr
	3hr		+	++	++	3hr		+	++	++
	6hr			NS	+	6hr			NS	+
	12hr				NS	12hr				NS
	24hr					24hr				

Table 4.2 Summary of changes in chemokine transcripts over time within infected DCs

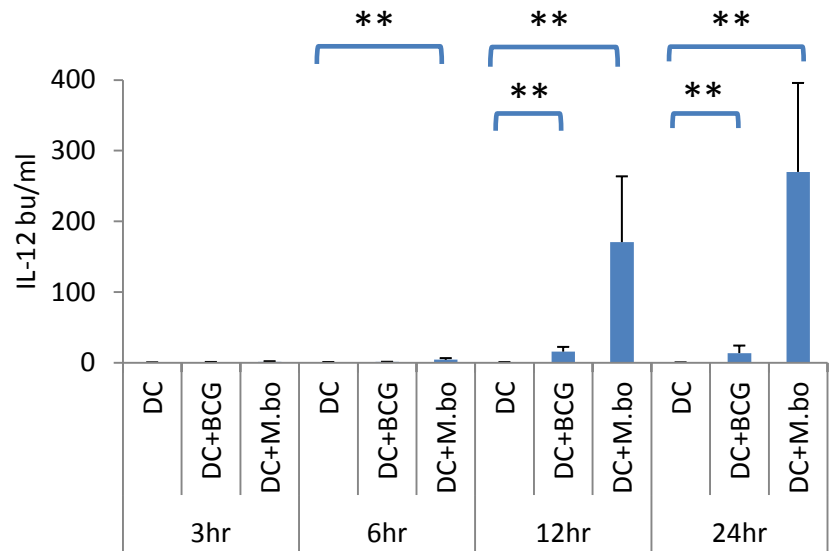
DCs infected with BCG or M.bovis were analysed for chemokine expression levels by quantitative PCR. Significance between the different time points (3/6/12/24 hours) was assessed using a GLM with Tukey post-hoc comparisons; $p < 0.05$ (+), $p < 0.01$ (++) or no significance (NS). Pooled data from four animals is summarised in the table above.

4.2.4 Cytokine production by infected DCs

Infection of DCs with *M.bovis* induced up-regulation of chemokine transcripts and recruitment of NK cells within *in vitro* chemotaxis assays. Previous research has suggested that upon mycobacterial infection, DCs are also able to produce cytokines (Hickman et al., 2002, Hope et al., 2004) such as IL-12 or IL-10 which can mediate the subsequent immune response and in this instance may influence the activation of recruited NK cells.

Supernatants were obtained at 3, 6, 12 and 24 hours post-infection (*M.bovis* or BCG) and were assayed for IL-12 and IL-10 protein levels by ELISA (2.8). Differences between infected and uninfected samples were evaluated using a GLM with Tukey post-hoc comparisons (95% CI). DCs infected with BCG produced IL-12 at significant levels (Figure 4.7.1) at 12 and 24 hours ($p < 0.001$) post-infection. However, infection with *M.bovis* resulted in a much larger induction of IL-12 production compared with BCG and levels were significantly elevated after 6, 12 and 24 hours ($p < 0.001$). Whilst significant increases in IL-10 were observed (Figure 4.7.2) after 6, 12 and 24 hours ($p < 0.001$) post-*M.bovis* infection, comparative to IL-12 the levels of protein detected were negligible.

4.7.1



4.7.2

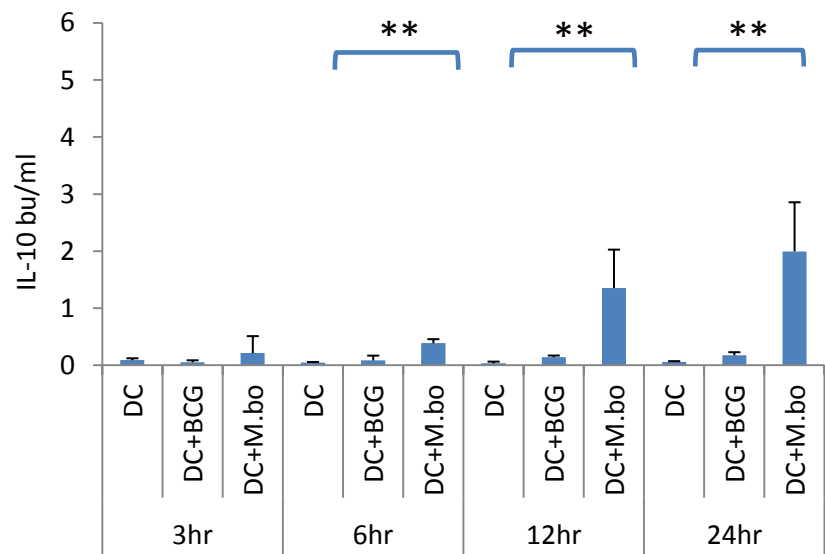


Figure 4.7 Cytokines produced by infected DCs

*Peripheral blood derived monocytes were cultured for 3 days to obtain DCs which were infected with BCG or M.bovis. Supernatants were retrieved at 3, 6, 12 and 24 hours post-infection with uninfected DCs at each time point serving as a negative control. Supernatants were subjected to analysis for IL-12 (Fig 4.6.1) and IL-10 (Fig 4.6.2) by ELISA. Data represents the average cytokine levels from 4 animals in biological units (bu) with error bars denoting the standard deviation between animals. Significance was assessed using log transformed data within a GLM with Tukey post-hoc comparisons (95% CI) $p < 0.05$ * $P < 0.01$ ***

4.3 Discussion

A prerequisite to the effective containment and development of protective immunity against mycobacterial pathogens is the recruitment of leukocytes to the site of infection as well as to draining lymph nodes. Chemokines are central to this process with gradients created within the local milieu or across endothelial junctions able to induce the directional migration and recruitment of cells bearing cognate receptors. As such, chemokines are produced by numerous cells in a highly complex and redundant system that functions to orchestrate the localisation of immune effector cells. Antigen presenting cells, prevalent within the respiratory tract are a significant source of inflammatory chemokines with alveolar macrophages reported to up-regulate expression as early as 4 hours following *M.bovis* infection (Widdison et al., 2011). Importantly, chemokines produced by maturing DCs function to recruit NK cells (Lande et al., 2003) enabling co-localisation and initiation of bi-directional interactions resulting in skewing of Th1 mediated immunity (Martin-Fontecha et al., 2004). Murine models have demonstrated the fundamental role that DCs play, with deficiencies resulting in aberrant recruitment of both NK cells and CD4+ T cells (Uchida et al., 2007). Experiments conducted herein sought to establish whether mycobacterially infected DCs mediated the recruitment of bovine NK cells and whether differential responses could be observed between subsets given that the patterns of chemokine receptor expression also varied significantly (3.2.4).

The release of chemokines by DCs is a dynamic process that occurs in a time-ordered fashion to induce the differential recruitment of leukocytes. Within this study, a range of chemokines were selected with roles in inflammation and lymphoid homing to determine expression levels over 24 hours following infection of DCs with *M.bovis* or BCG. It has been reported that the early secretion of inflammatory chemokines by maturing DCs (Piqueras et al., 2006, Sallusto et al., 2000, Sallusto et al., 1999) aids the accumulation of innate effector cells in attempts to rapidly control infection. DCs infected with *M.tuberculosis* up-regulated expression of CCL2, CCL3, CCL4 and CXCL8 within 3 hours of infection (Lande et al., 2003); receptors for which are widely expressed, in particular by granulocytes, macrophages and NK cells (Inngjerdigen et al., 2001, Ferrero et al., 2003). In a similar manner, transcript levels of inflammatory

chemokines CCL3, CCL4, CCL5, CXCL8 and CCL20 were augmented within 3-6 hours following infection of bovine DCs with *M.bovis* (Figure 4.6). In contrast *M.bovis* did not alter expression levels of CCL2 or CCL8; however these chemokines have been associated with anti-inflammatory immune responses (Chensue et al., 1996) and may not play a significant role during *M.bovis* infection. Maturing DCs sustained elevated expression of both CCL4 and CCL5 (Figure 4.6), a response likely to enable sustained recruitment of CCR5 expressing leukocytes (Sallusto et al., 1999) and levels of CCL3, CXCL8 and CCL20 continually increased over the 24 hour infection period (Figure 4.6). Interestingly, CCL20 was the most highly induced chemokine following *M.bovis* infection. Studies suggest that CCL20 plays a prominent role during an inflammatory immune response with rapid release by airway epithelial cells contributing to the early accumulation of DCs bearing CCR6 (Thorley et al., 2005). As such, elevated levels of CCL20 have readily been detected within supernatants from PBMCs and BAL fluid of TB infected patients (Lee et al., 2008).

The balance of chemokines produced by DCs changes during the advancing stages of maturation that occur following infection, to alter the populations of cells recruited. In particular, DCs secrete CXCL9, CXCL10 and CCL19 (Gustafsson et al., 2008, Lande et al., 2003) to promote the lymphoid homing of CXCR3 and CCR7 bearing T cells and NK cells (Yoneyama et al., 2002, Martin-Fontecha et al., 2004). It has been reported that *in vitro*, these chemokines are only detected at later time-points (8-48 hours) following infection of DCs (Piqueras et al., 2006, Sallusto et al., 1999). Within the 24 hour period, despite maturation, *M.bovis* infected DCs did not up-regulate expression of CXCL9 and CXCL10 with no detectable transcripts for CCL19. This may therefore be as a consequence of time and further experimentation would be required to determine whether the expression of lymphoid homing chemokines is augmented after 24 hours.

It is known that the nature of the maturation stimuli which DCs receive substantially influences the effector response. Hope et al., (2004) reported that DCs infected with BCG secreted reduced levels of IL-12 and IL-10 when compared with the levels observed with *M.bovis*; a response which has also been confirmed here (Figure 4.7). Similarly, a significantly reduced induction of chemokine expression by DCs infected with BCG was observed, with only CCL5 and CCL20 augmented at 6-12 hours post-

infection. Comparison of the transcript levels of CCL5 and CCL20 suggest that whilst up-regulated, the response to BCG was markedly reduced compared with *M.bovis*. However, it may be that the response to BCG is slower and both cytokines and chemokines may be augmented at time-points later than 24 hours. Alternatively BCG may induce the release of different chemokines which were not measured in this study. Further experimentation would need to be conducted to determine whether this is the case. In addition, whilst transcript levels appear to be significantly altered, there are no bovine antibodies to detect these chemokines therefore, it is not possible at this time to determine whether the observed levels equate to actual protein produced. In light of this factor, we utilised *in vitro* chemotaxis assays to clarify whether the observed induction of chemokine transcripts could be reflected within migratory responses.

Both granulocytes and monocytes migrated in response to supernatants from *M.bovis* infected DCs (Figure 4.1). This has previously also been reported to occur following *M.bovis* infection of alveolar macrophages (Widdison et al., 2008). Granulocytes are highly migratory and as such represent one of the first populations of cells to accumulate during an inflammatory immune response. Migration in this manner occurs as a consequence of CXCR1 and CXCR2 expression conferring responsiveness to CXCL8 (Ferrero et al., 2003). (Widdison et al., 2010) demonstrated that transcripts for CXCR1, CXCR2 and important inflammatory receptors could also be detected in monocytes derived from the peripheral blood of cattle. In addition, mycobacterially infected DCs rapidly produce CXCL8 (Lande et al., 2003) and data presented herein demonstrated significant increases in expression levels following *M.bovis* infection (Figure 4.6.5). Thus, the granulocyte/monocyte migration observed is likely to be as a consequence of inflammatory chemokines including CXCL8, produced by *M.bovis* infected DCs. Despite this fact, in agreement with previously published evidence (Ferrero et al., 2003, Widdison et al., 2008) significant lymphocyte migration was not observed and increasing the period of infection to potentially alter the balance of chemokines within the supernatant did not alter this response. Reports suggest that *in vitro*, lymphocytes require extensive stimulation prior to induction of chemotactic activity (Ferrero et al., 2003, Lande et al., 2003). Additionally, bovine $\gamma\delta$ T cells were

not responsive to supernatants but migrated when *M.bovis* infected DCs were left *in situ* (Price, 2009). Similarly, bovine NK cells could be recruited in this manner. It is not known why this is the case however, may be as a result of a continuous activatory signal delivered by the *M.bovis* infected DCs which may not be present or at high enough concentrations within supernatants. In addition, migrated NK cells may directly interact with DCs to potentiate chemokine release and enhance the further recruitment of additional NK cells.

NK cells are highly responsive to chemokines and prevalent both as resident populations and also as infiltrating cells during inflammatory immune responses. Murine models have demonstrated important roles of CCR1 (Shang et al., 2000), CCR5 (Khan et al., 2006) and CXCR3 (Martin-Fontecha et al., 2004, Vester et al., 1999) expression by NK cells enabling accumulation at sites of infection as well as within draining lymph nodes. Importantly, it has been shown that DC derived chemokines function to recruit NK cells. Within chemotaxis assays, significant migration of NK cells towards uninfected DCs was evident however; this response was highly variable between different groups of animals. Transcript analysis has revealed that DCs express high basal levels of important inflammatory chemokines CCL2, CCL3, CCL4, CCL5 and CXCL8 (Figure 4.5) and may therefore demonstrate chemokines which are constitutively secreted by DCs (Vissers et al., 2001). Data presented in Chapter 3 showed that NK cells derived from peripheral blood transcribe the chemokine receptors CCR1, CCR2, CCR3, CCR5, CXCR1 and CXCR2; expression of which would confer responsiveness to constitutively produced chemokines. It is possible that NK cells migrate in this manner to exert cytolytic responses upon autologous immature DCs; a mechanism by which NK cells can select appropriately primed DCs for the development of the immune response (Ferlazzo et al., 2003).

Following *M.bovis* infection, DCs recruited NK cells in significant numbers. The response was highly variable in chemotaxis assays that utilised the total NK cell population derived from peripheral blood which are predominantly CD2⁺ with a smaller sub-population of CD2⁻ NK cells. Detailed investigations utilising purified subsets clarified that the minor CD2⁻ NK cell population was preferentially recruited by *M.bovis* infected DCs (Figure 4.4). Variations in the migratory responses of subsets of

human NK cells have also been reported (Berahovich et al., 2006, Campbell et al., 2001) with CD56^{bright} NK cells selectively enriched at inflammatory sites (Dalbeth et al., 2004) and within draining lymph nodes (Fehniger et al., 2003). Bovine CD2- NK cells from peripheral blood preferentially expressed the majority of the inflammatory chemokine receptors including CCR2, CCR5, CCR6 and CXCR3 (3.2.4). In particular CCR5 is a promiscuous receptor and expression confers responsiveness to multiple inflammatory chemokines including CCL3, CCL4 and CCL5; transcripts for which were found to be augmented by *M.bovis* infected DCs (Figure 4.6). Expression of CCR5 was found to be implicit for the accumulation of NK cells to the site of parasitic (Khan et al., 2006) infection. In addition, *Klebsiella pneumoniae* infected human DCs recruited NK cells in a CCR5 dependent manner (Van Elssen et al., 2010). As such, it is possible that CCR5 and its ligands play a prominent role in the induced recruitment of bovine CD2- NK cells. There may also be a further role of CCR6; which was expressed at significantly higher levels by bovine CD2- NK cells, (3.2.4) and its ligand CCL20; transcription of which was highly induced following *M.bovis* infection of DCs (Figure 4.6.4).

In contrast to the responses observed with *M.bovis*, within the conditions investigated herein, DCs infected with BCG failed to recruit NK cells in significant numbers above the response that was already observed prior to infection. This is corroborated by the earlier finding that DCs infected with BCG did not up-regulate transcripts for select inflammatory chemokines. However, it has previously been demonstrated that CCL2, CCL3, CXCL10 and CCL22 can be readily detected within human PBMCs stimulated with BCG (Luo et al., 2007) and therefore, NK cells may still be able to respond to gradients of chemokines produced following administration of BCG. In addition, whilst it appears that there may be differences in the migratory response of NK cells to BCG or *M.bovis* infected DCs; these experiments utilised different groups of animals. We have previously observed significant variability between animals (Figure 4.3) and therefore, in order to further clarify true differences between BCG and *M.bovis*, migratory experiments would have to be repeated whilst accounting for this factor.

Interestingly, within chemotaxis assays bovine NK cells appeared to migrate in response to mycobacteria alone with a more prominent response observed within the CD2- population (Figure 4.4). Studies have suggested that NK cells express functional

formyl peptide receptors which confer migratory responsiveness to bacterial peptides (Kim et al., 2009). It is not known whether this is also the case for bovine NK cells and would require further investigation. In addition, NK cells can be directly activated by mycobacteria, without the need for co-stimulatory signals from accessory cells. Human NK cells cultured with BCG in the absence of a cytokine stimulus; up-regulated activation markers CD69 and CD25, increased production of IFN γ and TNF α , and NK mediated cytolytic activity was induced, with a more prominent effect on CD56^{bright} NK cells (Batoni et al., 2005, Esin et al., 2008, Esin et al., 2004, Marcenaro et al., 2008). Further research elucidated roles of TLR2 (Marcenaro et al., 2008), TLR3, TLR9 (Sivori et al., 2004) and NKp44 (Batoni et al., 2005, Esin et al., 2008) in the direct recognition of bacterial antigens by NK cells. Similar responses have also been observed within bovine NK cells directly responding to *Neospora caninum* to produce IFN γ (Boysen et al., 2006a). It is possible that the direct activation of NK cells in this manner may induce migratory responsiveness. In addition, emerging evidence suggests that NK cells are also an important source of chemokines and upon activation have been reported to secrete CCL3, CCL4 and CCL5 (Dorner et al., 2002, Nieto et al., 1998); chemokines which induce migration of cells bearing CCR5. Furthermore, discrete populations of NK cells produced CCL20 and functioned to recruit additional NK cells expressing CCR6 to maintain resident populations within mucosa associated lymphoid tissue (Cella et al., 2009). Thus, it is possible that NK cells directly activated by *M.bovis* may secrete chemokines and contribute to their own accumulation.

Importantly, percentages of CD2- NK cells recruited by *M.bovis* infected DCs were significantly higher compared with the numbers migrating in response to mycobacteria alone indicating an additional chemotactic response derived from infected DCs. In further investigations, definitive evidence for the role of chemokines could be provided by utilising NK cells incubated with *pertussis* toxin as a mechanism by which G-protein signalling may be inhibited. In previous studies, bovine CD2- NK cells have been reported to represent the more activated population with faster proliferation, IFN γ release and cytolytic activity following cytokine stimulation (Boysen et al., 2006b). Therefore, the selective recruitment of this population may have implications for

reciprocal interactions between NK cells and DCs and will be investigated further within this thesis.

5 Interactions of NK cells and DCs during *M.bovis* infection

5.1 Introduction

The precise requirements for protective immunity to mycobacterial infection are yet to be established however, the early production of IFN γ plays an important role in potentiating the innate immune response and effectively shaping adaptive immunity. NK cells are a significant source of IFN γ which functions to regulate macrophage bactericidal activity within *M.tuberculosis* infected lungs (Feng et al., 2006) as well as playing an implicit role in the priming of CD4+ and CD8+ T cells to polarise Th1 biased immunity (Mailliard et al., 2003, Martin-Fontecha et al., 2004). Increasingly the recruitment of NK cells and subsequent interplay with DCs is thought to play a fundamental role within this process. This may occur both at the site of infection as well as within draining lymph nodes with reciprocal interactions influencing the activation of both cell populations.

Immune interactions between NK cells and DCs were first described over a decade ago with mature DCs directly triggering cytolytic responses of NK cells to restrict tumour growth (Fernandez et al., 1999). The interaction has subsequently been the focus of intense research, with depletion of DCs found to result in the impaired activation of NK cells (Lucas et al., 2007). It has become apparent that initiation of effector responses of both NK cells and DCs requires multiple co-stimulatory signals (Fehniger et al., 1999a, Ortaldo and Young, 2003). NK cells produced IFN γ in response to *M.tuberculosis* infected DCs (Gerosa et al., 2002), however the response was only optimal following stimulation from DC derived cytokines as well as direct cell-cell contact. Importantly, it has been reported that the DC induced IFN γ production and proliferative responses of NK cells may be restricted to the CD56^{bright} sub-population, depletion of which resulted in a loss of IFN γ release (Ferlazzo et al., 2004a, Vitale et al., 2004). In addition, DCs are able to trigger NK cell derived cytolytic activity to augment the killing of *M.tuberculosis* infected monocytes and alveolar macrophages (Vankayalapati et al., 2005) or immature DCs (Hayakawa et al., 2004, Wilson et al., 1999, Yu et al., 2001). BCG infected DCs promoted NK cell derived lytic activity against autologous immature DCs (Ferlazzo et al., 2003), with susceptibility reduced upon maturation following up-

regulation of HLA class I (Ferlazzo et al., 2003, Ferlazzo et al., 2001). Consequently NK cells function to bias the immune response to those DCs which have undergone appropriate maturation.

Bacterially infected DC readily produce abundant levels of IL-12 (Hickman et al., 2002, Hope et al., 2004), which promote NK cell derived IFN γ production and cytolytic activity (Gerosa et al., 2002, Van Elssen et al., 2010). Roles for IL-18 (Artavanis-Tsakonas et al., 2003, Artavanis-Tsakonas and Riley, 2002, Bastos et al., 2008, Van Elssen et al., 2010), IL-15 (Lucas et al., 2007, Van Elssen et al., 2010) and TNF α (Humann and Lenz, 2010) have also been described, however only the inhibition of IL-12 functioned to completely abrogate NK cell proliferation and IFN γ release (Ferlazzo et al., 2004a, Gerosa et al., 2002, Van Elssen et al., 2010, Vitale et al., 2004). In addition, during antigen specific recall responses CD4 $^{+}$ T cell derived IL-2 induced NK cell effector responses which were further augmented by IL-12 derived from myeloid cells (Bihl et al., 2010, Horowitz et al., 2010a, Horowitz et al., 2010b).

Separation of NK cells from DCs has demonstrated that as well as soluble mediators, direct cell-cell contact is required for optimal activation of NK cells (Gerosa et al., 2002, Humann and Lenz, 2010, Vitale et al., 2004, Yu et al., 2001) with numerous studies attempting to elucidate the exact nature of the contact-dependent signals. In particular, NK cells in close proximity to DCs form immunological synapses to enable the polarised delivery of IL-12 (Borg et al., 2004) and potentially IL-18 (Semino et al., 2005) with an important role of CX3CR1 expression by NK cells to facilitate tethering to DCs (Pallandre et al., 2008). Furthermore, IL-15 bound to the surface of DCs may be trans-presented to promote proliferative responses of NK cells (Ferlazzo et al., 2004a, Humann and Lenz, 2010, Lucas et al., 2007). Additional interactions may include CD40/CD40L (Carbone et al., 1999) or activating KIR binding to MHC class I peptide complexes (Newman et al., 2006). Importantly, the recognition and killing of immature or infected DCs has been reported to be reliant on expression of CD94/NKG2A (Della Chiesa et al., 2003), NKp30 (Ferlazzo et al., 2002, Marcenaro et al., 2005) and potentially also NKG2D recognising MICA/B ligands expressed by DCs (Jinushi et al., 2003).

During reciprocal interactions, NK cells have been reported to influence the maturation and effector responses of DCs and in this manner act as a bridge between the innate and adaptive immune systems. NK cell derived TNF α and IFN γ functioned to promote the maturation of DCs (Mailliard et al., 2003), a process which also required NKp30 mediated signalling (Vitale et al., 2005). Consequently DCs matured in this manner produced elevated levels of CXCL9, CXCL10 (Lande et al., 2003) and IL-12 (Mailliard et al., 2003). Yet perhaps most significantly, IFN γ release by CD4 $^{+}$ (Van Elssen et al., 2010) and CD8 $^{+}$ T cells (Mailliard et al., 2003, Morandi et al., 2009) was augmented following DC interactions with NK cells.

Within the bovine system, it has been reported that NK cells secrete IFN γ and restrict mycobacterial replication within *M.bovis* infected macrophages (Denis et al., 2007). However the extent to which these reciprocal interactions may occur between bovine NK cells and DCs has previously not been investigated. It is known that *M.bovis* infected DCs are a major source of IL-12 (Hope et al., 2004) and IFN γ release from bovine NK cells was at least in part mediated by IL-12 derived from infected antigen presenting cells (Olsen et al., 2005). In addition BCG infected DCs induced the proliferation and IFN γ production from CD3 $^{-}$ CD8 $^{+}$ lymphocytes believed to be NK cells (Hope et al., 2002b); responses which required both cytokine production and cell-cell contact (Bastos et al., 2008). In light of these factors, experimental co-cultures were utilised to investigate whether reciprocal interactions may occur between blood derived NK cells and monocyte derived DCs during *M.bovis* infection. In particular, these studies focussed on whether the heterogeneity in the migratory responses observed between subsets of bovine NK cells could also be observed in their interactions with DCs.

5.2 Results

5.2.1 Activation of NK cells following culture with *M.bovis* infected DCs

To investigate the interactions of NK cells with *M.bovis* infected DCs, we utilised *in vitro* co-culture assays and initially determined whether there were alterations in the CD25 expression levels of NK cell subsets. The IL-2 receptor- α -chain (CD25) is widely expressed by lymphocytes and its up-regulation is commonly used as an indicator of T cell or NK cell activation. Monocyte derived DCs from four animals were infected with *M.bovis* and cultured with peripheral blood derived NK cells overnight prior to analysis of CD25 cell surface expression (2.12.1). NK cells cultured alone, with uninfected DCs or with *M.bovis*, served as control conditions.

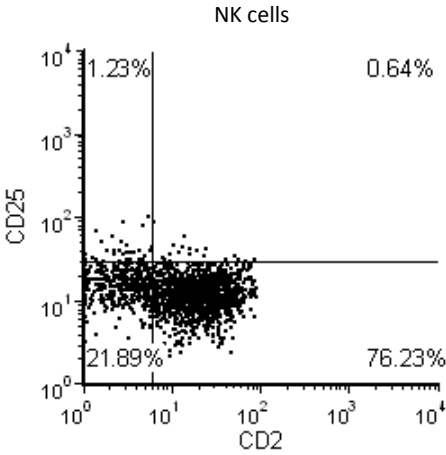
A very small percentage of CD2⁻ NK cells were found to constitutively express CD25; however this was not significantly higher than their CD2⁺ counterparts. Therefore, the majority of NK cells from peripheral blood did not express CD25 (Figure 5.1.1) and this was not significantly altered after either subset was cultured alone, with uninfected DCs or with *M.bovis* (Figure 5.1.3). In contrast, after NK cells were cultured with *M.bovis* infected DCs a significant increase in the percentages of CD2⁺ ($p < 0.001$) and CD2⁻ ($p < 0.001$) NK cells expressing CD25 was observed when compared with all other control conditions (Figure 5.1.2 and 5.1.3). Analysis of the proportions of CD25 positive NK cells within each subset, expressed as a percentage of the total proportion of CD2⁺ or CD2⁻ NK cells highlighted that similar percentages of NK cells within each subset were activated.

Detailed analysis of the mean fluorescence intensity of CD25 (Figure 5.1.4) indicated that whilst percentages of CD2⁺ and CD2⁻ NK cells expressing CD25 were similar, the intensity of expression by CD2⁻ NK cells was significantly augmented following culture with *M.bovis* infected DCs compared with control conditions ($p < 0.001$) and also compared with the intensities observed with CD2⁺ NK cells ($p < 0.001$) under all conditions. Furthermore, visual investigation of the FACS data (Figure 5.1.2) highlighted the presence of a population of CD2⁻ NK cells within the total CD2⁻ population (29% - gated in red) which was expressing CD25 at higher intensities after

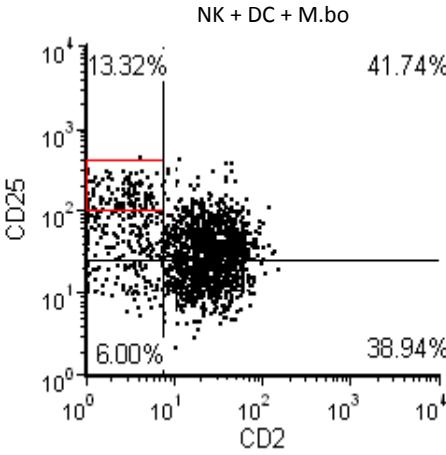
contact with *M.bovis* infected DCs and may therefore indicate differential levels of activation within this subset of NK cells.

Further phenotypic examination of CD25 expressing NK cells indicated no alterations in the expression levels of NK cell markers; CD16, CD27, CD8 $\alpha\alpha$, CD8 $\alpha\beta$ or CD62L. Additionally, no changes in NKp46 or CD2 expression levels were observed and no significant proliferation or cell death of either subset was observed after co-culture with *M.bovis* infected DCs (data not shown).

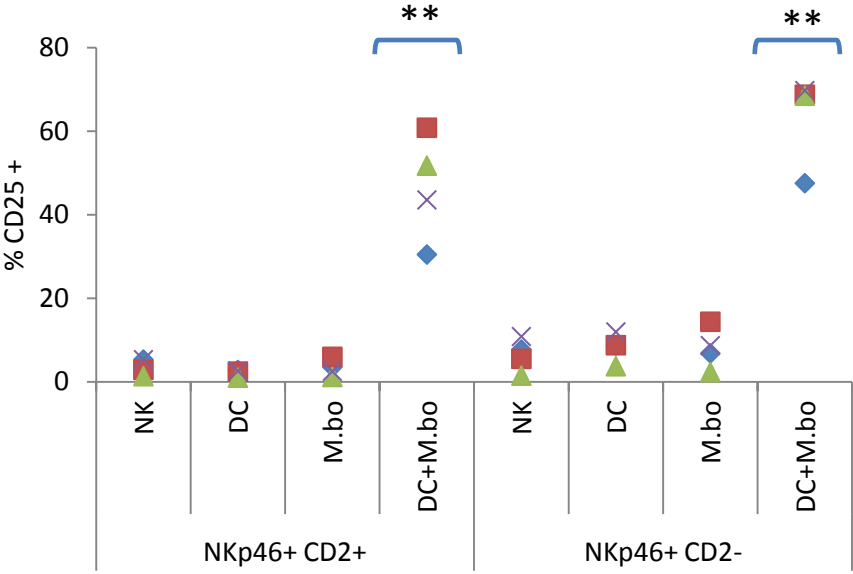
5.1.1



5.1.2



5.1.3



5.1.4

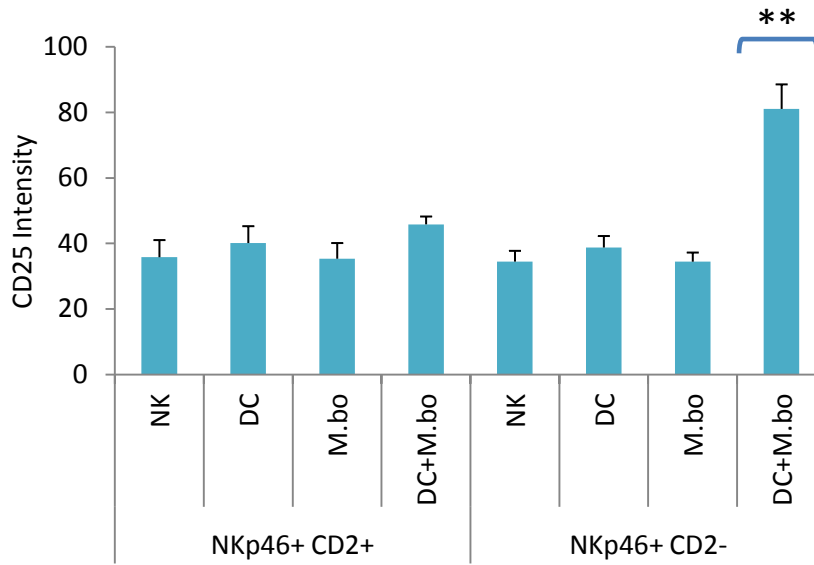


Figure 5.1 Activation of NK cells following co-culture with *M.bovis* infected DCs

Monocyte derived DCs were cultured for three days and infected with *M.bovis*. NK cells were purified from peripheral blood and cultured with the infected DCs at previously determined optimal ratios (2.10). Each culture condition was replicated in triplicate with NK cells cultured alone, with uninfected DCs or with *M.bovis* alone serving as controls. After overnight co-culture cells were labelled with mAbs for NKp46, CD2 and CD25 and analysed by flow cytometry. FACS plots from one representative animal display the percentages of CD2+ or CD2- NK cells expressing CD25 within the total gated NK cell population; NK cells alone (Fig 5.1.1) or NK cells after co-culture with *M.bovis* infected DCs (Fig 5.1.2) with a population of CD2- NK cells expressing high intensities of CD25 gated in red. Quadrants were set based on isotype controls and unstained cells. Pooled data from four animals (Fig 5.1.3) indicates the percentage of CD25 positive NK cells expressed as a percentage of the total CD2+ or CD2- population and the mean fluorescence intensity of CD25 (Fig 5.1.4) with error bars representing the standard deviation between animals. Significant changes in CD25 expression between culture conditions were assessed using log transformed data and a GLM with Tukey post-hoc comparisons (95%CI); $p < 0.05^*$, $p < 0.01^{**}$.

5.2.2 Cytokine production in co-cultures of NK cells and DCs

Upon activation, NK cells are able to produce immuno-modulatory cytokines such as IFN γ and TNF α and this contributes to their implicit role in the early appropriate priming of the subsequent immune response. DC derived cytokines are fundamental to the control of this response. Since *M.bovis* infected DCs appeared to induce the activation of NK cells, as determined by up-regulation of CD25 expression, further analysis was conducted to evaluate the cytokines produced by both NK cells and DCs to indicate which factors may be influencing the observed activation.

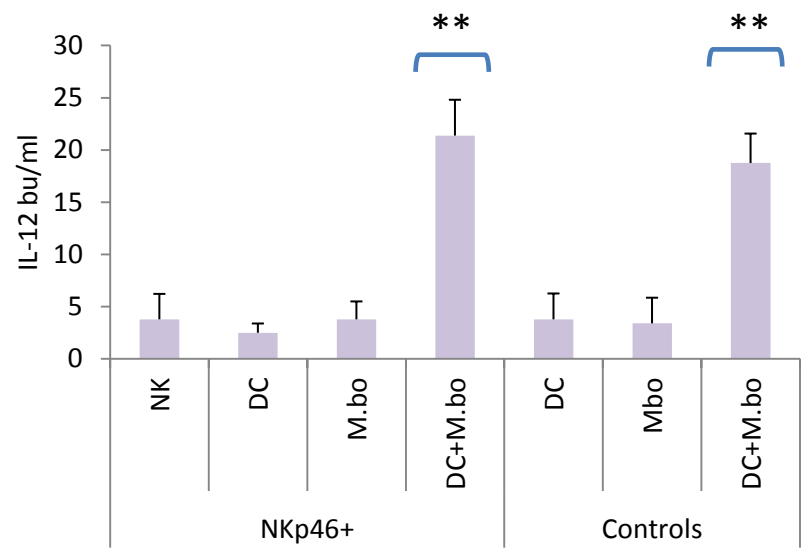
Supernatants were retrieved from co-culture experiments (5.2.1) and assayed for cytokines; IFN γ , TNF α , IL-12, IL-10, IL-6 and IL-2 by ELISA (2.8). Within initial experiments, peripheral blood derived NK cells isolated by positive selection of NKp46 expressing lymphocytes (2.4) were cultured with *M.bovis* infected DCs overnight prior to retrieval of supernatants. Further experiments involved use of NK cell subsets, identified and purified by differential expression of CD2 (2.4.1). NK cells cultured alone, with uninfected DCs or with *M.bovis* alone served as control conditions.

As highlighted previously (4.2.4), upon mycobacterial infection DCs produced elevated levels of the important inflammatory mediator IL-12 (Figures 5.2.1 and 5.2.2) when compared with the control conditions of DCs alone ($p=0.005$) or *M.bovis* alone ($p=0.003$). Within the co-culture conditions, either addition of total NK cells (Figure 5.2.1) or CD2+ and CD2- NK cell subsets (Figure 5.2.2) did not significantly influence the IL-12 levels that were already induced after infection of DCs with *M.bovis*. Neither IL-6 nor IL-2 were detected within any of the co-culture conditions and as previously described (4.2.4) only very low levels of IL-10 were found after infection. These levels were not altered after culture with NK cells (data not shown).

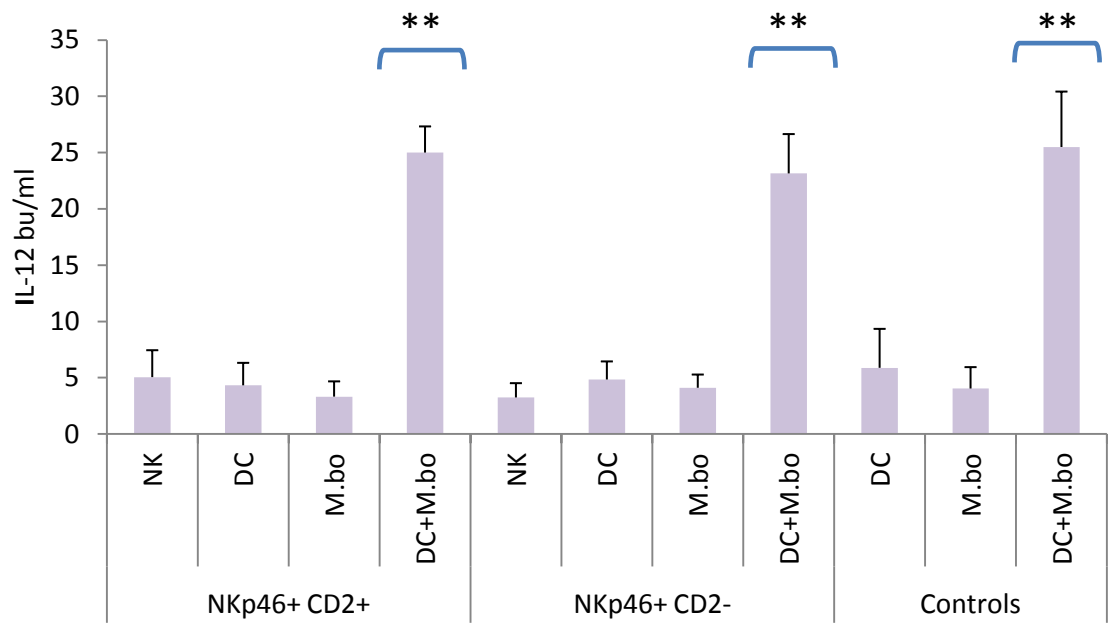
Analysis of supernatants by ELISA for NK cell derived cytokines indicated no detectable levels of TNF α . In contrast, total populations of NKp46+ NK cells produced significantly elevated levels of IFN γ but only after contact with *M.bovis* infected DCs (Figure 5.2.3) with no detectable levels produced within any of the control conditions of NK cells alone or in culture with uninfected DCs or *M.bovis* alone ($p<0.001$). Further analysis to determine the contribution of CD2+ and CD2- NK cell subsets to this response,

established that only CD2⁻ NK cells produced IFN γ ($p<0.001$) compared with all controls, with no detectable levels produced by CD2⁺ NK cells under any conditions (Figure 5.2.4). Furthermore, purification of CD2⁻ NK cells resulted in augmented levels of IFN γ (Figure 5.2.4) when compared with the levels observed with total NK cells (Figure 5.2.3), a reflection of an increase in the proportion of IFN γ producing cells within the purified cell co-cultures.

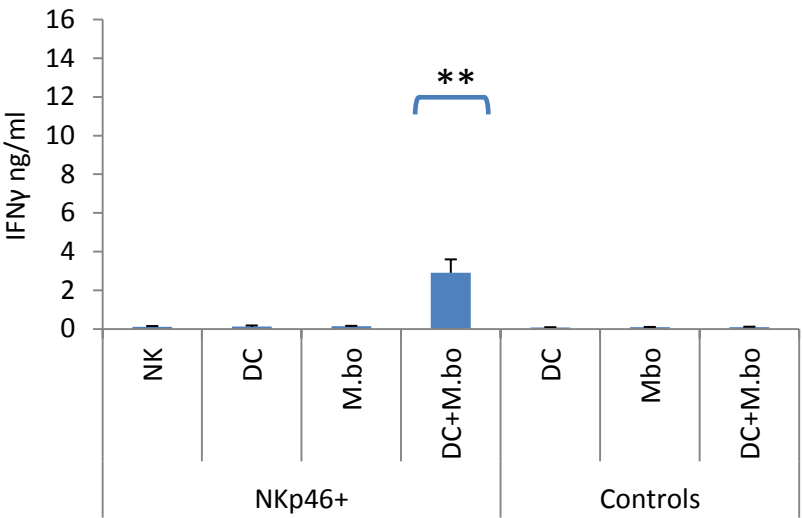
5.2.1



5.2.2



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5.2.4

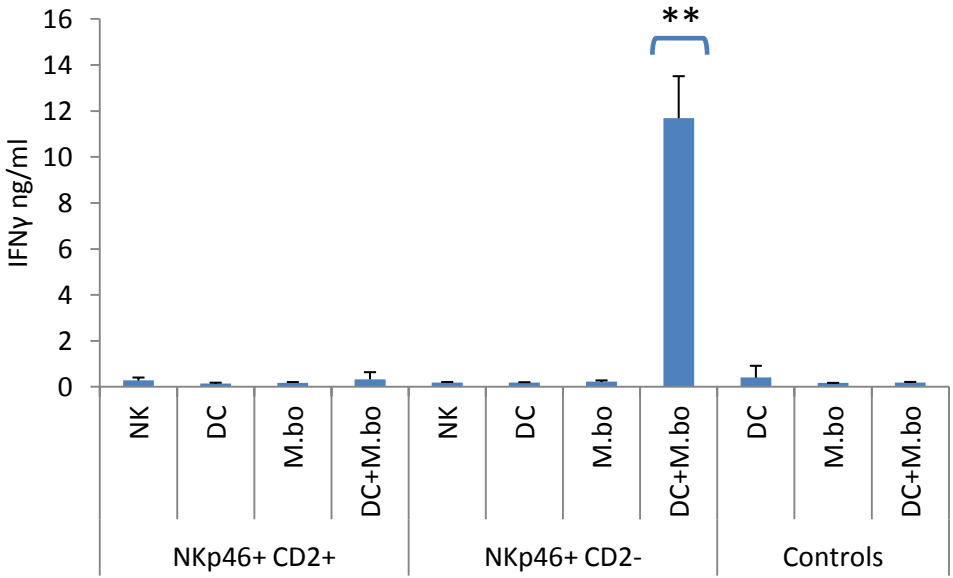


Figure 5.2 Cytokine production within co-cultures of NK cells and DCs

Monocyte derived DCs were cultured for three days and infected with M.bovis. Either total NK cells (Fig 5.2.1 and 5.2.3) or NK cell subsets (Fig 5.2.2 and 5.2.4) were purified from peripheral blood and cultured with the infected DCs at previously determined optimal ratios (2.10). Each culture condition was replicated in triplicate with NK cells cultured alone, with uninfected DCs or with M.bovis alone serving as controls. After overnight co-culture supernatants were retrieved and cytokine production was assayed by ELISA (2.8). Data represents the average levels of IL-12 bu/ml (Fig 5.2.1 and 5.2.2) or IFN γ ng/ml (Fig 5.2.3 and 5.2.4) from 4 animals with error bars representing the standard deviation between animals. Significance between co-culture conditions was assessed using log transformed data and a GLM with Tukey post-hoc comparisons (95% CI); $p < 0.05^*$, $p < 0.01^{**}$.

5.2.2.1 Assessing the IFN γ producing capacity of CD2+ NK cells

Studies of bovine NK cell subsets have illustrated that upon cytokine stimulation with IL-2 and IL-12, the CD2- subset appeared inherently better able to produce IFN γ when compared with CD2+ NK cells (Boysen et al., 2006b). Within co-culture assays, the predominant CD2+ NK cell subset found in peripheral blood was not able to produce detectable levels of IFN γ despite contact with *M.bovis* infected DCs. To determine whether purified CD2+ NK cells lacked the capacity to produce IFN γ , IL-12 and IL-18 stimulation of NK cell subsets (2.5) was incorporated as an additional condition and IFN γ and IL-12 levels within supernatants were measured by ELISA (2.8).

As previously observed, CD2+ NK cells did not produce IFN γ (Figure 5.3) despite the presence of secreted IL-12 from *M.bovis* infected DCs (data not shown). However, supplementation of NK cell activating cytokines IL-12 and IL-18 into the co-culture with the *M.bovis* infected DCs induced a significant increase in secretion of IFN γ ($p < 0.001$) up to levels observed with either IL-12 and IL-18 stimulation alone (CD2+ NK cells) or IL-12 and IL-18 stimulation of CD2- NK cells; indicating that the CD2+ NK cells were capable of IFN γ secretion.

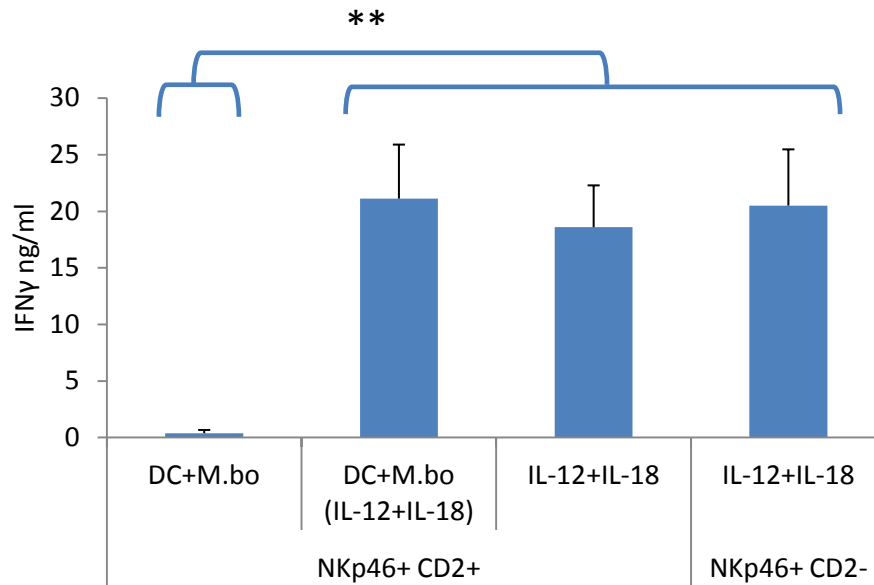


Figure 5.3 Assessing the IFN γ producing capacity of CD2 $^{+}$ NK cells

*Co-culture experiments were repeated with peripheral blood derived CD2 $^{+}$ NK cells co-cultured with *M.bovis* infected DCs, supplemented with or without IL-12 and IL-18 at optimal concentrations (2.5). Each culture condition was replicated in triplicate with cytokine stimulation of CD2 $^{+}$ and CD2 $^{-}$ NK cells serving as controls. Retrieved supernatants were assayed for IFN γ and IL-12 (data not shown) by ELISA (2.8). Data represents the average levels of IFN γ ng/ml (Fig 5.3) from 4 animals with error bars representing the standard deviation between animals. Significance between co-culture conditions was assessed using log transformed data and a GLM with Tukey post-hoc comparisons (95% CI); $p < 0.01^{**}$.*

5.2.2.2 The role of IL-12 in NK cell and DC interactions

DC derived cytokines such as IL-12 are considered particularly important to the induction of NK cell activation. Results herein have suggested that *M.bovis* infection results in IL-12 production (5.2.2) and that both CD2⁺ and CD2⁻ NK cell subsets supplemented with IL-12 along with IL-18 are able to produce IFN γ (5.2.2.1).

For this reason, the contribution of IL-12 to the observed IFN γ production by CD2⁻ NK cells in contact with *M.bovis* infected DCs was investigated (5.2.2). Blocking experiments were optimised using IL-12 stimulated NK cells supplemented with the IL-12 specific mAb CC326 (9.2) at a range of concentrations (0.1-100 μ g/ml) and analysis of IFN γ levels within supernatants. After cytokine stimulation, NK cells produced elevated levels of IFN γ (Figure 5.4). The subsequent addition of CC326 resulted in significantly decreased IFN γ levels at all tested concentrations, with 10 μ g/ml found to be sufficient to reduce IFN γ (>90% reduction) back to the basal levels produced by unstimulated NK cells. It has previously been shown that supplementation with isotype control antibodies does not influence IL-12 induced IFN γ secretion (Hope et al., 2002a) indicating that the blocking effect observed with CC326 was specifically inhibiting IL-12.

Introduction of CC326 at optimal concentrations into the co-culture induced effective blocking (Figure 5.5.1 and 5.5.3), with significantly reduced detectable IL-12 levels within supernatants of CD2⁻ NK cells cultured with *M.bovis* infected DCs ($p=0.046$) (49.3 ± 14.7 % reduction) and also with CD2⁻ NK cells stimulated with IL-12 and IL-18 ($p=0.031$) (61.5 ± 15.5 % reduction). Importantly, analysis of IFN γ levels produced under these conditions highlighted that CD2⁻ NK cells stimulated with IL-12 and IL-18 at optimal concentrations produced high levels of IFN γ and blocking of IL-12 resulted in only a marginal reduction ($3.9 \pm 6.1\%$) in the observed levels. In contrast, blocking of IL-12 produced by *M.bovis* infected DCs resulted in a substantial ($62.5 \pm 18.8\%$) and significant ($p=0.017$) reduction in the IFN γ produced by CD2⁻ NK cells (Figure 5.5.2 and 5.5.3).

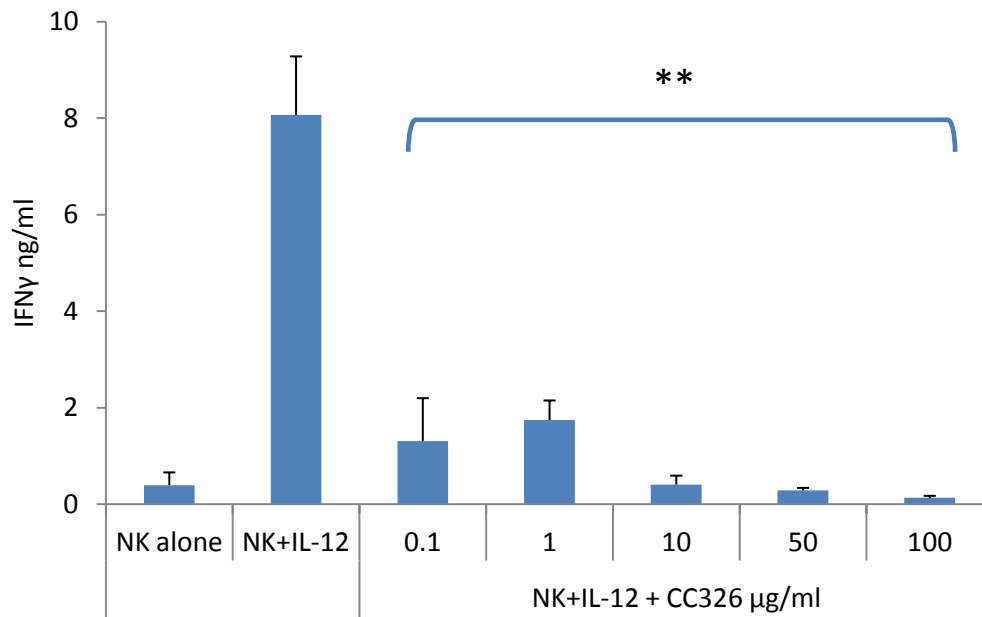
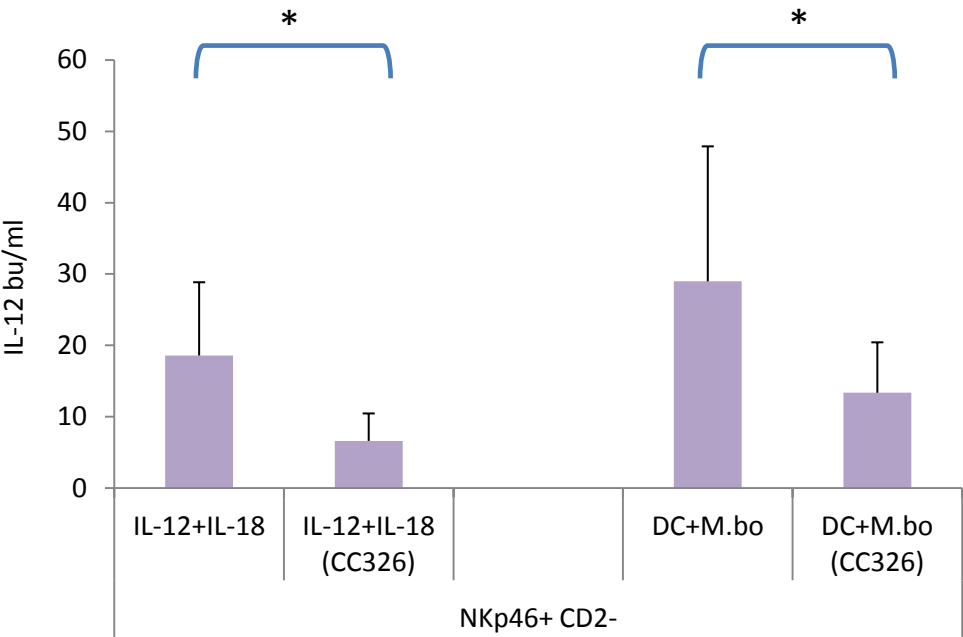


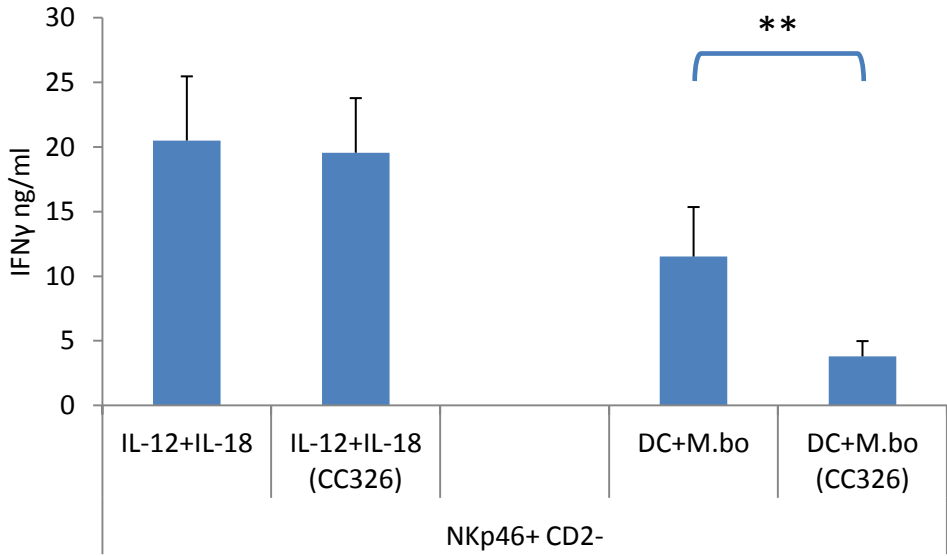
Figure 5.4 Blocking of IL-12 induced IFN γ production

NK cells isolated from peripheral blood were cultured with IL-12 at optimal concentrations (2.5) and supplemented with IL-12 blocking antibody - CC326 (9.2) at dilutions ranging from 0.1-100 μ g/ml. NK cells without stimulation were used as a control and supernatants retrieved were analysed for IFN γ levels by ELISA (2.8). Data represents the average IFN γ levels (ng/ml) from 2 animals with error bars denoting the standard deviation between animals. The efficacy of CC326 was assessed using log transformed data and a 1-Way ANOVA with Tukey post-hoc comparisons (95% CI); $p < 0.01^{**}$.

5.5.1



5.5.2



5.5.3

	% reduction in IL-12	% Loss of IFN γ
IL-12+IL-18 (CC326)	61.9% $\pm$ 15.5	3.9% $\pm$ 6.1
DC+M.bo (CC326)	49.3% $\pm$ 14.7	62.5% $\pm$ 18.8

Figure 5.5 Contribution of IL-12 to IFN γ production within co-culture

Co-culture experiments were repeated with peripheral blood derived CD2- NK cells co-cultured with M.bovis infected DCs with or without CC326 (10 μ g/ml). Each culture condition was replicated in triplicate with IL-12 and IL-18 cytokine stimulation of CD2- NK cells serving as a control. Supernatants were retrieved and analysed for cytokine levels by ELISA. Data represents the average levels of IL-12 bu/ml (Fig 5.5.1) and IFN γ ng/ml (Fig 5.5.2) from 4 animals with error bars representing the standard deviation between animals. The percentage reduction in IL-12 and IFN γ following supplementation with CC326 is summarised (Table 5.5.3) with efficacy of CC326 blocking assessed using log transformed data and pair-wise T-tests (95% CI); $p < 0.05^*$, $P < 0.01^{**}$.

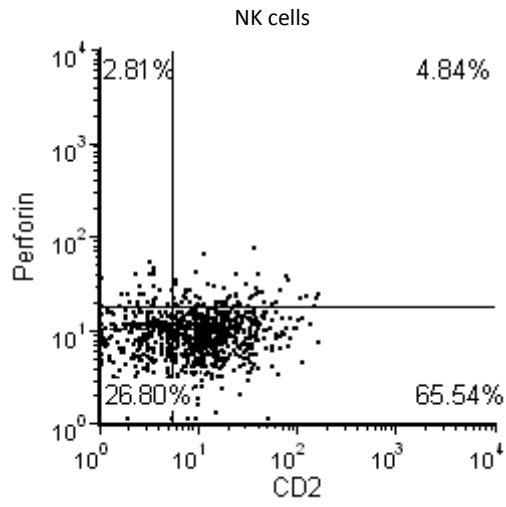
5.2.3 NK cell cytolytic activity

Fundamental to the role of NK cells within the innate immune system is their capacity to function as cytolytic lymphocytes, a response activated by the balance of signals received through activatory and inhibitory receptors resulting in the release of cytolytic proteins such as perforin and granzymes. For this reason, intracellular perforin levels of NK cells were analysed as a measure of their capacity to kill target cell populations. In particular, experiments were performed to determine whether contact with mycobacterially infected DCs was able to influence the cytolytic potential of NK cells.

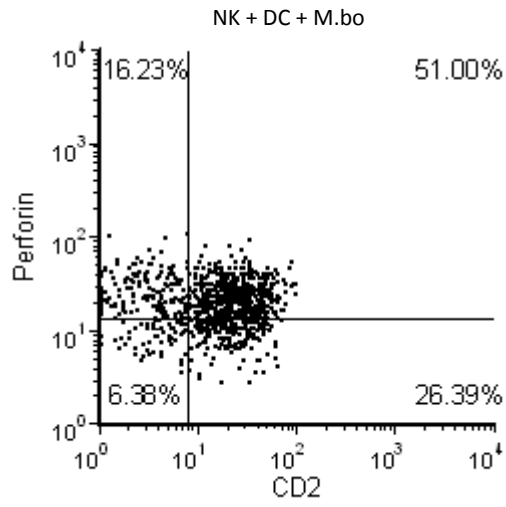
Monocyte derived DCs from four animals were infected with *M.bovis* and cultured with peripheral blood derived NK cells overnight prior to analysis of perforin expression (2.12.1). NK cells cultured alone, with uninfected DCs or with only *M.bovis* served as control conditions. With no stimulation, small percentages (5-15%) of both CD2+ and CD2- NK cells expressed detectable levels of perforin (Figure 5.6.1 and 5.6.3). Upon culture with uninfected DCs, a minor increase in the percentage of NK cells expressing perforin could be observed and this was found to be significantly so for CD2- NK cells ($p < 0.001$). Furthermore, a significantly higher proportion of both CD2+ ($p = 0.013$) and CD2- ($p < 0.001$) were found to express perforin in response to *M.bovis* alone (Figure 5.6.3) signifying a certain level of activation without DC involvement. However, within co-cultures with DCs infected with *M.bovis* the response was significantly augmented over all control conditions with 50-70% of both CD2+ ($p < 0.001$) and CD2- ($p < 0.001$) expressing cytolytic proteins (Figure 5.6.2 and 5.6.3) with no observed differences between NK cell subsets.

Additionally, detailed analysis of the mean fluorescence intensity levels indicated that after contact with infected DCs, the increase in the percentage of perforin positive NK cells was coupled with a significant increase in perforin intensity for both CD2+ ($p < 0.001$) and CD2- ($p < 0.001$) NK cells when compared with all control conditions (Figure 5.6.4).

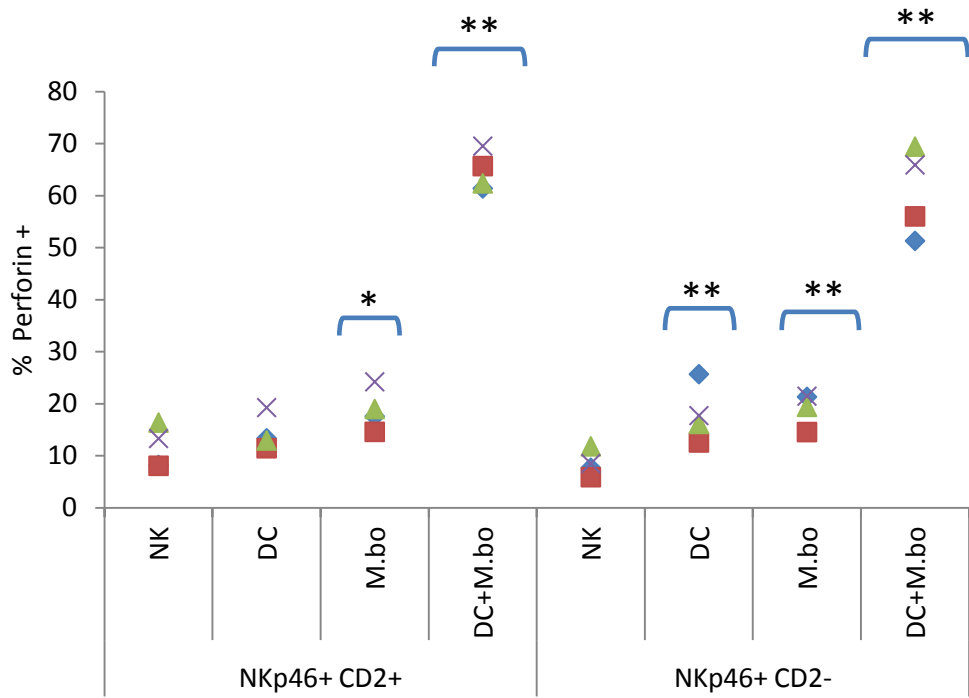
5.6.1



5.6.2



5.6.3



5.6.4

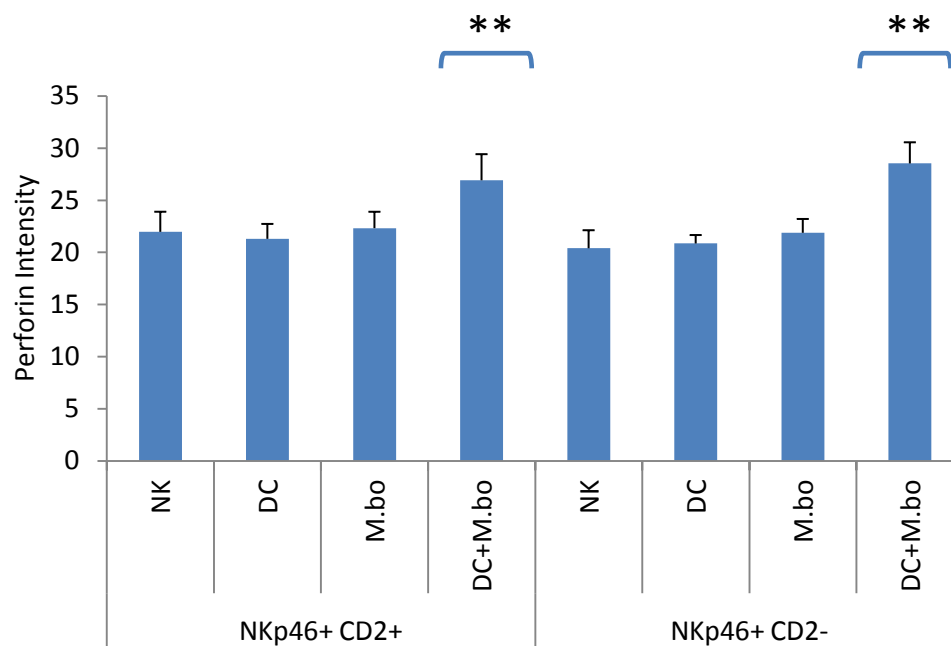


Figure 5.6 Perforin expression within co-cultures of NK cells and DCs

Monocyte derived DCs were cultured for three days and infected with *M.bovis*. NK cells were purified from peripheral blood and cultured with the infected DCs at previously determined optimal ratios (2.10). Each culture condition was replicated in triplicate with NK cells cultured alone, with uninfected DCs or with *M.bovis* alone serving as controls. After overnight co-culture cells were labelled with mAbs for NKp46, CD2 and perforin (2.12.1) and analysed by flow cytometry. FACS plots from one representative animal display the percentages of CD2+ or CD2- NK cells expressing perforin within the total NK cell population; NK cells alone (Fig 5.6.1) or NK cells after co-culture with *M.bovis* infected DCs (Fig 5.6.2). Pooled data from four animals (Fig 5.6.3) indicates the percentage of perforin positive cells expressed as a percentage of the total CD2+ or CD2- NK cell population and the mean fluorescence intensity of perforin (Fig 5.6.4) with error bars representing the standard deviation between animals. Significant changes in perforin production between culture conditions were assessed using log transformed data and a GLM with Tukey post-hoc comparisons (95%CI); $p < 0.05$ *, $p < 0.01$ **.

5.2.3.1.1 TLR expression by NK cell subsets

Investigation of perforin production by NK cells revealed that both CD2⁺ and CD2⁻ NK cells expressed elevated levels of cytolytic proteins in response to mycobacterial stimulation, independent of DC involvement. It has been suggested that NK cells express functional PRRs such as TLRs which enable direct responsiveness to bacterial PAMPs to induce NK cell cytolytic activity (Marcenaro et al., 2008).

To determine if this may also be the case with bovine NK cells, cDNA from peripheral blood derived NK cell subsets (3.2.4) was subjected to transcript analysis for TLRs 1-10 (9.5). Transcripts for all TLRs were detected on NK cells with TLR2 and TLR6 expressed at significantly higher levels than all other TLRs by both CD2⁺ and CD2⁻ NK cells (Figure 5.7). Similar to the patterns found with chemokine receptors, TLRs were also found to be differentially transcribed between subsets of NK cells with elevated levels of TLR1 ($p=0.040$) and TLR7 ($p=0.010$) on CD2⁻ NK cells and TLR3 ($p=0.002$) and TLR4 ($p=0.001$) on CD2⁺ NK cells. However, despite differences between subsets, transcript levels for these were significantly lower than those observed for TLR2 and TLR6 (Figure 5.7).

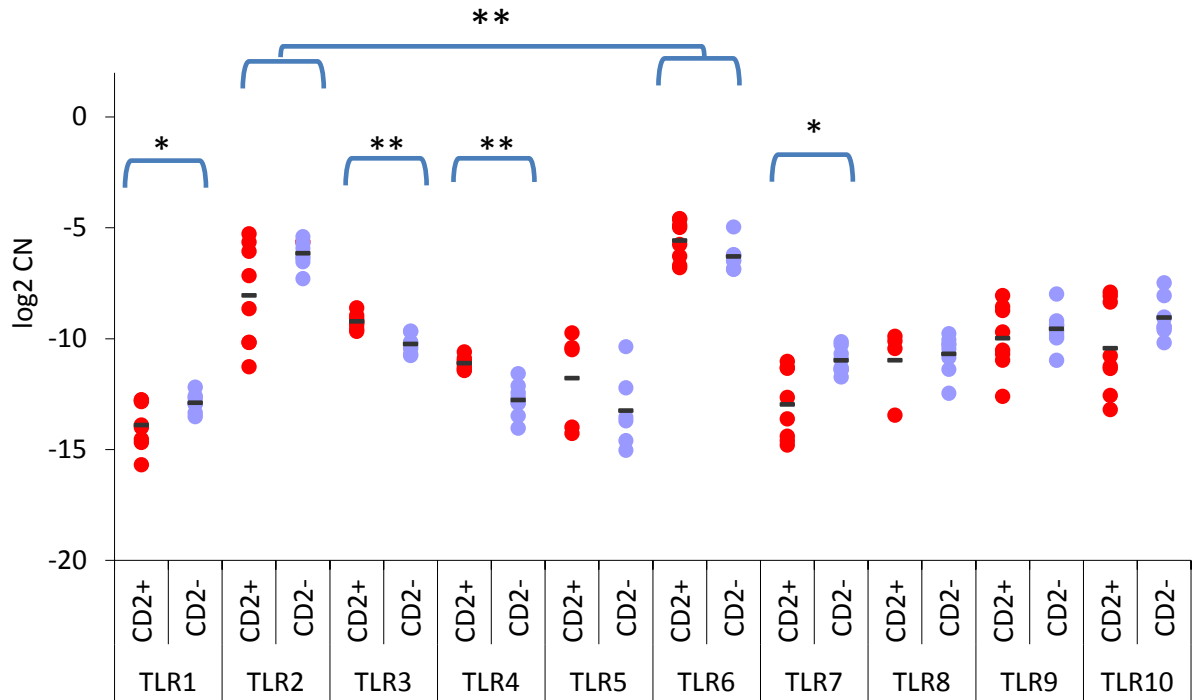


Figure 5.7 TLR expression by NK cell subsets

NK cell subsets (NKp46+ CD2+ and NKp46+ CD2-) were isolated from peripheral blood from eight animals as described (3.2.4). RNA was reverse transcribed and subjected to quantitative PCR analysis for the bovine TLRs. Each sample was tested in triplicate, quantified against a plasmid DNA standard curve and normalised to the housekeeping gene BoPo. Data represents the Log2 normalised copy number (CN), CD2+ NK cells are shown in red and CD2- in blue with each point denoting the transcriptional value obtained from one animal. Significance was assessed using the GLM and paired T-test $p < 0.05$ *, $p < 0.01$ **.

5.2.3.2 Killing Assays

Experimental examination of NK cells after contact with mycobacterially infected DCs demonstrated increased expression of cytolytic proteins and therefore, the potential induction of NK cell mediated killing activity. To determine whether DCs could directly activate a cytolytic response, NK cells from co-culture experiments were incubated with murine tumour P815 cells, known targets for perforin mediated killing by bovine NK cells (Boysen et al., 2006b).

Within initial killing assays, NK cells were incubated with P815 target cells at effector target (E:T) ratios ranging from 25:1 to 0.39:1 (Figure 5.8). Effective killing was determined by identifying percentages of P815 cells which incorporated propidium iodide (2.11) as an indicator of cell death. NK cells were able to kill P815 cells at all E:T ratios tested with no significant cell death observed when P815 cells were cultured without NK cells (data not shown). With increasing numbers of NK cells, the percentages of P815 cells killed also increased with 35-45% killing observed at 25:1. Effective killing was also observed at the E:T ratio of 12.5:1 and this ratio was selected for all subsequent killing assays.

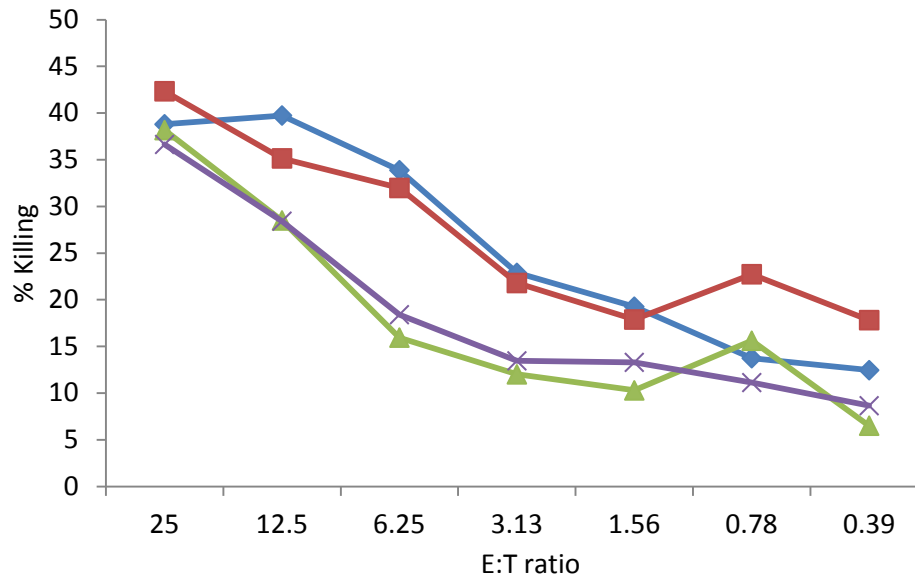


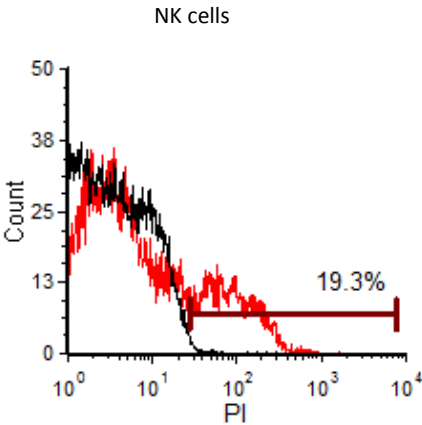
Figure 5.8 Optimising NK cell mediated killing assays

Peripheral blood derived NK cells were isolated by positive selection of NKp46 expressing lymphocytes (2.4). Target P815 cells were labelled with CFDA-SE membrane dye prior to incubation with activated NK cells in triplicate, at a range of effector to target (E:T) ratios for a 3 hour duration. Specific killing was analysed by labelling P815 cells with Propidium Iodide (PI - 25µg/ml) followed by analysis of the proportions of high FSC/SSC, CFDA-SE and PI positive target cells by flow cytometry. Data represents the percentage killing of P815 cells by activated NK cells from 4 animals at a range of E:T ratios.

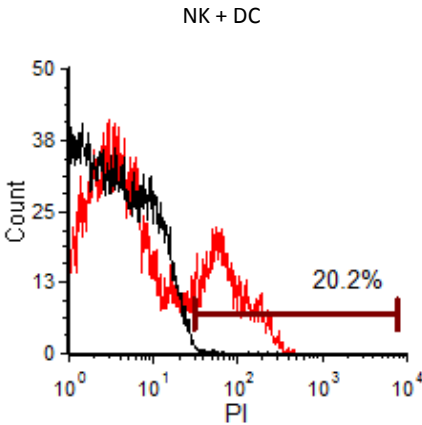
To establish whether the observed increase in perforin production within NK cells cultured with *M.bovis* infected DCs (Figure 5.6.3) translated into effective induction of cytolytic activity, NK cells from co-cultures were incubated with P815 target cells at the optimised E:T ratio of 12.5:1. Total NK cells, rather than subsets were used as perforin production was found to be equivalent between CD2+ and CD2- NK cells (Figure 5.6.3) following co-culture with *M.bovis* infected DCs.

No substantial cell death was observed within the control conditions where uninfected DCs or DCs infected with *M.bovis* were added to cytolytic assays, highlighting the requirement for NK cells for effective target cell killing (Figure 5.9.5). Without prior stimulation, NK cells were found to possess the innate capacity to induce significant killing of P815 target cells (Figure 5.9.1 and 5.9.5). Following culture of NK cells with uninfected DCs (Figure 5.9.2) or *M.bovis* alone (Figure 5.9.3), minor variations in killing activity were observed, but found to not be significant when compared with NK cells alone. In contrast, prior contact with *M.bovis* infected DCs augmented the killing capacity of NK cells with significantly higher percentages of killed/dead P815 cells (Figure 5.9.4 and 5.9.5) compared with all control conditions ($p < 0.001$).

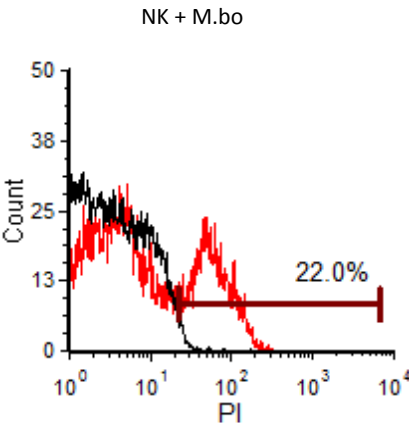
5.9.1



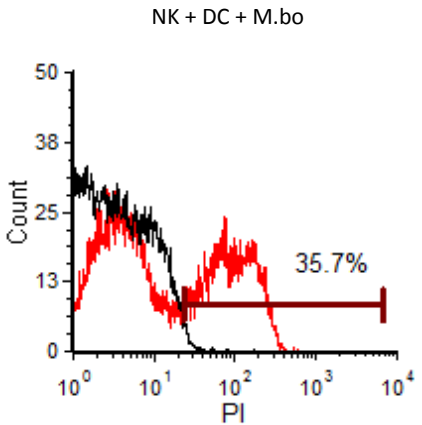
5.9.2



5.9.3



5.9.4



5.9.5

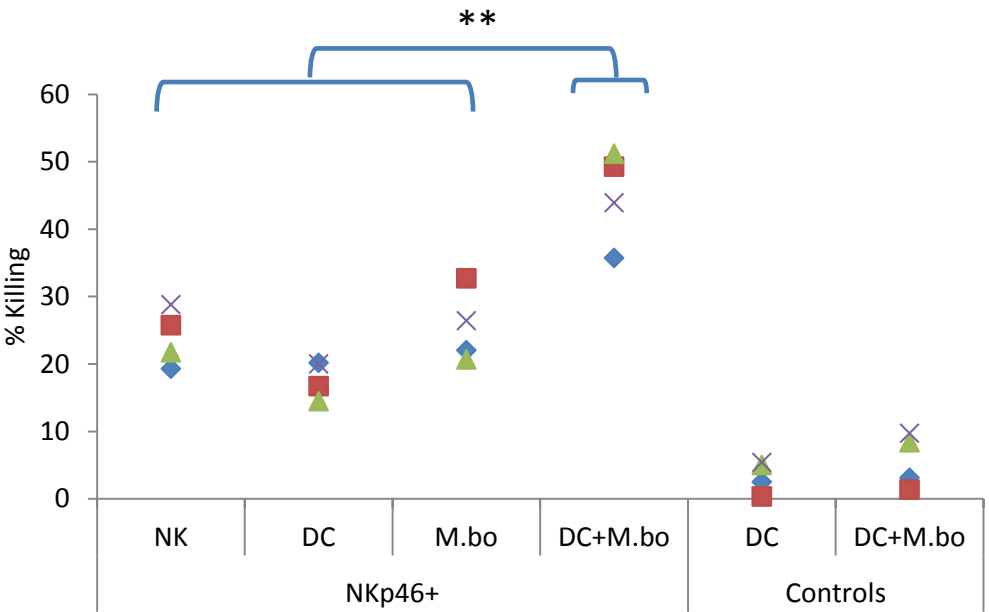


Figure 5.9 *M.bovis* infected DCs augment the killing capacity of NK cells

*Co-culture experiments were repeated with peripheral blood derived NK cells co-cultured with *M.bovis* infected DCs. Each culture condition was replicated in triplicate with NK cells cultured alone, with uninfected DCs or with *M.bovis* alone serving as controls. After overnight co-culture NK cells were retrieved and incubated for 3 hours with CFDA-SE labelled P815 target cells at an optimum (12.5:1) E:T ratio (Fig 5.8). NK cell specific killing was analysed by labelling P815 cells with Propidium Iodide (PI - 25µg/ml) followed by analysis of the proportions of high FSC/SSC, CFDA-SE and PI positive populations by flow cytometry. FACS plots from one representative animal display the percentages of CFDA-SE/ PI positive P815 cells after incubation with NK cells alone (Fig 5.9.1), NK cells with uninfected DCs (Fig 5.9.2), NK cells with *M.bovis* (Fig 5.9.3) or NK cells with *M.bovis* infected DCs (Fig 5.9.4). Pooled data from four animals (Fig 5.9.5) indicates the percentage killing with significance between conditions assessed using log transformed data and 1-way ANOVA's with Tukey post-hoc comparisons (95% CI); $p < 0.05^*$, $p < 0.01^{**}$.*

5.2.4 Contact dependency of NK cell activation

The *in vitro* co-culture assays demonstrated that DCs infected with *M.bovis* were able to functionally activate NK cells; with up-regulation of CD25 and perforin expression resulting in augmented NK cell specific cytolytic activity and cytokine production. DC derived cytokines released following infection were found to be implicit to the increased levels of IFN γ produced by CD2⁻ NK cells. Research has indicated that the interaction between NK cells and DCs may also be reliant on cell to cell contact to enable receptor-ligand interactions and directed cytokine delivery. To determine whether this was also the case with the observed activation of bovine NK cell subsets, co-culture assays were repeated with a culture well insert to prevent cell contact but to retain cytokine induced responses. Subsequent changes in CD25, perforin and IFN γ levels were assayed. Stimulation with IL-12 and IL-18 was incorporated as a culture condition as a positive indicator of cytokine movement through the transwell.

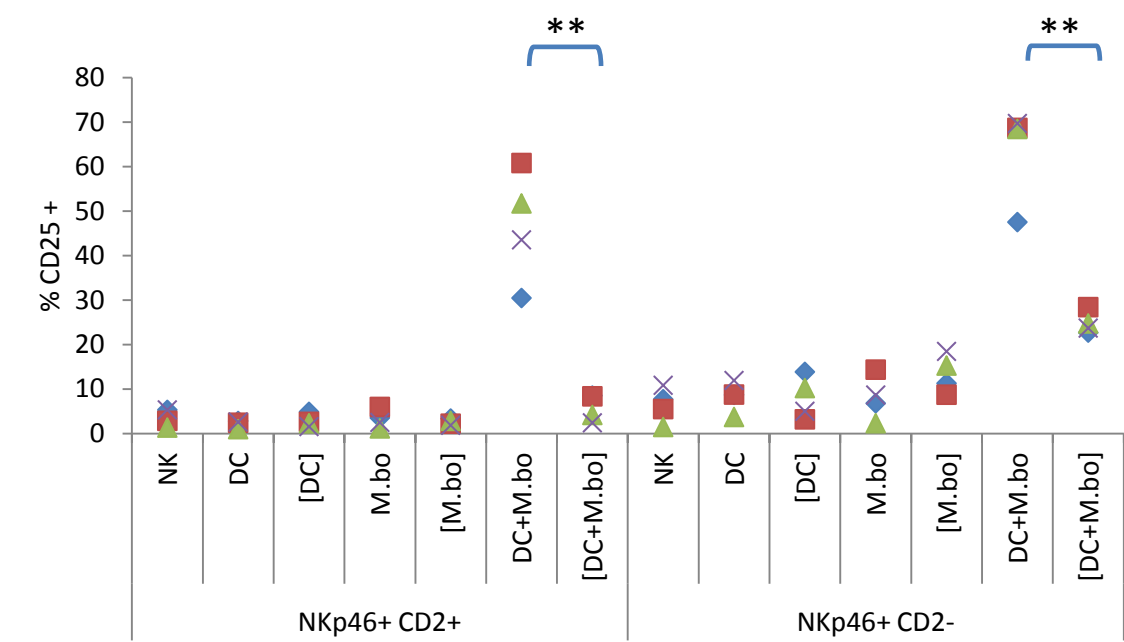
Analysis of CD25, perforin and IFN γ levels revealed a significant dependence on cell-cell contact for NK cell activation by *M.bovis* infected DCs. As expected, within IL-12 and IL-18 controls, no effect of transwell separation was observed (data not shown). However, separation of NK cells from *M.bovis* infected DCs (Figure 5.10.1) resulted in a significant loss in the percentage of CD25 positive CD2⁺ ($p=0.008$) and CD2⁻ NK cells ($p=0.001$). Additionally, blocking cell to cell contact significantly decreased the intensity of CD25 on CD2⁻ NK cells ($p=0.007$) (Figure 5.10.2). Comparison of NK cell subsets highlighted that the loss in the percentage of CD25 expressing CD2⁺ ($40.8\% \pm 13.3$) and CD2⁻ ($38.7\% \pm 9.5$) NK cells was similar (Table 5.10.3), however following transwell separation, there were significantly higher residual numbers of CD2⁻ NK cells expressing CD25 ($p=0.012$) compared with CD2⁺ NK cells (Figure 5.10.1). Therefore, loss of cell-cell contact had a more profound effect on the activation of CD2⁺ NK cells.

Similar experiments to determine the role of contact dependency on perforin production resulted in significantly lower percentages of perforin positive NK cells, after separation of DC ($p=0.014$), *M.bovis* ($p=0.005$), *M.bovis* infected DCs ($p<0.001$) from CD2⁺ NK cells and also DC ($p=0.024$), *M.bovis* ($p=0.030$) and *M.bovis* infected DCs ($p=0.045$) from CD2⁻ NK cells (Figure 5.11.1). Furthermore, there was an observed loss

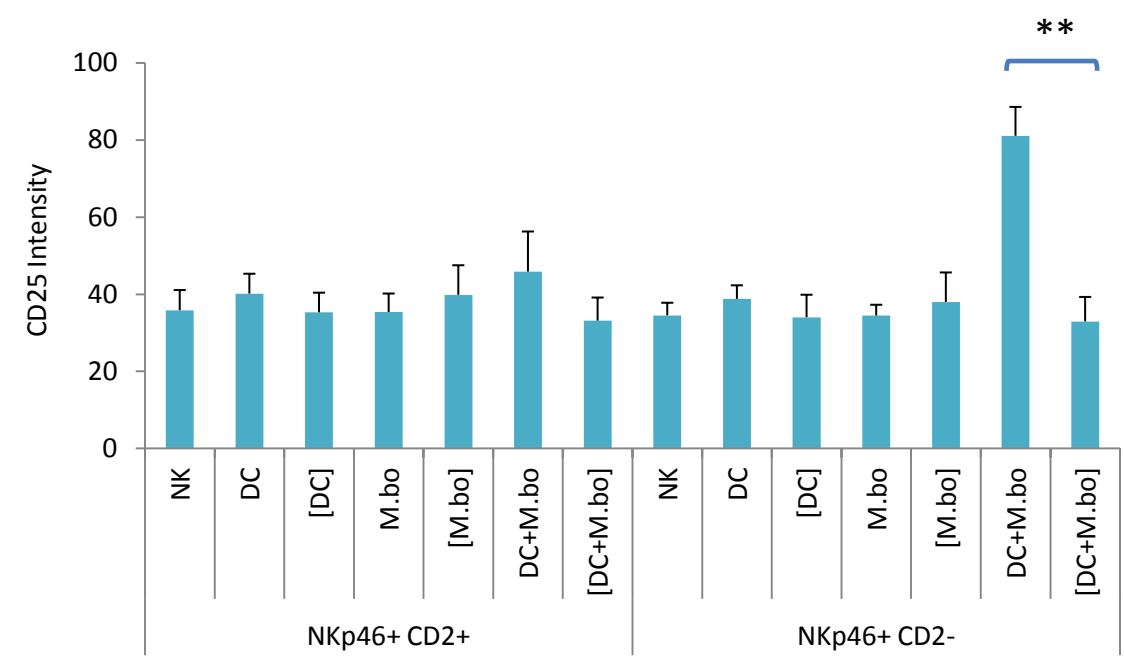
in the intensity of perforin expression following separation of both CD2+ or CD2- NK cells from *M.bovis* infected DCs, however this was found not to be significant due to large variations between animals (Figure 5.11.2). Comparison of NK cell subsets revealed that in a similar manner to CD25 expression, separation from *M.bovis* infected DCs appeared to affect CD2+ NK cells more profoundly with a larger reduction ($42.8\% \pm 4.7$) in the percentage of perforin positive NK cells (Table 5.11.3).

Within further co-culture assays the consequences of transwell separation on NK cell derived IFN γ production was investigated. As previously observed, CD2+ NK cells did not produce detectable levels of IFN γ after contact with *M.bovis* infected DCs and were only able to do so after IL-12 and IL-18 stimulation. In contrast, IFN γ secretion by CD2- NK cells was highly dependent on contact with *M.bovis* infected DCs with an almost complete loss ($p < 0.001$) of cytokine (Figure 5.12).

5.10.1



5.10.2



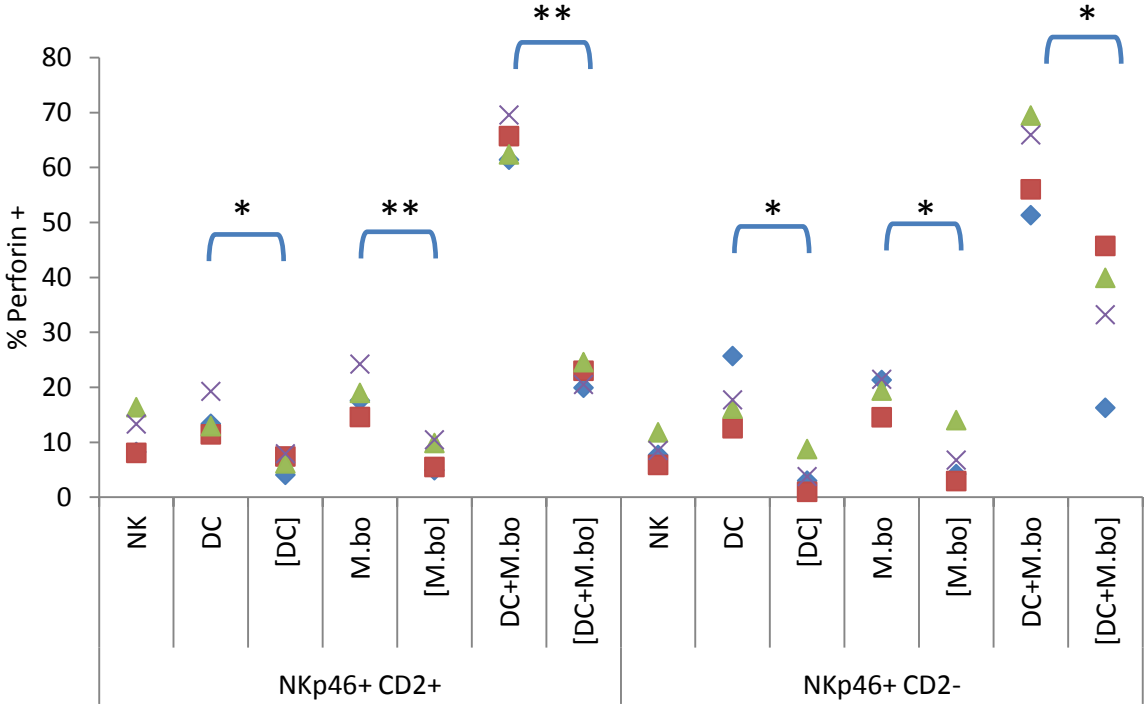
5.10.3

		Loss of % CD25 +ve NK cells	Loss of CD25 Intensity
NKp46+ CD2+	[DC]	0.6% ± 1.3	4.9 ± 5.6
	[M.bo]	0.7% ± 2.3	4.5 ± 7.9
	[DC+M.bo]	40.8% ± 13.3	12.7 ± 7.4
NKp46+ CD2-	[DC]	0.2% ± 7.1	4.8 ± 3.1
	[M.bo]	5.4% ± 8.2	3.5 ± 5.5
	[DC+M.bo]	38.7% ± 9.5	48.1 ± 13.3

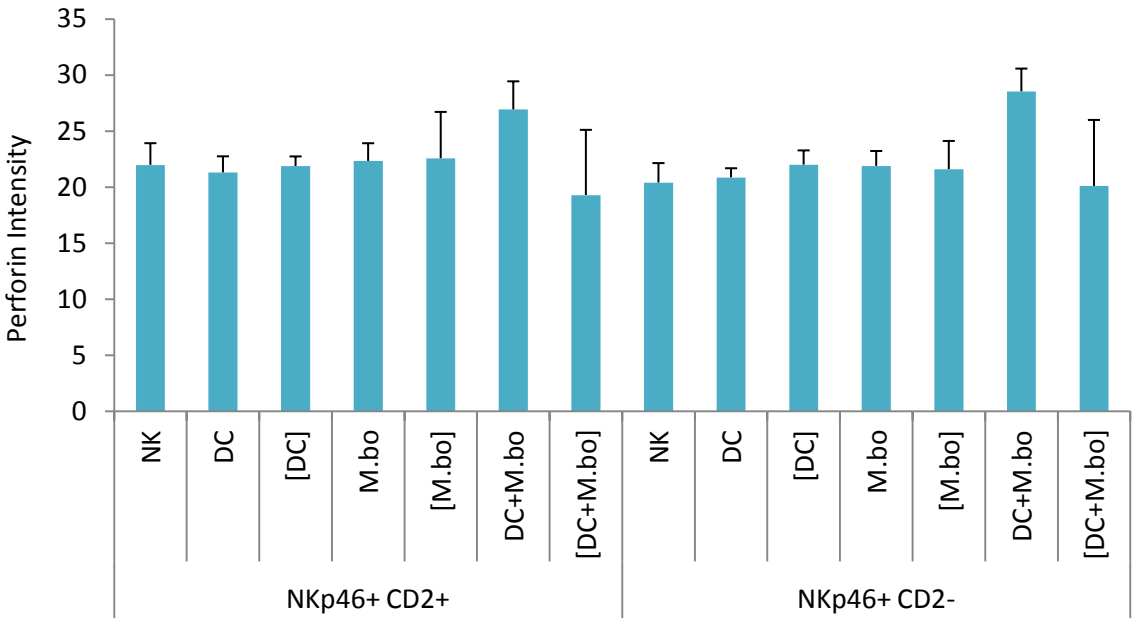
Figure 5.10 Loss of CD25 expression following separation of NK cells from DCs

Co-culture experiments with monocyte derived DCs infected with *M.bovis* and peripheral blood derived NK cell subsets were performed as described (2.10). *NK cells were either in contact with or separated from each culture condition using a 0.2µm culture well insert. Each culture condition was replicated in triplicate and NK cells were labelled with mAbs for NKp46, CD2 and CD25 and analysed by flow cytometry. Data is from four animals, representing the percentage of CD25 positive NK cells (Fig 5.10.1) expressed as a percentage of the total CD2+ or CD2- population and the mean fluorescence intensity (Fig 5.10.2) with error bars representing the standard deviation between animals. Transwell separation is indicated by []. Table 5.10.3 summarises the percentage loss of CD25 expressing NK cells and intensity as an average and standard deviation between four animals. Significant effects of transwell separation were analysed using log transformed data and the paired T-Test (95%CI); $p < 0.05^*$, $p < 0.01^{**}$.*

5.11.1



5.11.2



5.11.3

		Loss of % Perforin +ve NK cells	Loss of Perforin Intensity
NKp46+ CD2+	[DC]	7.9% ± 3.2	0.5 ± 1.9
	[M.bo]	11.2% ± 2.4	0.2 ± 2.8
	[DC+M.bo]	42.8% ± 4.7	7.6 ± 5.9
NKp46+ CD2-	[DC]	13.9% ± 6.5	4.4 ± 11.4
	[M.bo]	12.2% ± 5.1	0.3 ± 1.2
	[DC+M.bo]	26.9% ± 11.3	8.4 ± 6.1

Figure 5.11 Loss of Perforin expression following separation of NK cells from DCs

Co-culture experiments with monocyte derived DCs infected with *M.bovis* and peripheral blood derived NK cell subsets were performed as described (2.10). *NK cells were either in contact with or separated from each culture condition using a 0.2µm culture well insert. Each culture condition was replicated in triplicate and NK cells were labelled with mAbs for NKp46, CD2 and perforin and analysed by flow cytometry. Data is from four animals, representing the percentage of perforin positive NK cells (Fig 5.11.1) expressed as a percentage of the total CD2+ or CD2- population and the mean fluorescence intensity (Fig 5.11.2) with error bars representing the standard deviation between animals. Transwell separation is indicated by []. Table 5.11.3 summarises the percentage loss of perforin expressing NK cells and intensity as an average and standard deviation between four animals. Significant effects of transwell separation were analysed using log transformed data and the paired T-test (95%CI); $p < 0.05^*$, $p < 0.01^{**}$.*

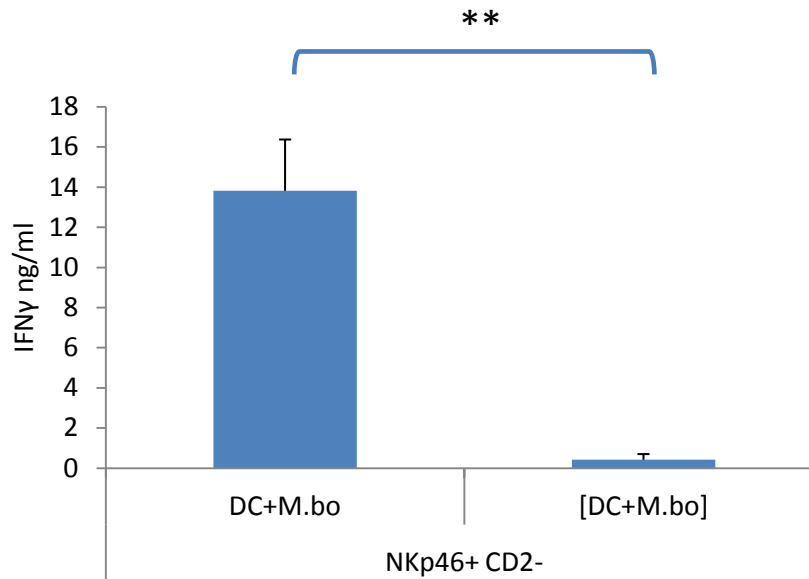


Figure 5.12 Loss of IFN γ release following separation of NK cells from DCs

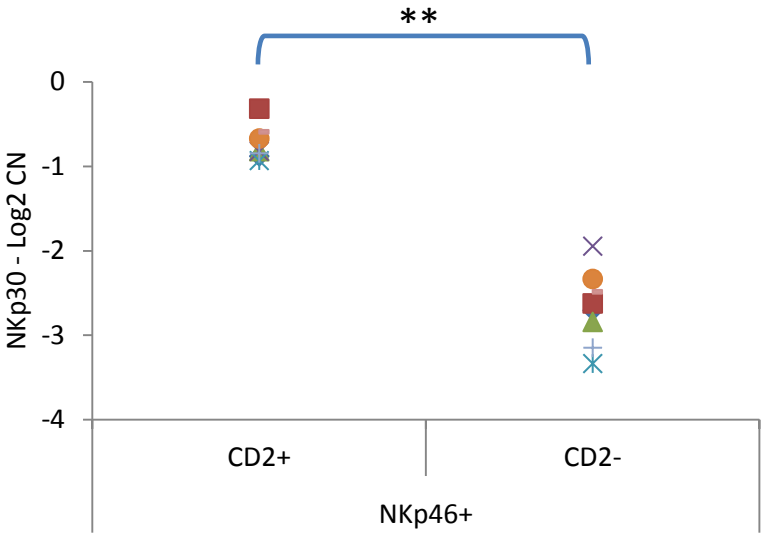
Co-culture experiments with monocyte derived DCs infected with *M.bovis* and peripheral blood derived NK cell subsets were performed as described (2.10). *CD2- NK cells were either in contact with or separated from M.bovis infected DCs using a 0.2 μ m culture well insert. Culture conditions were replicated in triplicate with cytokines (IL-12 and IL-18) alone serving as a control (data not shown). Supernatants were retrieved for analysis of IFN γ production by ELISA. Data represents the average levels of IFN γ ng/ml from four animals with error bars representing the standard deviation between animals. Transwell separation is indicated by []. Significance was determined using log transformed data and the paired T-Test (95%CI); $p<0.05^*$, $p<0.01^{**}$.*

5.2.5 Activatory and inhibitory receptor expression by NK cell subsets

Mycobacterially infected DCs have been shown to differentially influence effector functions of NK cell subsets with a role for both DC derived cytokines as well as cell-cell contact. Furthermore, CD2⁺ and CD2⁻ sub-populations of NK cells were found to differ in their individual requirements for contact with infected DCs, resulting in altered states of activation. It has been proposed that the capacity of an NK cell to respond in this manner is influenced in part by the balance of activatory and inhibitory receptor expression (Holmes et al., 2011, Ortaldo and Young, 2003). As disparity was observed between CD2⁺ and CD2⁻ NK cells, further transcriptional analysis was conducted to elucidate NK cell receptor expression patterns between subsets. Preliminary analysis of KIR (generic) expression by real time PCR indicated higher transcripts expressed by CD2⁺ NK cells (Dobromylskyj, 2009).

To further establish differences in NK cell receptors, quantitative PCR assays were designed to amplify genes for the NK cell receptors; NKp30 and Ly49 (3.2.3). Primers and probes were based on published sequences; NKp30 (NM_001040524), Ly49 (AY075101) and transcript levels expressed by peripheral blood derived NK cells were analysed. The natural cytotoxicity receptor, NKp30 was expressed at significantly elevated levels ($p < 0.001$) by CD2⁺ NK cells (Figure 5.13.1) compared to CD2⁻ NK cells, with transcription almost equating to house-keeping gene levels. In comparison, CD2⁻ NK cells expressed significantly higher levels of Ly49 ($p = 0.002$) (Figure 5.13.2) but transcript levels were considerably lower than those observed for NKp30.

5.13.1



5.13.2

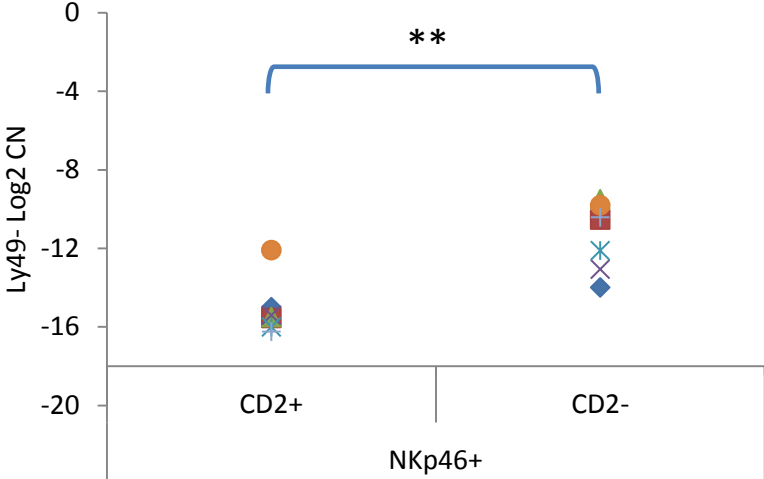


Figure 5.13 NKp30 and Ly49 expression by NK cell subsets

*NK cell subsets were isolated from peripheral blood from eight animals as described (3.2.4). RNA was reverse transcribed and subjected to quantitative PCR analysis. Each sample was tested in triplicate, quantified against a plasmid DNA standard curve and normalised to the housekeeping gene BoPo. Plasmid standards were kindly provided by Tim Connelly (NKp30) and Melanie Dobromylskyj (Ly49). Data represents the Log2 normalised copy number (CN) of NKp30 (Fig 5.13.1) and Ly49 (Fig 5.13.2) with each point denoting the transcriptional value obtained from one animal. Significance between NK cell subsets was assessed using the paired T-Test $p < 0.05$ *, $p < 0.01$ **.*

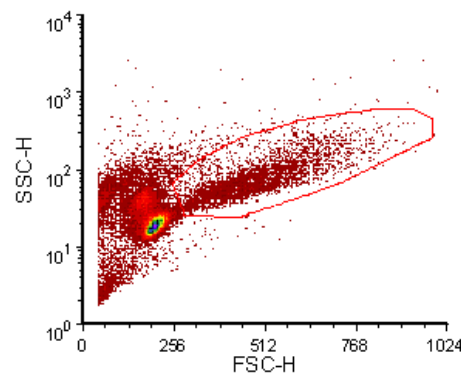
5.2.6 Reciprocal activation of DCs

Results herein have established that under conditions of mycobacterial infection, DCs are able to induce effector functions of NK cells including cytokine production and cytolytic activity involving both DC derived cytokines as well as cell-cell contact. It has been well documented that this interaction may be reciprocal with NK cells able to kill immature DCs or induce their maturation and in this manner promote the subsequent Th1 priming of the immune response.

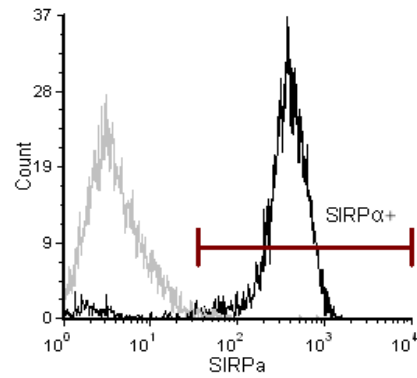
To ascertain whether NK cells were able to reciprocally activate DCs, co-cultures were repeated with DCs cultured with CD2+ or CD2- NK cell subsets either with or without *M.bovis* infection. DCs alone or DCs infected with *M.bovis* served as control conditions. DCs were identified within the co-cultures by gating a population of high FSC cells (Figure 5.14.1) which expressed SIRP α (Figure 5.14.2) and maturation of DCs was determined by analysis of cell surface expression levels of MHC class II, and co-stimulatory molecules CD40, CD80, CD86.

After contact with NK cells, either with or without the presence of *M.bovis*, there were no alterations in the percentage of DCs expressing MHC class II or any co-stimulatory molecules above the levels expressed by DCs alone (data not shown). As previously reported (Hope et al., 2004), the intensity of CD40 expression increased marginally after *M.bovis* infection of DCs (data not shown) and this remained unaltered upon addition of NK cells. Similarly, infection of DCs with *M.bovis* induced an elevated intensity of MHC class II expression on the cell surface of DCs (Figure 5.14.4 and 5.14.6) but this was found not to be significant compared to DCs alone (Figure 5.14.3 and 5.14.6). In contrast, when *M.bovis* infected DCs were in contact with CD2- NK cells (Figure 5.14.5 and 5.14.6), DCs significantly up regulated MHC class II with increased intensity of expression compared with uninfected DCs and CD2- NK cells ($p=0.002$) or DCs infected with *M.bovis* ($p=0.042$), or any response that was seen with infected DCs cultured with CD2+ NK cells ($p=0.044$).

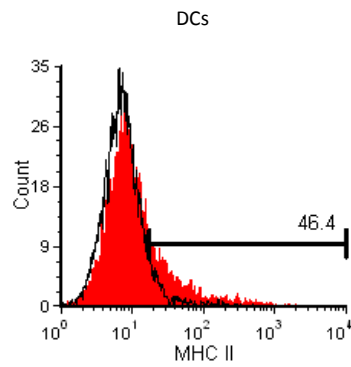
5.14.1



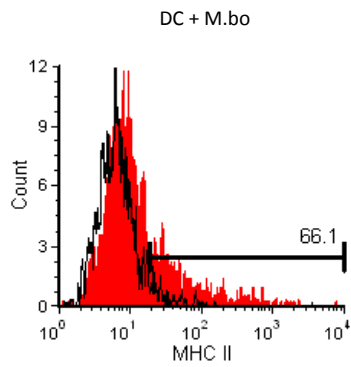
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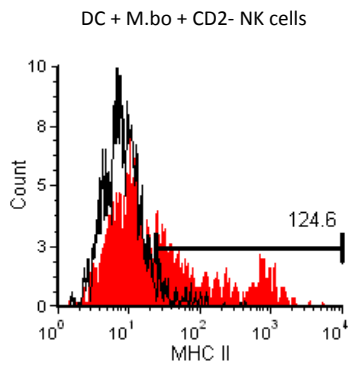
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5.14.4



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5.14.6

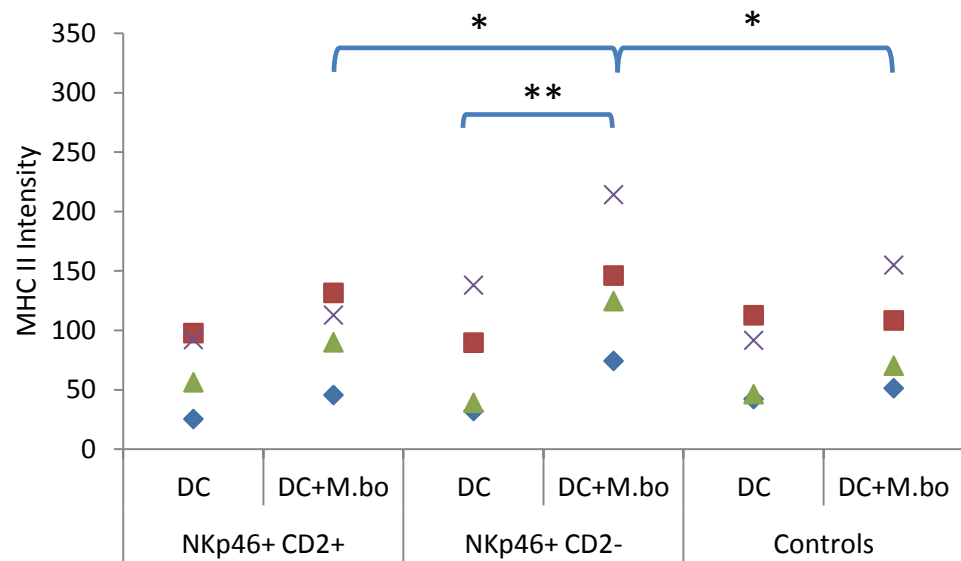


Figure 5.14 Reciprocal effects on DCs after culture with NK cells

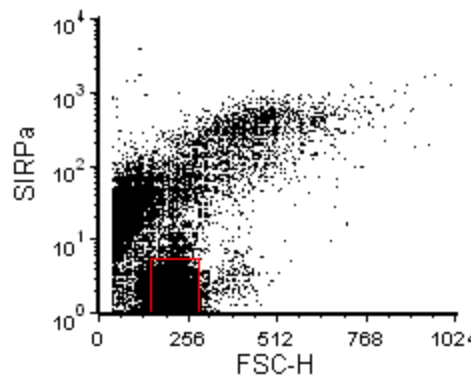
Co-culture experiments were performed with peripheral blood derived NK cells cultured with DCs and M.bovis. Each culture condition was replicated in triplicate with DCs cultured alone, with CD2+ or CD2- NK cells or with M.bovis alone serving as controls. After overnight co-culture cells were labelled with mAbs for SIRPα, MHC class II, CD40, CD80 and CD86 and analysed by flow cytometry. FACS plots from one representative animal are shown with DCs identified by high FSC (Fig 5.14.1) and SIRPα expression of gated cells (Fig 5.14.2). The intensity of MHC class II expression (red) of DCs alone (5.14.3) M.bovis infected DCs (Fig 5.14.4) or M.bovis infected DCs cultured with CD2- NK cells (Fig 5.14.5) is illustrated. Pooled data from four animals represents the mean fluorescence intensity of MHC class II (Fig 5.14.6). Significance between co-culture conditions were assessed using a GLM with Tukey post-hoc comparisons (95%CI); $p < 0.05^*$, $p < 0.01^{**}$.

5.2.7 *M.bovis* infected DCs augment MHC class II expression on CD2- NK cells

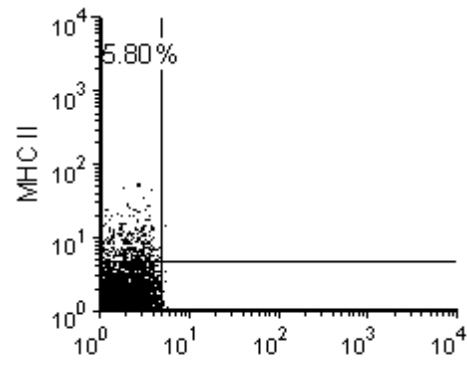
Recent reports suggest that discrete subsets of NK cells also express MHC class II proteins and this population preferentially expanded and was activated in the immune response to BCG infection (Evans et al., 2011). To determine whether this was also the case following *M.bovis* infection *in vitro*, MHC class II expression levels of NK cells after contact with infected DCs were analysed. Subsets of CD2+ or CD2- NK cells with uninfected DCs served as controls. During analysis of the mixed co-culture, NK cells were differentiated from DCs by excluding the high FSC/SIRPα positive population of DCs and positively gating the remaining live NK cell population (Figure 5.15.1). MHC class II expression within this population was subsequently analysed (Figure 5.15.2).

It has previously been observed that freshly isolated peripheral blood derived NK cells do not express MHC class II molecules (3.2.2). This was also the case when purified CD2+ NK cells had been in co-culture with uninfected DCs (Figure 5.15.3). Following infection with *M.bovis*, there was a very minor increase in the percentage of CD2+ NK cells expressing MHC class II however, this was not found to be significant. In comparison, small percentages of CD2- NK cells (~2%) expressed MHC class II after contact with DCs and the proportions of MHC class II positive CD2- NK cells were significantly augmented ($p=0.003$) after contact with *M.bovis* infected DCs (Figure 5.15.3)

5.15.1



5.15.2



5.15.3

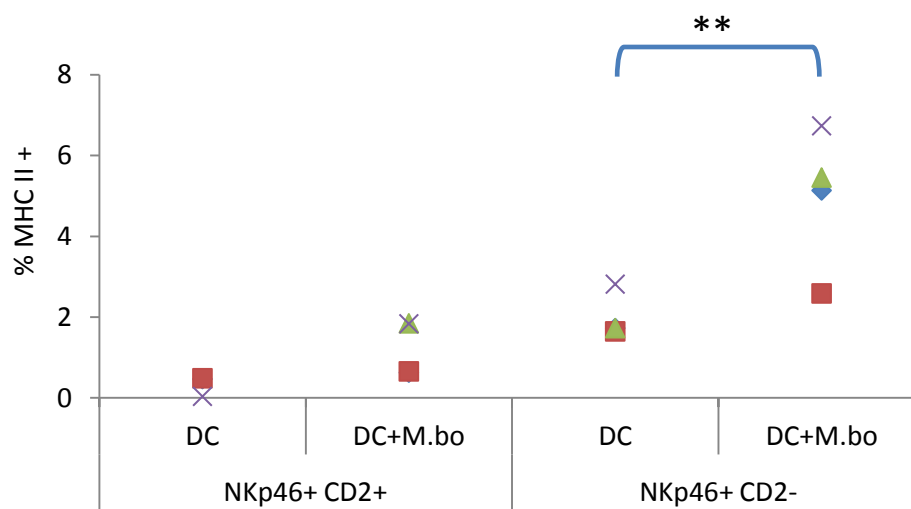


Figure 5.15 MHC class II expression by NK cell subsets following co-culture

Cells from co-culture experiments (Fig 5.14) were re-analysed to determine the percentages of NK cells expressing MHC class II with CD2+ or CD2- subsets cultured with uninfected DCs serving as controls. NK cells were identified by negative selection (gated in red) to eliminate the high FSC/SIRPα positive dendritic cell population (Fig 5.15.1) and MHC class II expression within the remaining NK cell population was analysed (Fig 5.15.2). FACS plots from one representative animal are shown. Pooled data from four animals demonstrates the percentage of NK cells expressing MHC class II (Fig 5.15.3). Significance between co-culture conditions was assessed using a GLM with Tukey post-hoc comparisons (95%CI); $p < 0.05^*$, $p < 0.01^{**}$.

5.3 Discussion

In the aftermath of pathogenic insult, reciprocal interactions between cells of the innate immune system and their resulting effector responses are implicit to the developing immune response. NK cells primarily function as cytolytic cells but are also a significant source of immuno-modulatory cytokines, functioning to bridge the innate and adaptive immune systems. Research within a number of disease models has demonstrated that the effector responses of NK cells may be induced or enhanced by co-stimulatory signals from DCs in the form of soluble stimuli or contact dependent receptor-ligand interactions. Importantly, within this bi-directional interaction NK cells may potentiate DC maturation with implications for the subsequent priming of adaptive immunity. Within this thesis, it has previously been demonstrated that *M.bovis* infected DCs selectively recruited CD2- bovine NK cells (4.2.2.2) and were a significant source of IL-12 (4.2.4); a known stimulant of NK cell activity. In continuation of these studies, *in vitro* co-culture assays were utilised to further illustrate that bovine NK cells and DCs reciprocally interact during *M.bovis* infection and to define important features and mechanisms of this interaction.

The IL-2 receptor- α -chain (CD25) is widely expressed by lymphocytes and its up-regulation is associated with an increased capacity to respond to cytokine stimulation. As such, analysis of CD25 expression levels is commonly used as an indicator of NK cell activation. Within these studies, large percentages (40-60%) of peripheral blood derived NK cells up-regulated expression of CD25 and secreted IFN γ after contact with *M.bovis* infected DCs. However, whilst similar percentages of NK cell subsets appeared to be activated, an enhanced intensity of CD25 expression was restricted to the CD2-sub-population of NK cells (Figure 5.2). Differential responses between populations of human NK cells have previously been reported with the CD56^{bright} subset preferentially proliferating and producing IFN γ following stimulation by DCs (Ferlazzo et al., 2004a, Vitale et al., 2004). In addition, murine CD27^{high} NK cells selectively migrated towards lymph nodes and interacted with DCs (Hayakawa and Smyth, 2006). In a similar manner, purification of subsets revealed that the IFN γ production by bovine NK cells in response to *M.bovis* infected DCs was restricted to the CD2- NK cell subset, with higher levels of IFN γ observed upon isolation of the subset when compared with the total NK

cell population. This is likely to be as a consequence of a change in proportions; within the total NK cell population the IFN γ producing CD2 $^{-}$ subset would only constitute 20-30% of the total cells whereas when separated, would represent the predominant population. (Feng et al., 2006) demonstrated that the early control of mycobacterial infection was reliant upon IFN γ derived from NK cells, functioning to promote the bactericidal activity of macrophages. Therefore, the proportions of CD2 $^{+}$ and CD2 $^{-}$ subsets within the tissues of the respiratory tract may have significant implications for the levels of IFN γ produced and the subsequent control of bacterial replication early following infection.

Importantly, data herein suggests that the CD2 $^{-}$ NK cell subset may be further composed of smaller heterogeneous sub-populations with differing responses during mycobacterial infection. Within chemotaxis assays, it was previously observed that 30-40% of CD2 $^{-}$ NK cells migrated towards *M.bovis* infected DCs (Figure 4.4). Of interest was the finding that, similar percentages (~30%) of NK cells within the CD2 $^{-}$ subset, also appeared to express CD25 at higher intensities and may therefore represent a discrete population of cells that preferentially migrate towards and interact with DCs during mycobacterial infection. Similar responses have been reported in humans with small sub-populations of HLA-DR $^{+}$ NK cells preferentially secreting IFN γ following BCG infection (Evans et al., 2011). Interestingly, an increase in expression of MHC class II by CD2 $^{-}$ NK cells following co-culture with *M.bovis* infected DCs was also observed. However, further investigations would be required to determine if the migrating, MHC class II positive and IFN γ producing CD2 $^{-}$ NK cells represent corresponding populations.

The reasons for the observed disparity between the IFN γ producing capacities of bovine NK cell subsets are not known. It has previously been suggested that heterogeneity in IFN γ production by NK cells directly correlated with levels of DC derived IL-12 (Newman et al., 2006). However, analysis of IL-12 and IL-10 protein content within supernatants revealed that both CD2 $^{+}$ and CD2 $^{-}$ NK cells received equivalent levels of these soluble signals from *M.bovis* infected DCs. In addition, upon optimal supplementation with IL-12 and IL-18, CD2 $^{+}$ NK cells produced IFN γ with levels equivalent to those produced by CD2 $^{-}$ NK cells. Boysen et al., (2006b) previously reported that CD2 $^{+}$ bovine NK cells proliferated in the presence of IL-2 however; the

response was slower when compared with CD2⁻ NK cells. Therefore, during interactions with mycobacterially infected DCs, the activation requirements of bovine NK cell subsets appear to be significantly different with CD2⁺ NK cells potentially requiring a larger or more prolonged stimulus to induce IFN γ production.

NK cells are prolific killers with release of cytolytic proteins functioning to control the spread of infection. Bovine NK cells constitutively produce perforin and predominantly function as cytolytic cells through perforin mediated mechanisms (Boysen et al., 2006b, Endsley et al., 2006, Graham et al., 2009). These findings were confirmed with small percentages (10-20%) of both CD2⁺ and CD2⁻ NK cells found to express perforin and both populations able to kill P815 tumour target cells. Upon contact with uninfected DCs, the percentage of CD2⁻ NK cells expressing perforin was significantly augmented. Autologous immature DCs are susceptible to NK cell mediated lysis as a consequence of low HLA class I expression (Ferlazzo et al., 2003, Ferlazzo et al., 2001, Wilson et al., 1999). Killing in this manner functions as an editing process such that NK cells bias the immune response to those DCs which have undergone appropriate maturation (Ferlazzo et al., 2002). The observed induction of perforin expression may therefore represent a mechanism by which CD2⁻ NK cells are able to kill autologous immature DCs.

Similarly, small percentages of both CD2⁺ and CD2⁻ NK cells also increased expression of perforin after stimulation with *M.bovis* alone. The induction of migratory responses of NK cells exposed to mycobacteria, independent of antigen presenting cell involvement was also observed previously (Figure 4.4). NK cells are known to respond directly to microbial stimuli, with BCG able to induce proliferative and cytolytic activity of human NK cells (Batoni et al., 2005, Esin et al., 2008, Esin et al., 2004, Marcenaro et al., 2008). This represents mechanisms by which cytolytic effector cells can be rapidly activated to a certain extent following infection, without complete dependency upon co-stimulatory signals. Within these human studies BCG was directly recognised via NKp44 (Esin et al., 2008) and TLR2 (Marcenaro et al., 2008). The bovine orthologue for NKp44 has not been identified within the genome, however by quantitative PCR transcripts for all the TLRs were found within bovine NK cells with significantly elevated expression of TLR2 and TLR6; receptors with a known capacity to bind

mycobacterial PAMPs. NK cell cytolytic activity can be induced via signalling through TLRs (Sivori et al., 2004); therefore the observed increase in perforin may be as a result of direct activation in this manner. However, cytolytic assays herein illustrated that the small induction in perforin expression by mycobacteria alone did not augment the capacity of NK cells to kill P815 tumour target cells. In contrast, in the presence of *M.bovis* infected DCs, a much larger increase in the expression and intensity of perforin coincided with augmented cytolytic activity. Importantly, the response was not restricted to either subset of NK cells. Evidence therefore suggests that fully functional effector responses of bovine NK cells require multiple signals received from mycobacteria alone as well as antigen presenting cells. It has previously been reported that bovine NK cells were able to control *M.bovis* replication within macrophages (Denis et al., 2007). In further investigations, it would therefore be interesting to determine whether NK cells activated by DCs may in turn function to kill mycobacterially infected DCs or macrophages as a means to directly control bacterial expansion.

Data herein has demonstrated that during *M.bovis* infection, the induction of NK cell derived IFN γ release and cytolytic activity was reliant upon co-stimulatory signals received from DCs. Numerous studies have illustrated that DC derived cytokines are fundamental to the cross-talk between NK cells and DCs. Mycobacterially infected DCs are a significant source of IL-12 (Hickman et al., 2002, Hope et al., 2004) which is implicit to the early innate release of IFN γ within the lungs of *M.tuberculosis* infected mice (Feng et al., 2006) and effective Th1 polarisation of the immune response (Hickman et al., 2002). Analysis of protein levels within supernatants confirmed that NK cells in co-culture with *M.bovis* infected DCs were exposed to IL-12. Olsen et al., (2005) reported that the release of IFN γ by bovine NK cells was partially reliant on IL-12 derived from antigen presenting cells exposed to mycobacterial antigens. This finding was confirmed herein, with blocking of IL-12 resulting in a substantial (62%) decline in the level of IFN γ produced by CD2- NK cells responding to *M.bovis* infected DCs. However, the partial decline suggests that other factors also influence the IFN γ producing ability of CD2- NK cells. Roles for TNF α (Humann and Lenz, 2010), IFN α (Newman et al., 2006), IL-18 (Artavanis-Tsakonas et al., 2003, Artavanis-Tsakonas and

Riley, 2002, Bastos et al., 2008, Van Elssen et al., 2010), IL-15 (Lucas et al., 2007) and IL-2 (Granucci et al., 2004) have also been described in the induction of DC derived effector responses of NK cells however, *M.bovis* infected bovine DCs did not appear to produce detectable levels of TNF α or IL-2. Levels of IL-15 and IL-18 could not be confirmed as at present there are no bovine antibodies to detect these cytokines. In addition, whilst transcripts for IL-18 were previously detected within bovine DCs, expression levels did not change significantly following *M.bovis* infection (Thom, 2005). Furthermore, indirect evidence suggests that there may be a minimal role for IL-18 within these co-culture conditions. During blocking experiments NK cells exposed to both IL-12 and IL-18 were not substantially affected by the loss of IL-12 with only a very small decrease (3%) in the levels of IFN γ produced. Titration experiments have previously demonstrated that very low levels of IL-12 are able to synergise highly effectively with IL-18 to induce optimal IFN γ release of bovine WC1+ $\gamma\delta$ T cells (Price et al., 2007). As such in the presence of IL-18, a significant loss in IL-12 does not substantially influence the levels of IFN γ produced. In contrast, blocking of IL-12 produced by *M.bovis* infected DCs substantially reduced the levels of IFN γ produced by CD2- NK cells in co-culture with *M.bovis* infected DCs; suggesting that IL-18 may not have been present or may have been present at low levels and could therefore not compensate for the loss of IL-12. Van Elssen et al., (2010) reported that DC derived IL-12 also influenced the cytolytic activity of NK cells and further experimentation would be required to determine if this was also the case within these assays.

Bovine CD2- NK cells in co-culture with *M.bovis* infected DCs partially retained the capacity to produce IFN γ despite the loss of IL-12. Whilst this may be explained by the presence of additional cytokine stimuli, studies have reported that optimal induction of NK cell effector responses require direct receptor-ligand interactions. Experiments utilising transwell separation demonstrated that subsets of NK cells and their effector responses had differential contact requirements. In this manner, both CD2+ and CD2- NK cells were found to require contact with *M.bovis* infected DCs to maintain expression of CD25 and perforin. However, CD2+ NK cells appeared more reliant upon direct interactions with DCs with larger percentages of CD2- NK cells remaining activated and expressing perforin despite separation from *M.bovis* infected DCs. In

contrast however, secretion of IFN γ by CD2⁻ NK cells was found to be completely reliant upon cell-cell contact with a complete loss upon transwell separation from *M.bovis* infected DCs. Therefore, despite the presence of cytokines, in the absence of contact dependent signals, CD2⁻ NK cells were rendered unresponsive. Within TB infection, elevated levels of IFN γ may contribute to immuno-pathological states observed during chronic infection. It is likely that the requirement of CD2⁻ NK cells for multiple signals prior to the release of IFN γ illustrates a control mechanism preventing excessive secretion in response to cytokines alone. Similarly, CD2⁺ NK cells constitute the predominant population within peripheral blood and therefore the requirement for contact with infected DCs may represent a mechanism by which cytolytic activity is controlled.

It is increasingly apparent that the interactions between NK cells and DCs during mycobacterial infection are highly complex and diverse. Bovine NK cell subsets appear to differ substantially in their requirements for cytokines and cell-cell contact to initiate effector responses. These observations are not restricted to the bovine system with divergences in IFN γ production reported within human NK cell population (De Maria et al., 2011, Evans et al., 2011, Ferlazzo et al., 2004a, Vitale et al., 2004) and also between donors (Artavanis-Tsakonas et al., 2003, Artavanis-Tsakonas and Riley, 2002). Emerging evidence has suggested that variations in NK cell responses may in part be explained by the existence of a threshold of activation. Bovine CD2⁻ NK cells required cytokines and contact dependent stimuli from *M.bovis* infected DCs to produce IFN γ whilst remained activated and maintained perforin expression with cytokines alone. In comparison CD2⁺ NK cells were unable to secrete IFN γ unless additional cytokine stimuli were provided and were entirely dependent on cytokines and cell-cell contact to remain activated and maintain a cytolytic status. Therefore, subsets of bovine NK cells may possess differing thresholds of activation that govern responsiveness during mycobacterial infection. It has been proposed that thresholds are determined by the balance of expression between activatory and inhibitory receptors (Holmes et al., 2011, Ortaldo and Young, 2003) and it is likely that deciphering the precise components will prove a significant challenge for future studies. The study of bovine NK cell receptors is still in its infancy. Multiple KIR genes that may encode activatory

and inhibitory receptors have been identified (Dobromylskyj and Ellis, 2007, Storset et al., 2003). In addition cattle also possess a polymorphic Ly49 gene that encodes inhibitory receptors (Dobromylskyj et al., 2009, McQueen et al., 2002). Importantly, within initial studies transcript analysis revealed significant divergences may exist between the expression levels of activatory and inhibitory receptors between bovine NK cell subsets. Ly49 was found to be preferentially transcribed by CD2⁻ NK cells. In comparison the activatory NKp30 receptor with known involvement in the recognition of immature DCs (Ferlazzo et al., 2002), was expressed at higher levels by CD2⁺ NK cells. However, substantial research will be required before it is known whether the differential levels of expression influence the divergent responses observed during interactions with *M.bovis* infected DCs.

Perhaps more important however is the reciprocal interaction between NK cells and DCs, reported to influence the progression and development of the adaptive immune response. Murine studies have elucidated that the effective control of mycobacterial infection is reliant upon transport of antigen by migratory DCs to draining lymph nodes (Marino et al., 2004) to effectively prime CD4⁺ T cells and subsequently polarise Th1 biased immune responses. NK cells play an implicit role in this process, functioning to promote the maturation of DCs (Lande et al., 2003, Mailliard et al., 2003, Marcenaro et al., 2009, Morandi et al., 2009, Van Elssen et al., 2010) such that DCs that had been in contact with NK cells were better able to induce IFN γ release by CD4⁺ T cells (Van Elssen et al., 2010). Herein, we observed a significant increase in the intensity of MHC class II expression on DCs, however importantly the response was restricted to DCs that had been in contact with CD2⁻ NK cells. It is possible therefore that the CD2⁻ NK cell subset in particular plays a prominent role in modulating adaptive immune responses however, further experiments would need to be conducted to determine whether the observed increase in intensity of MHC class II translated into an augmented capacity to present antigen and prime T cell responses during mycobacterial infection.

In summary, co-culture assays herein have demonstrated evidence for reciprocal activating signals between bovine NK cells and *M.bovis* infected DCs that are initiated by both cell contact and soluble mediators. In addition, the substantial heterogeneity

observed between the activation requirements of bovine NK cell subsets is likely to influence their capacity to respond and interact with DCs during mycobacterial infection with implications for the innate control of mycobacterial expansion as well as the developing adaptive immune response.

6 Distributions of NK cells *in vivo* during mycobacterial infection

6.1 Introduction

Bovine NK cells are localised in numerous compartments ideally placed to rapidly respond in the innate immune response to antigenic insult with heterogeneous subsets differentially distributed within these compartments. CD2- NK cells, demonstrated herein to preferentially migrate towards and interact with mycobacterially infected DCs, constitute the minor blood derived NK cell population but are found in significant numbers within the parafollicular and medullary regions of lymph nodes (Boysen et al., 2008). Similarly, both human CD56^{bright} (Fehniger et al., 2003, Ferlazzo et al., 2004b) and murine NK cells form resident populations within these regions, however percentages are significantly lower within murine lymph nodes (Bajenoff et al., 2006). Despite this, the murine model has delivered significant insight into the *in vivo* migratory behaviour of NK cells. During steady-state conditions, small percentages of NK cells within murine lymph nodes exist in a motile state with a large proportion in contact with resident networks of DCs (Bajenoff et al., 2006).

During an inflammatory immune response NK cells accumulate at the site of infection and also within draining lymph nodes. Administration of LPS matured DCs to mice, enhanced the *in vivo* migratory responses of NK cells such that significant numbers were recruited from the periphery to draining lymph nodes (Martin-Fontecha et al., 2004) to influence the polarisation of Th1 immune responses. In a similar manner, intranasally inoculated BCG is rapidly phagocytosed by infiltrating DCs (Reljic et al., 2005) and both BCG and *M.tuberculosis* infection induced increases in the absolute numbers of activated, IFN γ producing NK cells within the lungs (Feng et al., 2006, Junqueira-Kipnis et al., 2003, Saunders et al., 2002, Saxena et al., 2002). A similar response was also seen following administration of mycobacterial antigens resulting in increased percentages of activated NK cells within localised lymphoid tissue (Hall et al., 2010) with redistribution to more central regions of the parafollicular areas (Beuneu et al., 2009, Hall et al., 2010). Consequently, activated populations of NK cells can also be detected in the periphery; however within mice this depends on the entry of NK cells to draining lymph nodes to enable interactions with DCs (Lucas et al., 2007).

Mycobacterial infection in cattle influences the proportions and distributions of innate effector cells highlighting involvement within the developing immune response. Following BCG vaccination percentages of circulating $\gamma\delta$ T cells increased (Buza et al., 2009), prior to the development of antigen specific immunity (Buddle et al., 1995). In addition IFN γ producing WC1+ $\gamma\delta$ T cells accumulated in the lungs and tonsils upon intranasal infection with BCG (Price et al., 2010) and activated $\gamma\delta$ T cells have been detected in the periphery of *M.bovis* infected cattle (Kennedy et al., 2002). Despite this, the depletion of WC1+ $\gamma\delta$ T cells did not significantly influence the pathogenesis or progression of *M.bovis* infection highlighting redundancy within the innate immune response (Kennedy et al., 2002). This is largely believed to be derived from NK cells due to functional overlap with $\gamma\delta$ T cells, however very little is known of the *in vivo* distributions and responses of bovine NK cells under inflammatory conditions. Percentages of NK cells within the blood were reported to decrease early following parasitic infection with *Neospora caninum* followed by an increase in numbers of IFN γ producing, cytolytic NK cells detected at later time-points (Klevar et al., 2007). However, in the only reported study of NK cells and mycobacterial responses in cattle, NK cell numbers in the peripheral blood were not altered at 6 weeks post-BCG vaccination (Denis et al., 2007).

Within this thesis it has previously been demonstrated *in vitro* that bovine NK cells are responsive during mycobacterial infection with elevated migration and reciprocal interactions with infected DCs. In initial experiments, we sought to comprehensively describe the distribution of NK cell subsets within the tissues and lymph nodes of the head, respiratory tract and prescapular lymph nodes; regions which would be directly involved in the immune response to respiratory pathogens or vaccination. In subsequent experiments, this study sought to investigate whether administration of mycobacteria influenced the percentages or effector responses of the NK cell populations within these compartments and also within the peripheral circulation.

6.2 Results

6.2.1 Intranasal BCG inoculation

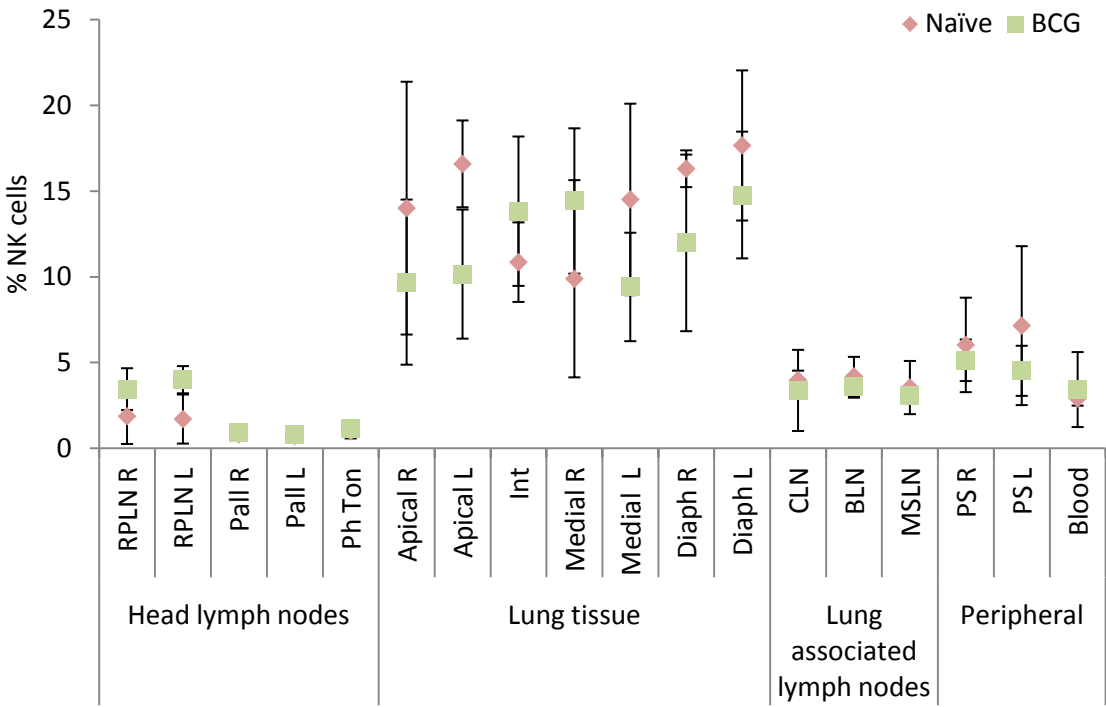
Experiments were conducted to ascertain percentages of NK cells and proportions of subsets within tissues and lymph nodes from the head and respiratory tract (Table 2.1) and to determine whether mycobacterial exposure altered these levels. Calves received BCG Pasteur delivered intranasally (2.2.2) to mimic the natural route of mycobacterial infection and tissues and lymph nodes were retrieved after 7 days at post-mortem with naïve age matched calves serving as controls. Percentages of NK cells and proportions of CD2+ and CD2- subsets were determined by flow cytometry following labelling of lymphocytes for NKp46 and CD2 (2.12.1).

Tissues derived from the apical to diaphragmatic regions of the lungs were found to contain the highest percentages of NKp46+ cells, with 10-20% of lymphocytes constituting the NK cell population (Figure 6.1.1). When compared, no significant differences were found between the different lobes of the lung and numbers were not altered after exposure to BCG. In contrast, tissues and lymph nodes of the head contained fewer percentages of NK cells (1-5%), similar to levels seen within lung associated and peripheral lymph nodes. Overall the percentages of NK cells varied considerably between animals and administration of BCG did not significantly influence this level in the majority of tissues or lymph nodes. However, within retropharyngeal lymph nodes (Figure 6.1.2) which drain the site of inoculation, calves given BCG were found to contain higher percentages of NK cells when compared with naïve animals and this was significant on the draining (left) side ($p=0.040$).

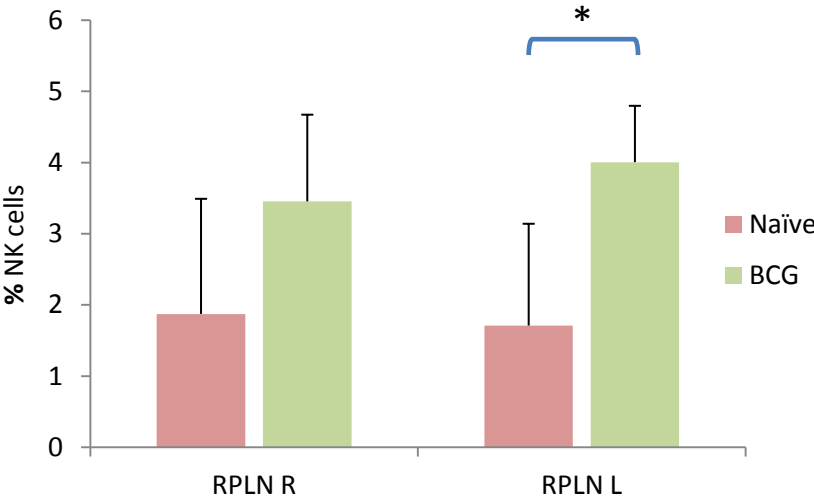
Detailed analysis of the proportions of NK cell subsets within these tissues highlighted considerable variations (Figure 6.1.3). As has been described previously, CD2- NK cells constituted the predominant population within all lymph nodes examined. In contrast tonsillar and lung tissues were found to contain a more equal distribution of CD2+ and CD2- NK cells with a minor predominance (50-55%) of the CD2+ NK cell population. Generally, NK cell populations within tissues contained higher proportions of CD2- cells when compared with the percentages found within peripheral blood. These

proportions were not found to be significantly altered within any of the tissues examined after administration of BCG (Figure 6.1.4).

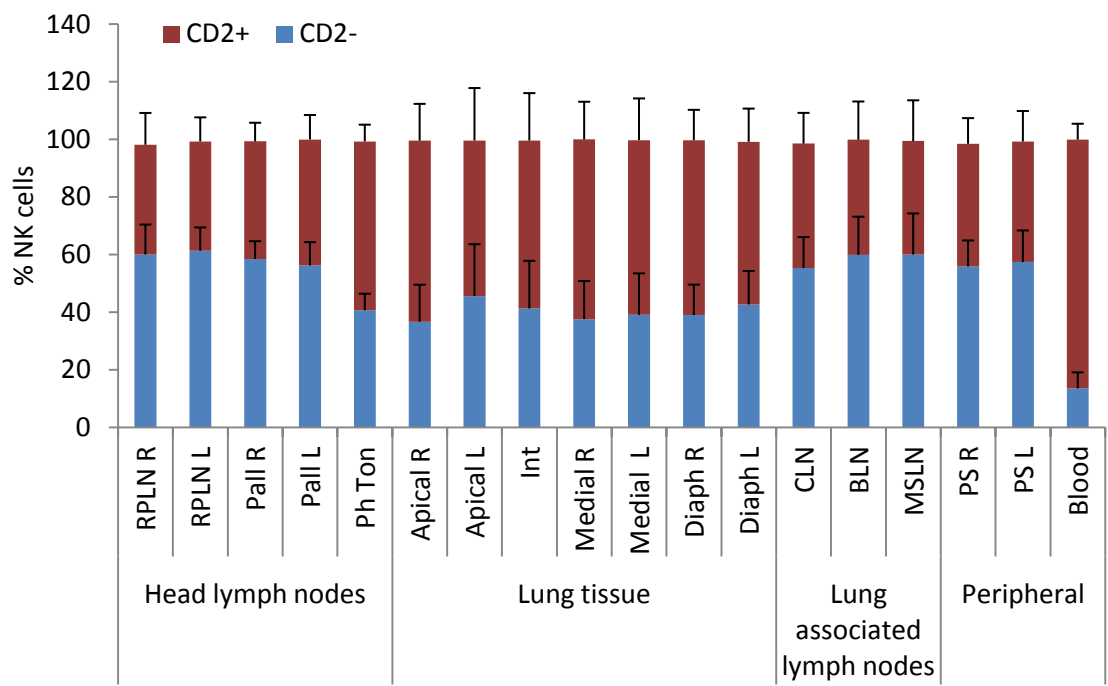
6.1.1



6.1.2



6.1.3



6.1.4

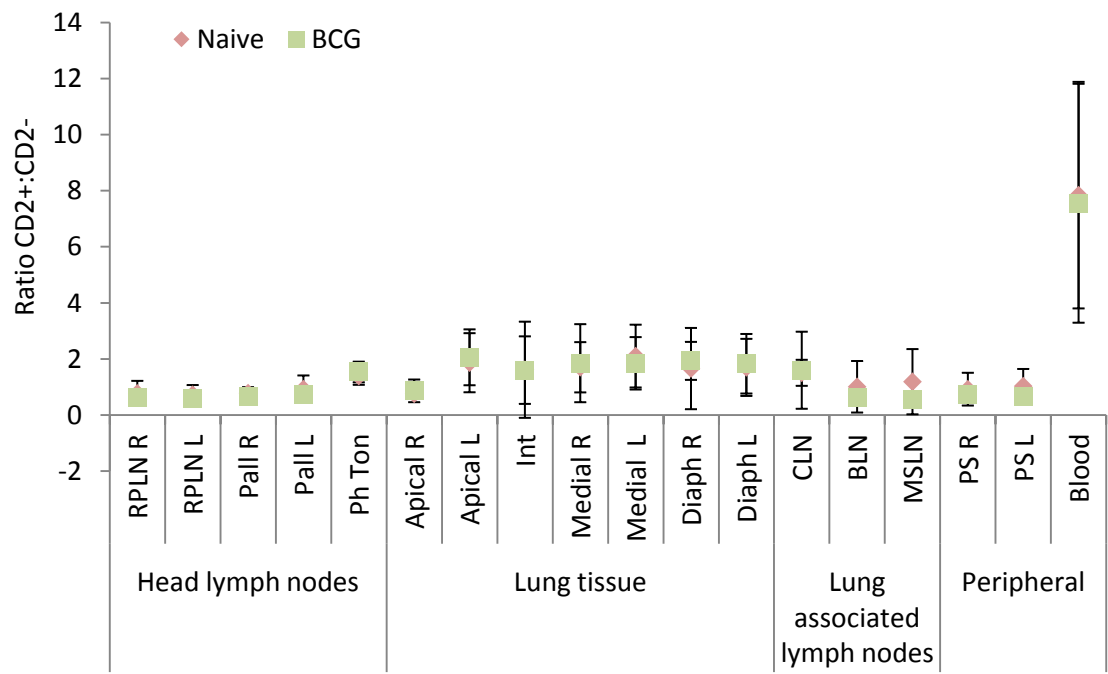


Figure 6.1 Percentages of NK cells within head and respiratory tract tissues post-intranasal BCG inoculation

Calves were inoculated with BCG Pasteur delivered intranasally and tissues and lymph nodes from the head and lungs were obtained (Table 2.1) at post-mortem 7 days post-inoculation. Naive animals served as controls. Percentages of NK cells and subsets were determined by identifying NKp46+ lymphocytes and CD2 expression levels by flow cytometry. Data represents the average percentages of NK cells from 3 naive animals (pink) and 5 BCG inoculated (green) animals (Fig 6.1.1) with percentages of NK cells within RPLNs shown in more detail (6.1.2). Proportions of CD2+ (red) and CD2- (blue) NK cell subsets within tissues were determined (Figure 6.1.3) and ratios of CD2+:CD2- NK cells compared (Fig 6.1.4). Error bars denote the standard deviation between animals. Significance was assessed using paired T-Tests and a GLM with Tukey post-hoc comparisons (95% CI); $p < 0.05^*$.

6.2.2 Influence of subcutaneous BCG vaccination on NK cells within draining lymph nodes.

Experiments conducted to determine the effects of intranasal BCG administration indicated small yet significant increases in the percentages of NK cells within draining lymph nodes (Left RPLN Figure 6.1.2). As the immune response may have been substantially dispersed amongst the lymphoid tissues found within the head and respiratory tract, further studies were conducted to ascertain how BCG administration may influence NK cell numbers and functionality within specific draining lymph nodes. Calves were vaccinated with BCG Pasteur (2.2.1), administered subcutaneously to the left shoulder and the prescapular draining lymph node was obtained at post-mortem, initially at 7 days post-vaccination. Lymph nodes from the right shoulder served as an internal control.

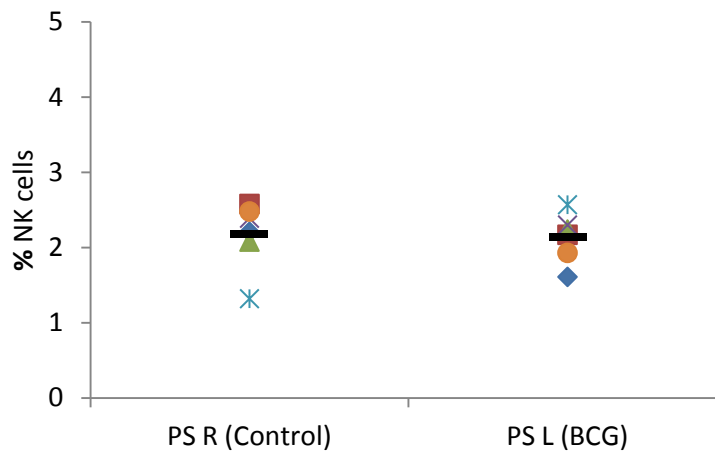
Within the total lymphocyte population found within prescapular lymph nodes, NK cells constituted between 1-3% of cells (Figure 6.2.1) with a predominance of CD2- NK cells within this population (Figure 6.2.2). Comparison of numbers between the control and BCG administered draining lymph nodes resulted in no observed differences in the overall percentages of NK cells (Figure 6.2.1). Additionally, BCG administration did not alter the proportions of NK cell subsets (Figure 6.2.2).

Studies have indicated that the DC induced recruitment of NK cells to draining lymph nodes may occur at early time points post-challenge and be transient in nature (Martin-Fontecha et al., 2004). For this reason, subcutaneous administration of BCG Pasteur was repeated within additional calves and prescapular lymph nodes were obtained at day 1 and day 3 post-challenge. Similar to day 7, NK cells numbers and proportions of NK cell subsets were not influenced by BCG at earlier time points (data not shown).

As vaccination with BCG appeared not to induce the overall recruitment of NK cells to draining lymph nodes, further investigations were conducted to ascertain whether any phenotypical differences could be observed within the populations of NK cells. Calves were vaccinated with BCG Pasteur to the left shoulder and the right and left prescapular lymph nodes obtained 7 days post-challenge were analysed for CD25 cell

surface expression and intracellular perforin and IFN γ expression (2.12). Large percentages of both CD2⁺ and CD2⁻ NK cells (30-40%) were found to constitutively express CD25 as has been reported previously (Boysen et al., 2008). However, BCG administration did not appear to influence these expression levels (data not shown). Additionally only a small percentage of NK cells from lymph nodes expressed detectable levels of perforin with none found to be expressing IFN γ . In a similar manner to CD25; perforin or IFN γ production was not significantly altered by BCG vaccination.

6.2.1



6.2.2

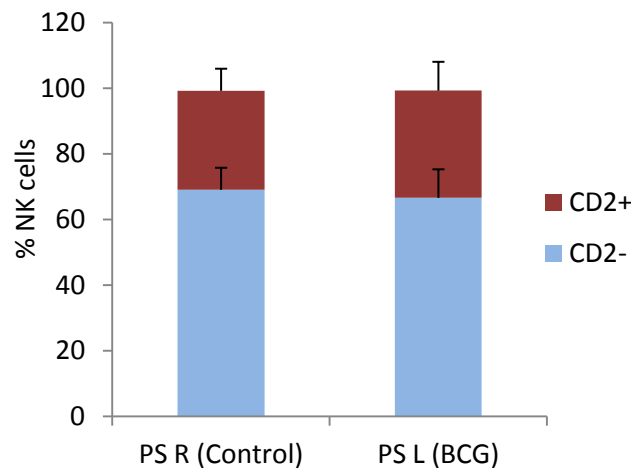


Figure 6.2 Percentages of NK cells within prescapular lymph nodes following BCG vaccination

Calves received 1×10^6 cfu BCG Pasteur delivered subcutaneously to the left shoulder and draining prescapular lymph nodes (PS) were obtained at post-mortem 7 days post-challenge with nodes from the right shoulder serving as controls. Percentages of NK cells and subsets were determined by identifying NKp46+ lymphocytes and CD2 expression levels by flow cytometry. Data represents the percentages of NK cells from six animals within the BCG vaccinated left (L) PS and the control right (R) PS (Fig 6.2.1). Proportions of CD2+ (red) and CD2- (blue) NK cell subsets were determined (Fig 6.2.2) with error bars denoting the standard deviation between animals. Significance was assessed using paired T-Tests and a GLM with Tukey post-hoc comparisons (95% CI).

6.2.3 BCG vaccination induced alterations in the percentages of circulating NK cells

Preliminary investigations to ascertain whether NK cells were recruited *in vivo* post-BCG inoculation resulted in varying responses. Small increases in the percentages of NK cells were observed within retropharyngeal lymph nodes draining the site of intranasal inoculation however, a similar increase was not observed within draining prescapular lymph nodes following subcutaneous BCG vaccination. For this reason, a more global approach was undertaken. Vaccination experiments were repeated with calves receiving BCG Pasteur administered subcutaneously to the left shoulder and age matched naïve animals serving as controls (2.2.1). Percentages of NK cells within blood were monitored every day post-vaccination up until seven days followed by every fortnight thereafter for twelve weeks. Whole blood stimulation (week 7 post-vaccination) with *M.bovis* derived purified protein (PPDb) confirmed the presence of antigen specific IFN γ responses in all vaccinated animals as an indication of successful delivery of BCG (data not shown).

No significant changes in NK cell numbers were observed within the first week post-vaccination (data not shown) or at any time points within naïve animals (Figure 6.3). However, two weeks following vaccination there was an observed increase in the percentages of circulating NK cells compared to naïve animals, with a peak in NK cell numbers at four weeks. At this time, percentages of NK cells within vaccinated calves were significantly higher than those found prior to vaccination ($p=0.037$) and also compared to naïve animals ($p=0.008$). Further analysis of the proportions of NK cell subsets, by determining CD2 expression levels highlighted no significant effect of BCG vaccination on the proportions of CD2 $^{+}$ or CD2 $^{-}$ NK cells within the total NK cell population (data not shown).

Detailed examination of intracellular molecule expression (2.12.1) indicated only a small percentage of NK cells expressing perforin. Over the twelve week sampling period, variations in perforin expression levels were observed however, as this was the case in both control and vaccinated animals, it is likely to reflect age related changes in expression, as previously described (Graham et al., 2009) rather than a response to

vaccination. Additionally, NK cells from both naïve and vaccinated animals did not express intracellular IFN γ at any of the time-points assayed (data not shown).

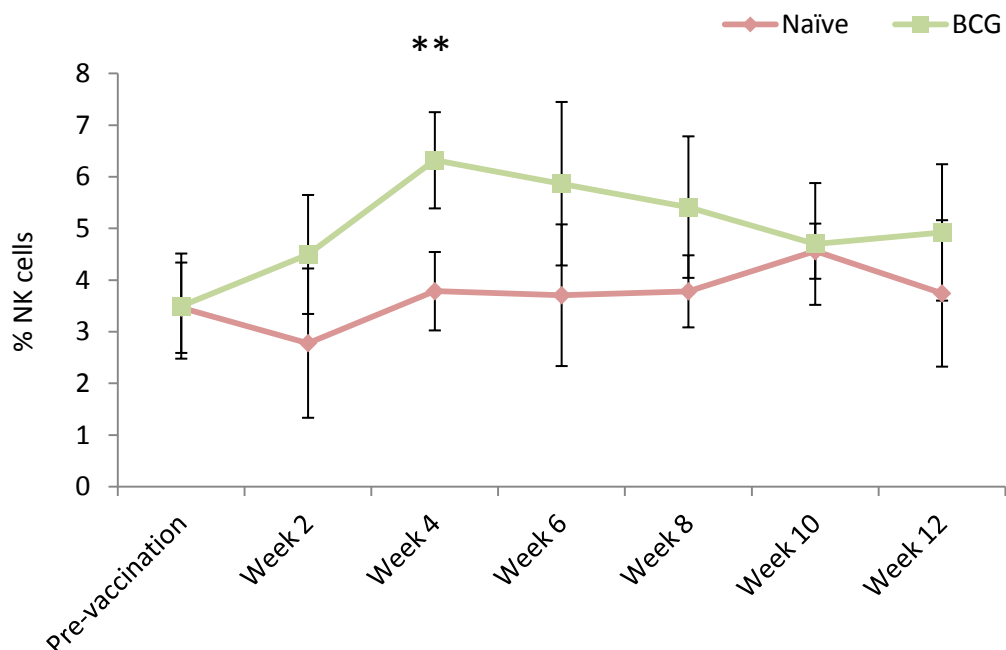


Figure 6.3 BCG vaccination induced changes in percentages of circulating NK cells

Peripheral blood samples were obtained from eight animals; four of which received BCG Pasteur delivered subcutaneously to the left shoulder and four naïve animals which were age-matched controls. Percentages of NK cells were determined by identifying NKp46+ lymphocytes pre-vaccination, each day up to seven days post-vaccination (data not shown) and every fortnight thereafter for twelve weeks. Data represents the average percentages of NK cells within the total lymphocyte population from BCG vaccinated (green) and naïve (pink) animals and error bars denote the standard deviation between animals. Significance was assessed using T-Tests and GLM with Tukey post-hoc comparisons (95% CI); $p < 0.05^*$, $p < 0.01^{**}$.

6.2.4 *M.bovis* induced alterations in the cytokine production and percentages of circulating NK cells

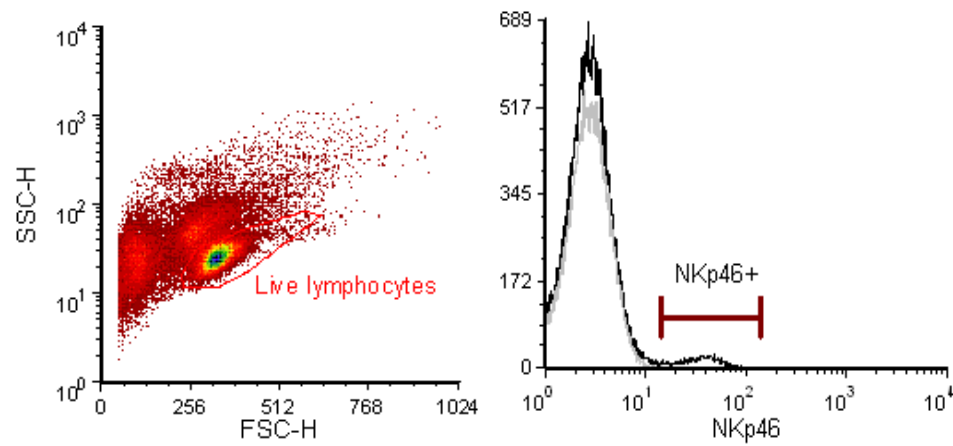
Further *in vivo* experimentation sought to determine how infection with virulent *M.bovis* may induce early innate immune responses and in particular whether alterations in NK cell numbers and activation status could be observed. Peripheral blood samples were obtained from five animals intra-tracheally challenged with *M.bovis* (2.2.3) and percentages of NK cells (Figure 6.4.2) and proportions of NK cell subsets were analysed between days 2 and week 3 post-infection. Animals were slaughtered at week 12 and post-mortem examination of head and respiratory tract tissues to assess the size and severity of lesions revealed that all animals had lesions typical of bovine TB infection (9.8).

Following *M.bovis* infection, alterations were observed in the percentages of NK cells (Figure 6.4.3) with significantly fewer circulating cells at both day 5 ($p=0.009$) and week 2 ($p=0.017$) when compared with percentages of NK cells prior to infection. By week 3 post-infection, cell numbers had recovered and were similar to pre-infection levels. Detailed analysis of the proportions of NK cell subsets, by determining CD2 expression levels highlighted alterations in the CD2⁻ population (Figure 6.4.4). In particular the proportion of CD2⁻ NK cells appeared to be significantly increased at day 2 post-infection ($p=0.014$) compared with pre-infection levels, however the response was very small and no other alterations were observed at any other time point.

In vitro data within co-culture experiments proposed significant alterations in NK cell activatory responses following mycobacterial infection (5.2). To determine whether this was also the case *in vivo*, expression of intracellular perforin and IFN γ by peripheral blood derived NK cells were assayed at early time-points following experimental challenge. As has previously been shown, small percentages of circulating NK cells were expressing perforin, however the observed levels did not change following infection. In contrast, significant changes in the percentages of NK cells producing IFN γ were observed. By day 2 (Figure 6.5.1) post-infection small percentages of CD2⁻ NK cells were found to be producing detectable levels of IFN γ . This was also the case at day 5 (Figure 6.5.2) and was significantly augmented by week

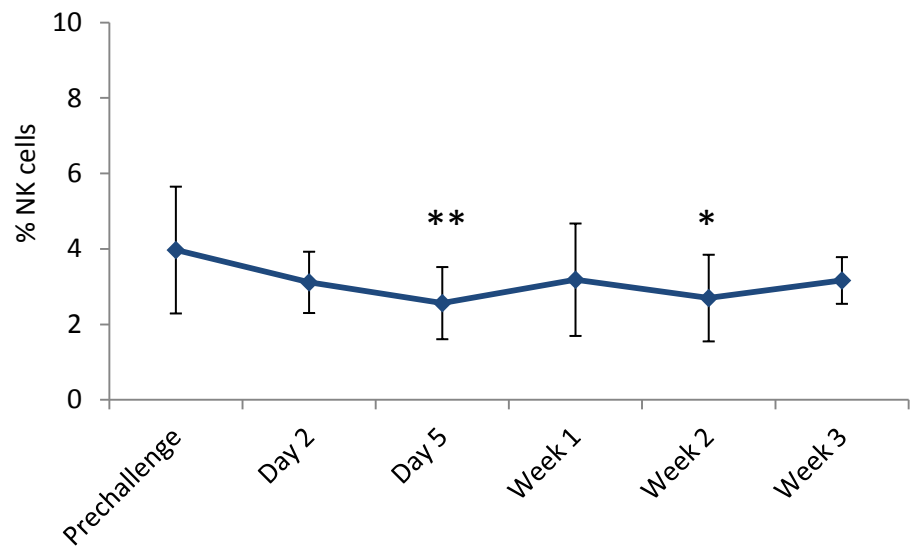
2 (Figure 6.5.3) when compared with the percentages at day 2 ($p=0.030$) and day 5 ($p=0.003$) (Figure 6.5.4). By contrast, CD2+ NK cells did not express IFN γ at any of the time-points analysed post-mycobacterial challenge.

6.4.1



6.4.2

6.4.3



6.4.4

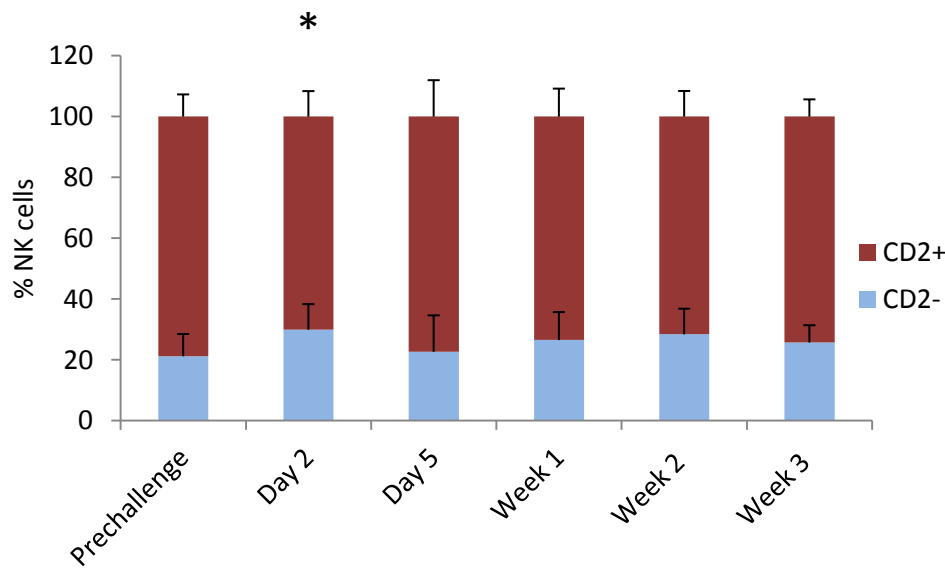
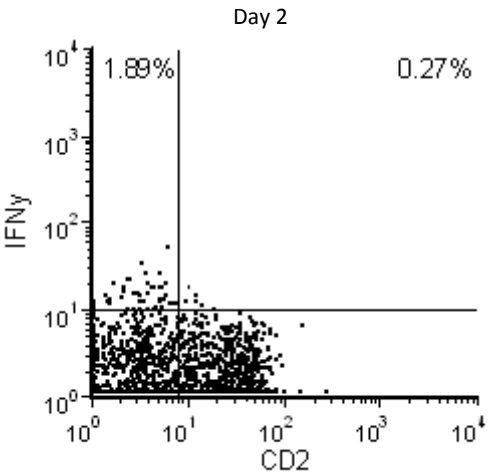


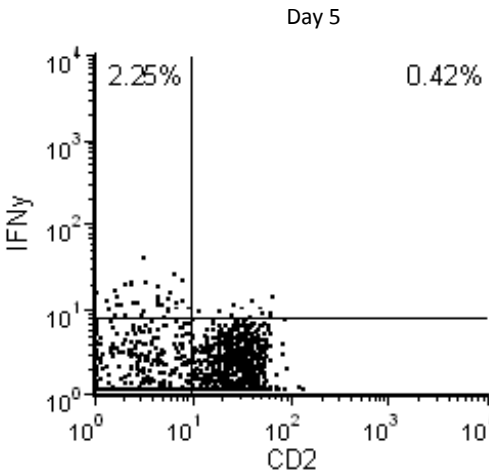
Figure 6.4 *M.bovis* induced changes in percentages of circulating NK cells

*Peripheral blood samples were obtained from five animals intra-tracheally challenged with *M.bovis* at time points between days 2 and week 3 post- infection (2.2.3). PBMCs were isolated, live cells identified by their FSC and SSC properties (Fig 6.4.1) and percentages of NK cells (Fig 6.4.2 and 6.4.3) and proportions of CD2+ and CD2- NK cell subsets (6.4.4) were determined by labelling lymphocytes with mAbs for NKp46 and CD2 and analysis by flow cytometry with pre-challenge data serving as a control. Data represents the average values obtained from five animals with error bars denoting the standard deviation between animals. Significant changes in the numbers of NK cells and the proportions of subsets were assessed using log transformed data and a 1-Way ANOVA or GLM with Tukey post-hoc comparisons (95% CI); $p, 0.05^*, p < 0.01^{**}$.*

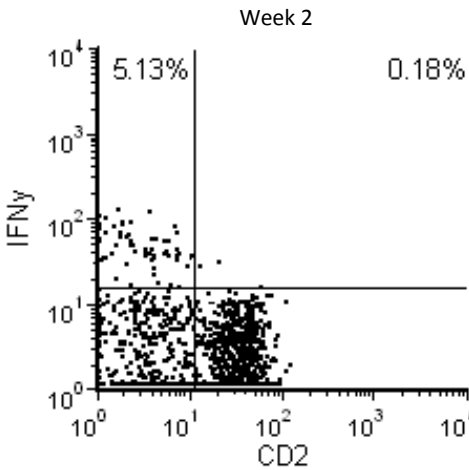
6.5.1



6.5.2



6.5.3



6.5.4

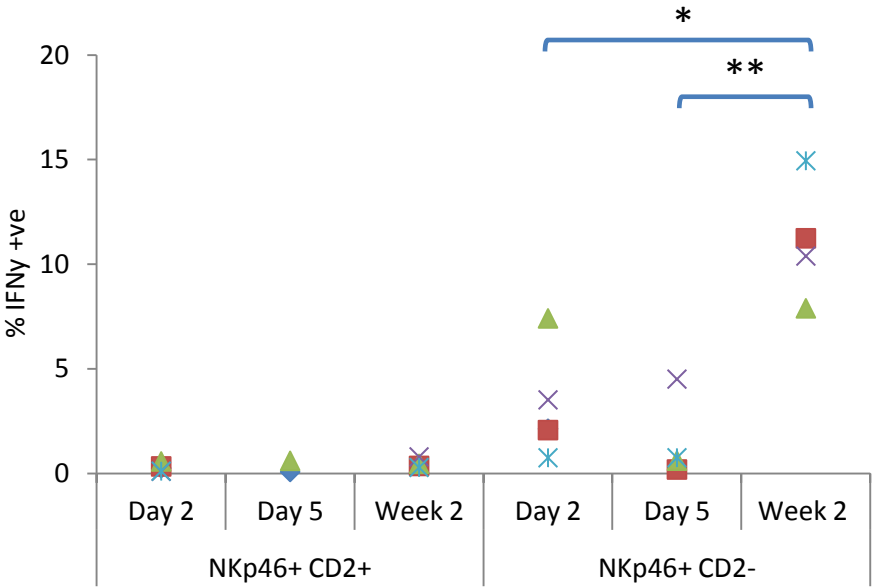


Figure 6.5 *M.bovis* induced changes in cytokine production by NK cells

*Proportions of NK cell subsets producing IFN γ and Perforin from *M.bovis* infected animals were determined by overnight incubation of PBMCs with TCM supplemented with BFA (10 μ g/ml) followed by intracellular cytokine staining (2.12.1.1). FACS plots from one representative animal are shown with proportions of CD2 $^{+}$ and CD2 $^{-}$ NK cells producing IFN γ at days 2 (Fig 6.5.1), day 5 (Fig 6.5.2) and week 2 (Fig 6.5.3) post- challenge. Pooled data from five animals (Fig 6.5.4) represents the percentage of IFN γ positive NK cell subsets expressed as a percentage of the total CD2 $^{+}$ or CD2 $^{-}$ population. Significant changes in the proportions of IFN γ producing NK cells were assessed using log transformed data and a GLM with Tukey post-hoc comparisons (95% CI); $p < 0.05^{*}$, $p < 0.01^{**}$.*

6.3 Discussion

The virulent airborne pathogen, *M.bovis* predominantly infects via the respiratory route with naturally infected cattle presenting with granulomatous lesions within the respiratory tract and head. Innate effector cells such as NK cells and $\gamma\delta$ T cells are likely to be amongst the first lymphocytic populations to respond to infected antigen presenting cells within these localities. It has previously been documented that NK cells play prominent role during mycobacterial infection (Feng et al., 2006) and that NK cell derived IFN γ in particular is required for effective Th1 polarisation of the immune response (Martin-Fontecha et al., 2004). Under steady state conditions, as well as forming a constituent of the peripheral blood derived population, NK cells are also prevalent within a number of tissues. Bovine NK cells have been identified in the spleen, liver, lung and also within mucosal and non-mucosal lymph nodes (Boysen et al., 2008, Goff et al., 2006, Storset et al., 2004).

Within this study, tissues and lymph nodes of the head and respiratory tract were comprehensively analysed with the view that these would be the regions directly afflicted by *M.bovis* infection. The highest percentages of NK cells were found within the apical to diaphragmatic region of the lung constituting 10-20% of the total lymphocyte population. Similarly innate lymphocytes are commonly found in significant numbers within tissues underlying mucosal surfaces such as the gut or uterine mucosa, to provide rapid innate defence mechanisms within areas that may be exposed to a high level of antigenic insult. In comparison, the head and lung associated lymph nodes contained smaller percentages (1-5%) of NK cells. Populations of NK cells have also been identified within human lymph nodes (Fehniger et al., 2003, Ferlazzo et al., 2004b) however, in contrast are largely excluded from lymph nodes in mice (Bajenoff et al., 2006, Gregoire et al., 2007). It has been hypothesised that the prevalence of NK cells within lymph nodes of cattle may be a reflection of the higher environmental microbial burden to which cattle are exposed to, resulting in a primed, CD25 expressing and cytolytic resident population (Boysen et al., 2008).

Studies within this thesis have demonstrated significant heterogeneity in the responses of bovine NK cell subsets; with differential migration and cytokine release.

In particular, the production of IFN γ in response to *M.bovis* infected DCs was determined by the proportion of CD2- NK cells present within the total NK cell population. Therefore, a greater knowledge of the distribution patterns of bovine NK cell subsets within the tissues afflicted by mycobacterial infection may provide further insight into the pathogenesis and progression of infection. Bovine CD2- NK cells are found in predominant numbers within lymph nodes and it was confirmed that this was also the case within lymph nodes of the head and respiratory tract. It has previously been reported that IL-2 rich environments, as is the case within lymph nodes, may favour the expansion and persistence of CD2- NK cells (Boysen et al., 2008). In addition, within this thesis transcripts for CCR7 and CXCR3 were found at significantly higher levels within CD2- NK cells; expression of which would confer the capacity to home towards CXCL10, CCL19 and CCL21 rich lymphoid tissue. In contrast, the proportions of CD2+ and CD2- NK cells within the lungs and tonsillar regions were far more equal with a slight predominance of CD2+ NK cells. Within co-cultures of *M.bovis* infected DCs CD2+ NK cells appeared to possess a higher threshold for activation compared with CD2- NK cells, requiring both cytokines and cell-cell contact prior to activation and induction of cytolytic activity (5.2.4). In addition IFN γ release was only induced after optimal cytokine stimulation (5.2.2.1). The environment within the lungs and airways is regarded as being relatively anergic, with bovine alveolar macrophages reported to require multiple sequential signals in the form of LPS and IFN γ to promote bactericidal activity against *M.bovis* infection (Aldwell et al., 1997). Similarly it might be hypothesised that a larger proportion of CD2+ NK cells that require multiple stimuli prior to activation may represent a control mechanism to prevent the development of large inflammatory immune responses within the airways.

Models that administer antigen by mimicking the natural route of infection provide a useful insight into the progression of an immune response within the tissues that would be specifically afflicted by mycobacterial infection. Within mice, intranasally delivered BCG was rapidly taken up by DCs (Reljic et al., 2005) with an increase in the absolute number of NK cells within the lung parenchyma (Saxena et al., 2002). Similarly, following infection with *M.tuberculosis*, NK cells were recruited to the lungs contributing to local IFN γ production (Junqueira-Kipnis et al., 2003). Corresponding

studies in cattle demonstrated increased numbers of IFN γ producing WC1+ $\gamma\delta$ T cells within the pharyngeal tonsils and lung tissue 7 days after intranasal delivery of BCG (Price et al., 2010). Despite this, within similar studies we observed only a small increase in the percentage of both CD2+ and CD2- NK cells within the left retropharyngeal lymph node with no change in any other tissues within the head or respiratory tract following intranasal BCG inoculation. The retropharyngeal lymph nodes drain the tonsillar region and have been reported as the primary site of lesion formation, containing the highest bacterial counts following experimental infection with *M.bovis* (Griffin et al., 2006). Within our intranasal challenge studies, BCG was administered to the left nostril and the small yet significant changes in the NK cell population observed on this side may therefore be a reflection of elevated bacterial exposure and the ensuing immune response at this site. In further investigations, analysis of prescapular lymph nodes that specifically drain the site of subcutaneous BCG vaccination did not reveal any changes in the percentages, proportions of subsets or functional activity of NK cells at time-points ranging from 1 day to 2 weeks post-vaccination.

However, we observed a significant increase in the percentages of circulating NK cells at 2 to 4 weeks post-vaccination; time-points that correspond with early innate immune responses that may occur prior to the development of antigen specific immunity (Buddle et al., 1995, Hope et al., 2005b). Similarly Buza et al., (2009) also detected increased numbers of circulating $\gamma\delta$ T cells in cattle as early as 1 week following BCG vaccination. It should however be noted that in future studies, the absolute numbers of NK cells would need to be considered in order to fully clarify whether the observed change in the percentages of circulating NK cells post-vaccination are a true reflection of changes in NK cell numbers rather than as a consequence of changes in other lymphocytic populations.

Substantial evidence suggests that BCG primes the innate immune response, with NK cells implicated as a source of early innate IFN γ (Hope et al., 2002b, Olsen et al., 2005). Herein, whilst changes in the circulating numbers of NK cells could be detected, within these studies we could not confirm substantial lymph node involvement. This however, does not completely discount a role of NK cells within lymph nodes. Murine

studies reported changes in the absolute numbers of NK cells (Saxena et al., 2002) upon intranasal BCG administration. We analysed the percentage of NK cells in relation to the total lymphocytic population and therefore changes in the absolute numbers would not have been detected. In addition, NK cells are largely excluded from murine lymph nodes and therefore recruitment during an inflammatory immune response is readily detectable. In comparison lymph nodes within cattle contain relatively large pre-existing resident populations. It has been reported that NK cells within activated lymph nodes are significantly more mobile resulting in a more wide-spread distribution and multiple transient interactions with DCs (Beuneu et al., 2009, Hall et al., 2010). In addition, upon intranasal inoculation with BCG, local re-distributions of resident $\gamma\delta$ T cells within lymphoid tissue were observed (Price et al., 2010). It might therefore be hypothesised that during mycobacterial infection, rather than a large influx or expansion of NK cells, local resident populations may be re-distributed to regions where interactions with DCs may ensue prior to release into the circulation. Further investigations utilising immuno-fluorescent analysis would need to be conducted to determine if this was the case.

In further preliminary studies, changes in NK cell numbers and phenotype were readily detectable in the periphery of *M.bovis* infected cattle. Similar responses have also been described within the bovine $\gamma\delta$ T cell population (Kennedy et al., 2002). We observed an early decrease in the percentage of circulating NK cells, potentially signifying re-distribution and accumulation at the site of infection. In order to confirm this, tissues from *M.bovis* infected cattle would need to be analysed in detail by flow cytometry or immuno-fluorescent microscopy. Of interest was the finding that at time-points prior to the decrease in total NK cells, a transient yet significant increase in the CD2- NK cell population was detected. Within *in vitro* studies, this thesis has demonstrated that CD2- NK cells are selectively recruited by *M.bovis* infected DCs and preferentially secrete IFN γ whilst reciprocally promoting DC maturation. As such, it may be this subset which is selectively released into the circulation or stimulated to proliferate in preparation for the immune response to *M.bovis* infection.

Furthermore, evidence herein suggests that CD2- NK cells may play a prominent role during the early phases of infection, with IFN γ producing CD2- NK cells detected in the

periphery 2 days following *M.bovis* infection with increasing percentages over time. This may contribute to early attempts to control infection however, all animals within the *M.bovis* challenge studies were infected as evidenced by the development of lesions within the head and respiratory tract at post-mortem. It is therefore not known to what extent the observed responses are associated with protective immunity. Studies suggest that high circulating numbers of innate effector cells such as NK cells and $\gamma\delta$ T cells contribute to the enhanced efficacy that is observed when BCG is administered to neonates (Buddle et al., 1995, Hope et al., 2011, Hope et al., 2005b). Therefore, future studies comparing vaccinated/infected animals versus infected animals would need to be conducted to determine whether any differences in the IFN γ responses of CD2- NK cells influence the severity of *M.bovis* infection and potentially correlate with protection.

Tuberculosis is a chronic respiratory illness caused by highly virulent mycobacterial species belonging to the *M.tb* complex. Whilst *M.tuberculosis* infects only humans, *M.bovis* has much broader host range (Thoen et al., 2006) and is the primary cause of TB within cattle. The increasing incidence of bovine TB poses a considerable burden to the UK economy as well as a potential health-risk through zoonotic transmission to humans. Control ‘test and slaughter’ strategies have been in place for over 50 years but have failed to curb the rise in infections particularly apparent in the South West regions of the UK. This at least in part may be due to the existence of a reservoir of infection with *M.bovis* endemic within the wild badger population. Shedding of *M.bovis* from infected badgers contributes to the persistence of infection resulting in repeated herd breakdowns and spread of bovine TB into new areas of the UK (Krebs et al., 1998, Smith et al., 2006). As a consequence, a number of strategies have been explored that attempt to control *M.bovis* infection in badgers as an indirect means to reduce the incidences within cattle. Badger culling trials conducted over a number of years concluded that whilst localised culling may be effective, long-term repeated initiatives would be required to continually remove infected badger populations over much larger areas. However, this strategy may not be logistically or financially feasible (Jenkins et al., 2010) and highlights the need for alternative control measures to be established. The vaccination of badgers with BCG has been licensed in the UK, with trials currently being conducted within regions of high incidences. However, it has been proposed that the most effective means of controlling bovine TB infection will be through development of an effective vaccination strategy for cattle (Krebs et al., 1998).

The attenuated strain of *M.bovis* BCG is widely administered in humans throughout the world and still remains the most effective vaccine to prevent the most severe forms of TB. Despite this, the efficacy that is observed following BCG vaccination is highly variable and is most effective when administered to infants (Fine, 1995, Marchant et al., 1999). This is also true for cattle with a number of studies demonstrating enhanced protection against *M.bovis* challenge following neonatal vaccination (Buddle et al., 1995, Hope et al., 2011, Hope et al., 2005b). The reasons for

this are not completely understood however it is believed that exposure to environmental mycobacteria may inappropriately prime the immune system and influence the subsequent immune response to BCG vaccination (Buddle et al., 2002, Fine, 1995). Therefore the vaccination of neonates prior to substantial exposure may circumvent these issues. In addition, neonatal calves possess high circulating percentages of innate effector cells such as NK cells and $\gamma\delta$ T cells that may contribute to an enhanced innate and subsequent adaptive immune response following BCG vaccination.

However, BCG vaccinated cattle respond non-specifically in the tuberculin skin test that is used throughout the UK to identify *M.bovis* infected cattle and as a consequence, under current EU legislation the administration of BCG to cattle is prohibited. This has been the driving force for substantial research within the field, in attempts to identify novel vaccine candidates or alternative diagnostic strategies. It is clear that concurrent research into the fundamental immune responses that occur during *M.bovis* infection are also required in order to underpin the future development of preventative strategies against bovine TB infection.

Research within this thesis has focussed on the potential for innate immune interactions to influence immune responses in cattle during *M.bovis* infection. It is widely accepted that the innate immune system not only functions as a front-line defence mechanism but also plays an implicit role in the shaping of adaptive immunity. In particular, small sub-populations of cytolytic lymphocytes known as NK cells can reciprocally interact with DCs to potentiate the priming of T cell responses. The precise mechanisms that confer protective immunity to mycobacterial infection have not been established however, the early release of IFN γ and Th1 polarisation is of fundamental importance (Cooper et al., 1993, Flynn et al., 1993, Orme, 2004, Welsh et al., 2005). This thesis therefore explored the hypothesis that bovine NK cells also play a significant role in the innate immune response to *M.bovis* infection. Previous research has defined phenotypically and functionally distinct subsets of bovine NK cells however their roles during infection have not been investigated. The main findings from this study are summarised in Figure 7.1.

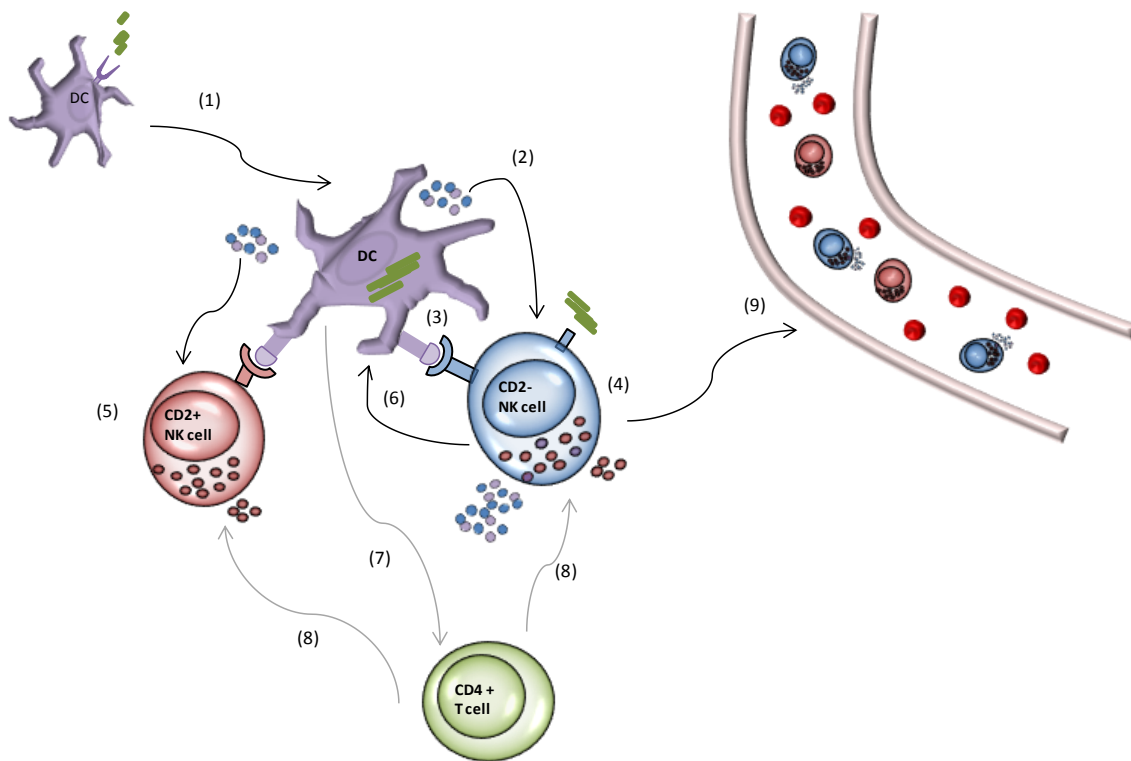


Figure 7.1 The reciprocal interactions of *M.bovis* infected DCs and bovine NK cells

*DCs lining the trachea or resident within the lungs are likely to be amongst the primary populations responding to mycobacterial infection (1). NK cells form resident populations within the lung parenchyma and lymph nodes highlighting the potential for reciprocal NK DC interactions to occur both at the site of infection as well as within draining lymph nodes. DCs infected with *M.bovis* undergo a process of maturation resulting in augmented expression of inflammatory chemokines and selective recruitment of bovine NKp46+ CD2- NK cells. DC derived cytokines (IL-12) (2) alongside direct cell-cell contact (3) contribute to the activation of CD2+ and CD2- NK cells, however the precise contact dependent signals are yet to be determined. Upon activation, both CD2+ and CD2- NK cells up-regulate expression of perforin which corresponds to an increased capacity to kill target cell populations. In contrast, only CD2- NK cells secrete IFN γ (4). Evidence suggests that CD2+ NK cells (5) are more reliant on cell contact and cytokine stimulation to remain activated. During reciprocal interactions, CD2- NK cells enhance the maturation of DCs with increased intensity of MHC class II expression (6). Studies are required to determine whether this translates into an increased capacity to prime or activate adaptive immune responses during mycobacterial infection (7/8). The prominent role of NK cells during the innate immune response is evidenced by an increase in circulating numbers of NK cells following BCG vaccination and IFN γ expressing CD2- NK cells detectable within the periphery at early time points following *M.bovis* infection (9).*

Substantial differences in the patterns of chemokine receptor expression between CD2+ and CD2- bovine NK cells derived from peripheral blood were demonstrated herein. Bovine CD2- NK cells transcribed higher levels of important inflammatory and lymphoid homing chemokine receptors when compared with the levels observed for CD2+ NK cells. These differences appeared to influence the migratory capacity with *M.bovis* infected DCs selectively recruiting CD2- NK cells within *in vitro* chemotaxis assays. Further analysis utilising co-culture assays highlighted the ability of *M.bovis* infected DCs to induce the effector responses of bovine NK cells in a cytokine and cell-cell contact dependent manner. These interactions were reciprocal in nature with an enhanced intensity of MHC class II expression by *M.bovis* infected DCs following contact with bovine CD2- NK cells. Importantly, during this interaction only CD2- NK cells secreted IFN γ and significant differences in the activation requirements between both subsets of NK cells were observed. In addition, IFN γ expressing CD2- NK cells could be detected in the peripheral circulation of cattle as early as 2 days following infection with *M.bovis*.

Whilst, this study has demonstrated significant involvement of bovine NK cells during *M.bovis* infection, the relevance of these responses *in vivo* in terms of protective immunity is not known. Murine studies have described the importance of NK cell derived IFN γ release, required for induction of cell mediated immunity (Martin-Fontecha et al., 2004). The role of IFN γ in mycobacterial infection is a debated issue amongst researchers. It is believed that early innate secretion of IFN γ contributes to the control of bacterial replication as well enabling effective Th1 polarisation. However, during advanced stages of *M.bovis* infection in cattle, elevated levels of IFN γ positively correlate with an increased severity of infection (Vordermeier et al., 2002). It might therefore be hypothesised that at early stages following both vaccination and infection, the effective recruitment of bovine CD2- NK cells and subsequent interactions with DCs may function to potentiate Th1 immune responses in the development of protective immunity. Conversely, during advanced stages of infection the resident populations of NK cells within the lymph nodes and tissues of the head and respiratory tract may continue to interact with *M.bovis* infected DCs in an un-regulated manner to contribute to the immuno-pathological state associated with

chronic TB infection. It is possible that the balance of CD2+ and CD2- NK cells within tissues afflicted by mycobacterial infection may be pivotal in determining the extent of damage, with a larger population of CD2- NK cells potentially more detrimental. Clearly, substantial research will be required to determine whether the observed responses *in vitro* influence the balance between protection or progressive infection.

Interestingly, within *in vitro* assays the responses of BCG infected DCs and NK cells were substantially different. Whilst *M.bovis* infection of DCs augmented the transcription of a number of inflammatory and lymphoid homing chemokines, the same was not true for BCG. In a similar manner, DCs infected with BCG secreted reduced levels of IL-12 and IL-10 when compared with the levels observed with *M.bovis*. In addition, the intranasal or subcutaneous administration of BCG did not appear to substantially influence the percentages of NK cells within local tissues or draining lymph nodes. Published reports have defined the implicit nature of DC derived chemokines in the recruitment of NK cells, with the resulting interaction fundamental for the effective development of Th1-dominated immune responses (Martin-Fontecha et al., 2004). It might therefore be speculated that the inability of BCG infected DCs to promote the recruitment of NK cells may contribute to the varied efficacy that is often observed following vaccination. However, a more comprehensive study of the responses of bovine NK cell subsets with BCG both *in vitro* and *in vivo* would need to be conducted before this conclusion can be made.

Nevertheless, the potential for targeting the NK cell and DC interaction for vaccine induced protective immunity is gaining precedence in a number of disease models. Adjuvants may be incorporated into vaccines to induce HIV specific NK cell effector responses (reviewed by (Altfeld et al., 2011)). In addition murine studies have described the capability of DC based vaccines in the induction of NK cell mediated anti-tumour responses (Shimizu and Fujii, 2009). Perhaps most significantly however, is the finding that during reciprocal interactions NK cells function to enhance the maturation of DCs and therefore directly influence adaptive immune responses. In particular, it has previously been reported that DCs produced elevated levels of IL-12, increased expression of CCR7 and induced stronger T cell mediated IFN γ responses after contact with NK cells (Mailliard et al., 2003, Morandi et al., 2009, Van Elssen et al., 2010). The

findings within these studies have formed the basis for research into DC based vaccination strategies that promote NK cell and DC interactions to skew Th1 mediated immune responses. Given that protective immunity to mycobacterial infection is also reliant upon cell mediated CD4+ and CD8+ T cell responses, the interaction between bovine CD2- NK cells and DCs may represent an important target for future vaccination strategies against bovine TB. Particularly, since this interaction appeared to enhance the expression of levels of MHC Class II on DCs which may facilitate T cell stimulation. Importantly, murine studies have demonstrated the potential for targeting DCs for protective immunity against TB infection. Immunisation with DCs pulsed with viral vectors expressing mycobacterial antigens induced CD4+ and CD8+ T cell responses, conferring significant protection upon *M.tuberculosis* challenge (McShane et al., 2002). However, the involvement of NK cells within these responses was not determined and highlights the need for studies to investigate this interaction further as a factor that may influence protective efficacy.

An important consideration in the development of a vaccine to target this pathway appears to be that the precise maturation stimuli utilised substantially influences the capacity of DCs to recruit and interact with NK cells (Gustafsson et al., 2008). Within murine studies, Martin-Fontecha et al., (2004) demonstrated that only certain adjuvants were able to promote T cell priming and this was dependent upon the ability of matured DCs to recruit NK cells to draining lymph nodes. Signalling via TLRs expressed by DCs appears to be an important prerequisite with ligands for TLR4 stimulating the secretion of CCL5, the recruitment of NK cells (Van Elsen et al., 2010) and subsequent IFN γ release (Zanoni et al., 2005).

The targeting of DCs for vaccination is a particularly attractive prospect in cattle and has been the focus of substantial research in recent years. The development of a bovine cannulation model enables the acquisition of afferent lymph containing *ex vivo* DCs that drain the site of subcutaneous vaccination (Hope et al., 2006). Evidence suggests that DCs within the skin of cattle exist as heterogeneous sub-populations that differ in their capacity to stimulate T cell responses (Howard et al., 1997). In particular, only a small subset of DCs that constitute on average about 20% of the total DC population were able to phagocytose BCG and were subsequently poorly able to

stimulate antigen specific CD4+ T cell proliferative responses (Hope JC, unpublished). As such, there may be potential to incorporate adjuvants alongside BCG to promote the recruitment and interaction between this sub-population of DCs and CD2- NK cells as a strategy to enhance T cell mediated immunity.

In summary, this thesis has presented evidence to suggest that through interactions with DCs, bovine NK cells participate in the early innate immune responses to *M.bovis* infection. The distinct roles that NK cell subsets play are likely to be of significant importance in particular, in the shaping of adaptive immune responses. Further research is required to determine precisely how the responses observed herein influence the developing immune response. A greater understanding of the mechanisms by which NK cell and DC interaction confer protective immunity may underpin the future development of targeted prophylactic strategies in which this interaction can be exploited to our advantage.

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9.1 Media

Tissue culture medium (TCM)

RPMI 1640 with Glutamax + 25mM HEPES

10% foetal calf serum

5x10⁻⁵M 2-mercaptoethanol

Gentamicin (50µg/ml)

Dendritic cell culture medium

RPMI 1640 with Glutamax + 25mM HEPES

25mM HEPES

10% foetal calf serum

5x10⁻⁵M 2-mercaptoethanol

Gentamicin (50µg/ml)*

IL-4 (rbo IL-4) 200u/ml

GM-CSF (rbo GM-CSF) 0.2u/ml (Werling et al., 1999)

**Gentamicin not included in infected cultures*

Phosphate-buffered saline (PBS) pH6.8

Sodium chloride - 170 mM

Potassium chloride - 3.4 mM

Sodium phosphate - 9.2 mM

Potassium phosphate -1.8 mM

PBS/BSA/Azide

Bovine serum albumin - 10 g/l

Sodium azide - 0.1 % (w/v)

PBS

FACS Flow/BSA (0.5%)

Bovine serum albumin -5 g/l

FACS Flow - 0.22µm filtered

Ammonium chloride lysis buffer (0.1M)

Ammonium chloride - 8.29 g/l
Potassium hydrogen carbonate - 11 g/l
EDTA (disodium salt) - 37 mg/l
1M Sodium hydroxide - pH 7.2

Middlebrook 7H9 medium

Middlebrook 7H9 broth base - 4.7g
Tween 80 - 0.5ml
Sodium Pyruvate - 4.1g
OADC Supplements - 100ml
Super Q Water - 900ml
Middlebrook 7H9, containing OADC, 0.05% Tween 80 and pyruvate (4.16 g/l) –
Microbiological Services, IAH

Middlebrook 7H10 agar

Middlebrook 7H10 agar base - 19.0g
Super Q water - 900ml
Following autoclaving: Oleic acid, bovine albumin, dextrose, beef catalase
(OADC Supplements) - 100ml

Middlebrook 7H11 agar

Mycobacteria 7H11 agar - 18.9g
0.6% Malachite Green solution - 1ml
Super Q water - 770ml
Following autoclaving: Amoxicillin (59mg/ml) - 2ml, Polymixin B (100,000U/ml)
- 2ml, Trimethoprim (5mg/ml)- 2ml, Amphotericin B (2.5mg/ml)-20ml, Lysed
sheep blood - 5ml, Foetal calf serum - 100ml, OADC Supplements - 100ml

Guanidine Thiocyanate buffer

Guanidine thiocyanate 4M
tri-sodium citrate pH7 – 250mM
Tween 80 0.5%
Sodium N-lauryl sarcosine 0.5%
Super Q water - 100ml
Supplemented with 0.1M 2-mercaptoethanol prior to use

9.2 Monoclonal antibodies

Antigen	Antibody	Isotype	Specificity	Supplier
NKp46	CD335	IgG1	Mouse anti-bovine	AbD Serotec, UK
	CD335-PE			
	CD335-FITC			
CD2	ILA43	IgG2a	Mouse anti-bovine	IAH
	CC42-647	IgG1		IAH; Directly conjugated
CD3	MM1A	IgG1	Mouse anti-bovine	VMRD, Washington
CD25	ILA111	IgG2a	Mouse anti-bovine	IAH
	CACT108A			VMRD, Washington
IFNγ	CC330-PE	IgG1	Mouse anti-bovine	IAH; Directly conjugated
	CC330			IAH
	CC302			IAH
TNFα	CC327	IgG2b	Mouse anti-bovine	IAH
	CC328	IgG2a		
IL-10	CC318	IgG2b	Mouse anti-bovine	IAH
	CC320	IgG1		
IL-12	CC301	IgG2a	Mouse anti-bovine	IAH
	CC326	IgG2b		
IL-6	CC310	IgG2b	Mouse anti-bovine	IAH
CD4	CC30	IgG1	Mouse anti-bovine	IAH
CD8$\alpha\alpha$	CC63	IgG2a	Mouse anti-bovine	IAH
CD8$\alpha\beta$	CC58	IgG1	Mouse anti-bovine	IAH
$\gamma\delta$ TCR	GB21A	IgG2b	Mouse anti-bovine	VMRD, Washington
WC1	CC39	IgG1	Mouse anti-bovine	IAH
CD62L	CC32	IgG1	Mouse anti-bovine	IAH
MHC II	CC158	IgG2a	Mouse anti-bovine	IAH
SIRPα	ILA24	IgG1	Mouse anti-bovine	IAH
	CD172a-RPE/647	IgG2b		AbD Serotec, UK
CD40	ILA156	IgG1	Mouse anti-bovine	ILRI Kenya, N McHugh
CD80	N32/52-3	IgG1	Mouse anti-bovine	ILRI Kenya, N McHugh
CD86	ILA190	IgG1	Mouse anti-bovine	ILRI Kenya, N McHugh
Perforin	Perforin-FITC	IgG2b	Mouse anti-human	BD Pharmingen
	Perforin-PE			
CD16	KD1	IgG2a	Mouse anti-human	Dr D Pende, Italy
CD27	MT271	IgG1	Mouse anti-human	BD Pharmingen
CD14	CCG33	IgG1	Mouse anti-bovine	IAH
CD11b	CC94	IgG1	Mouse anti-bovine	IAH
CD21	CC21	IgG1	Mouse anti-bovine	IAH

9.3 Isotype controls

Specificity	Supplier
Mouse IgG1 AF 488 conjugate	Serotec Ltd
Mouse IgG1 AF 647 conjugate	CALTAG Laboratories
Mouse anti-human IgG2b RPE conjugate	BD Pharmingen
Mouse anti-human IgG2b AF 488 conjugate	
Mouse anti-chicken Bu-1 (AV20-IgG1)	Dr C Butter, IAH (Hope et al., 2000)
Mouse anti-chicken CD4 (AV29-IgG2b)	
Mouse anti-chicken spleen cells (AV37-IgG2a)	

9.4 Fluorochromes

Specificity	Supplier
Goat anti mouse IgG-RPE	Southern Biotech
Goat anti mouse IgG1-FITC	
Goat anti mouse IgG2a-FITC	Zenon® labelling kit - Invitrogen
Goat anti mouse IgG2a-FITC	

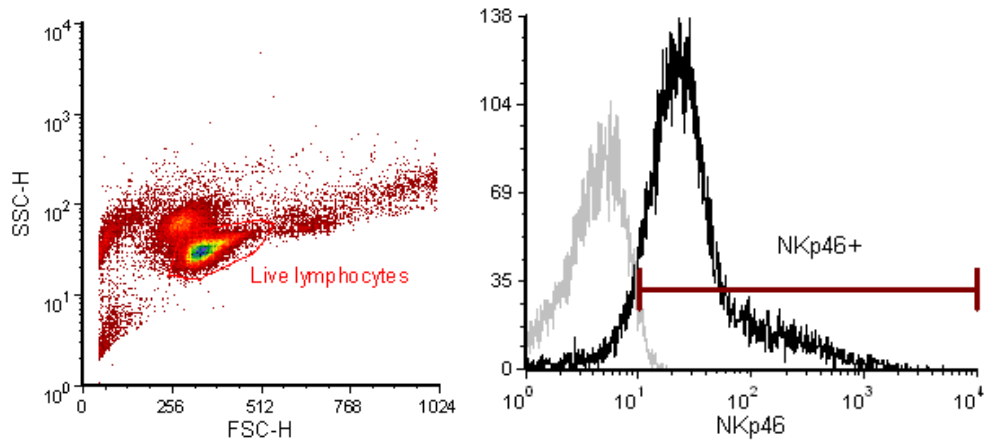
9.5 Primer and Probe sequences

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Probe (5' FAM - 3' TAMRA)
CCR1	ATGGGCTTGACAGCGAGAT	CAGACGACAATGCTGGTGAT	AGTCCGAGCCCGCAGAGCAA
CCR2	CACAATGTGCTTCCCACATC	GAGCTGTGCTTCGATTTGTC	CCCTGCCGAAAGACCAGCGT
CCR3	CAAATGCACTTGAAGGCTGA	CAACGAAGGCATAGATCACG	TCACCAGCATGGCCAGGTCC
CCR4	GCCTCTGCAAGCTGATTTCT	AGTCAAGGTCCTGGCTCTCA	TACCTGGCCATCGTGCACGC
CCR5	TGTCGCAACGAGAAGAAGAA	TGCAGTTATTCAAGCCAAAGAA	CGTCCTTCTCCTGAGCACCTTCCA
CCR6	CTGCTCTTCGGCTTCTTCAT	AGTTCTGCGCCTGGACTAAG	CCGCTGGTGTTCATGATCTTCTGC
CCR7	AGCCAATGAAGAACGTGTTG	AATCGTCCGTGACCTCATCT	TTTCCAGGTGTGCCTCTGCCA
CCR8	TTCTGGGTCCCTTTCAACAC	CTCATGACACACCCATCCAG	TTCCCTGCATGACATGCACGTC
CCR9	CACAGAAGCCACAAGCCTAA	TCTCACAGAAGTAGTCCGTGAAG	TGCCGTCGTACTIONCATGGGC
CCR10	CCGCAGAACAGGTCTCCT	GGGAGACACTGGGTTGGA	TCCAGGCCTTCAGTCGCGC
CXCR1	GGGTTTGAGGATGAGTTTGAA	CAG TGCTTATCTCACAGGGACT	TCTTCTGTGGGTGGCGTGCC
CXCR2	ACC TGCCCTCTATTCTAACAGA	GCA TGCTTATTGATA TCCAGAATTT	CTGCCCCATGTGCGGCCAG
CXCR3	TGAGATGAGTGAACGCCAAG	GCACAAAGAGGAGGCTGTAGA	CCTGCCACAGGACTTCAGCC
CXCR4	TTGCTGACATCAAGGAGGTG	ACCACGATGTGCTGAACTG	TCCCAGCGACCTGTGGCTAGTG
CXCR5	ATGAACTACCCTCTCACTCTGGA	CTCCCTAATTCCTGTACAGGTC	CATGGACCTCATGAACTACAACCTGGA
CXCR6	TTCCGGAAGCACAAGTCTCT	GATGAGCTTCACGAGGTTGA	CAGGAACACCGCCACCACCA
CX3CR1	CCATCCTGACCACCTCAGTT	CATCTCCATCGCTGGTGTA	TCCCGCCTGCTCCTTTGTGA
XCR1	TCCTGTACTGCCTGGTGTTC	GTCTGAGAGGCACAGGTTGA	TCAGCCTGGTGGGCAACAGC

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Probe (5' FAM - 3' TAMRA)
Chemokines			
CCL2	GCTCAGCCAGATGCAATTAAC	CACTTGCTGCTGGTGACTCT	TCCCAAGTCGCCTGCTGCTATACA
CCL3	GCCTGCTGCTTCTCCTATGT	TTTGAGACCAGCAGCCAGT	CCCGGCAGCTTTCTCGCAA
CCL4	ATGAAGCTCTGCGTGACTGT	TCACAAAGTTGCGAGGAATC	CGCTCTCAGCACCAATGGGC
CCL5	CCTGCTGCTTGCCTATATCT	AGCACTTGCTGCTGGTGTAG	CCGCACCCACGTCCAGGAG
CCL8	GCTCAGCCAGATTCAGTTTCT	TGTAGCTGTCCAGCTTCTTGA	CCCAATCACCTGCTGCTTTAGTGTGA
CCL20	TCAGAAGCAAGCAACTTCGAC	GATGTCACAGGCTTCATTGG	CCAGCTGCTGTGTGAAGCCCA
CXCL8	TGAAGCTGCAGTTCTGTCAA	TGGAAAGGTGTGGAATGTGT	TGAGTACAGAACTTCGATGCCAATGCA
CXCL9	CAAGCAAAGCAGTTTGGTGT	CCACAGGCTGATGCAAATAC	CTGGGCCTTGCCAAGCTGCT
CXCL10	ACTCTGAGTCAAGGTGTACCTCTCT	GGATTGACTTGCAAGGAATGA	CGCTGTTCTGCATCGAGATCA
NK cell receptors			
NKp30	AGCTGCACATCTGGGACAC	GAGGTCCTTCTCCACCAC	CGACACCCAGGCCCAGCAC
Ly49	CAAAGACCTGACAAGACTGAGG	TTGCAGTGACTCTTGGGATC	AAGAGTCTTCAGTGCCCTGGCATG
Toll-like receptors			
TLR1	GCACCACAGTGAGTCTGGAA	CAACTGGAGGATCGTGAAGA	TGTGTGCTTGATGATAATGGGTGTCCT
TLR2	ACGACGCCTTCGTGTCCTAC	GCTCCTGGACCATGAGGTTT	CGAGCGGGATTCTACTGGGTGG
TLR3	AAAGAGTTCTCTCCTGGGTGTT	TGCTCAGGGACAGATTCTCA	CAATGCCAAGCTGAGCCCCA
TLR4	TGGAGGACATGCCAGTGCT	CACCGACACACTGATGATCGT	AGTTTCAGGAACGCCACTTGTCAGCTG
TLR5	CTAGACCTGGGTGGAAGTCAG	AGGGATGAAGGTAAAGACTCTGAA	TTCCTGTGGTCTCTCCGATGCTG
TLR6	CCTGCCCATCTGTAAGGAAT	TAGGTGCAAGTGAGCAATGG	TTGGCAACTTGACCCAACTGAATTTT
TLR7	GCTGAAGACTGTCCCTGAGA	TTTGAGCTGAGGTCCAGATG	TCCAAGTGTCCCGCAGCCTC
TLR8	TCCACATTTGAAACGAAGACC	ACATCGGTGAGTCTGGGAAC	CCTGACGTTGAGTTTCTGTCCATCC
TLR9	CACCATCTTCAACGACCTGA	CTTCTCCAGGGACACCAGAC	TCCTTCGCCCCACCTGCACCT
TLR10	TGGTTGGATGGTCAGATTCA	CAGGGCAAATCAAAGTGGA	CCATTGTGGTTGTCATGCTCGTTCT
Housekeeping gene			
BoPo	GCACAATTGAAATCCTGAGTG	GGGTTGTAGATGCTGCCATT	AGCGAAGCCACGCTGCTGAA

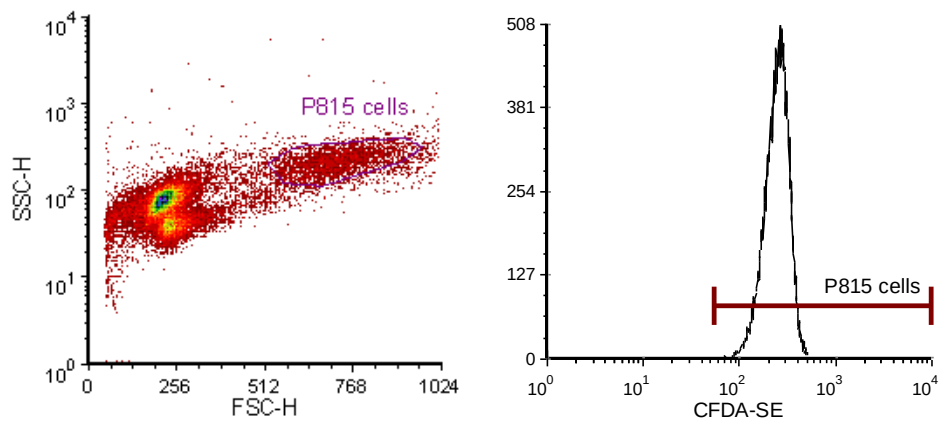
9.6 Gating strategies

9.6.1 Identification of NK cells in co-culture



Live lymphocytes were identified by their FSC/SSC properties and NK cells within co-cultures were differentiated by identifying NKp46 positive lymphocytes

9.6.2 Identification of P815 cells in killing assays with NK cells



P815 target cells were identified by their FSC/SSC properties and CFDA-SE positive cells

9.7 Statistics results (P values)

Comparison of chemokine receptor transcripts between NK cell subsets in the blood and prescapular lymph nodes

	Blood CD2+ vs CD2-	Prescapular lymph node CD2+ vs CD2-
CCR1	0.0013	0.0011
CCR2	0.0006	0.1563
CCR3	0.8901	0.8528
CCR4	0.6560	0.1191
CCR5	0.0054	0.6144
CCR6	0.0334	0.2662
CCR7	0.0007	0.0030
CCR8	< 0.0001	0.0094
CCR9	0.6973	0.0572
CCR10	Not enough data	Not enough data
CXCR1	0.3141	0.5805
CXCR2	0.1202	0.6512
CXCR3	0.0004	0.2695
CXCR4	0.0009	0.4896
CXCR5	< 0.0001	0.9584
CXCR6	< 0.0001	0.2433
CX3CR1	< 0.0001	0.0002
XCR1	0.4887	0.8184

Comparison of chemokine receptor transcripts between the blood and prescapular lymph nodes

	CD2+ Blood vs PSLN	CD2- Blood vs PSLN
CCR1	0.0821	0.4714
CCR2	0.0088	0.3824
CCR3	0.0317	0.0271
CCR4	0.0299	0.1615
CCR5	0.0015	0.009
CCR6	0.0011	0.0109
CCR7	0.0004	0.0529
CCR8	0.6468	0.5686
CCR9	0.5881	0.0002
CCR10	Not enough data	0.1802
CXCR1	0.0648	0.0171
CXCR2	0.4841	0.2365
CXCR3	< 0.0001	0.0143
CXCR4	< 0.0001	0.0012
CXCR5	< 0.0001	< 0.0001
CXCR6	0.0033	< 0.0001
CX3CR1	0.0014	0.0146
XCR1	0.6557	0.9877

Comparison of toll-like receptor transcripts between NK cell subsets in the blood

	Blood CD2+ vs CD2-
TLR1	0.0457
TLR2	0.0787
TLR3	0.0002
TLR4	0.0010
TLR5	0.5843
TLR6	0.0988
TLR7	0.0196
TLR8	0.8904
TLR9	0.5286
TLR10	0.1211

Comparison of chemokines transcribed by infected DCs – each time point compared with negative control.

		CCL2 (MCP-1)	CCL3 (MIP1 α)	CCL4 (MIP1 β)	CCL5 (RANTES)	CCL8 (MCP-2)	CCL20 (MIP3 α)	CXCL8 (IL-8)	CXCL9 (MIG)	CXCL10 (IP10)
DC + M.bo	3hr	0.808	0.000	0.000	0.001	0.211	0.000	0.808	0.313	0.022
	6hr	0.931	0.016	0.000	0.032	1.000	0.000	0.012	1.000	1.000
	12hr	0.856	0.000	0.000	0.000	0.938	0.000	0.000	1.000	0.998
	24hr	0.827	0.000	0.004	0.000	0.922	0.000	0.000	1.000	0.541
DC + BCG	3hr	0.999	1.000	0.811	0.455	1.000	1.000	1.000	1.000	0.987
	6hr	0.990	0.746	0.160	0.999	0.996	0.009	0.599	0.423	1.000
	12hr	1.000	0.932	0.176	0.023	1.000	0.000	0.947	1.000	0.981
	24hr	0.964	0.554	0.993	0.000	1.000	0.000	0.141	0.994	0.879

Comparison of chemokines transcribed by infected DCs – time-point comparison for each chemokine

DC + M.bo	<u>CCL3</u>	3h	6h	12h	24h	<u>CCL4</u>	3h	6h	12h	24h
	3h		0.985	0.006	0.000	3h		1.000	0.747	1.000
	6h			0.236	0.006	6h			0.221	1.000
	12h				0.984	12h				0.568
	24h					24h				
	<u>CCL5</u>	3h	6h	12h	24h	<u>CCL20</u>	3h	6h	12h	24h
	3h		0.782	1.000	0.923	3h		0.048	0.000	0.000
	6h			0.429	0.032	6h			0.000	0.000
	12h				0.997	12h				1.000
	24h					24h				
DC + BCG	<u>CXCL8</u>	3h	6h	12h	24h	<u>CCL20</u>	3h	6h	12h	24h
	3h		0.962	0.022	0.001	3h		0.013	0.000	0.000
	6h			0.388	0.036	6h			0.094	0.030
	12h				0.988	12h				0.999
	24h					24h				
	<u>CCL5</u>	3h	6h	12h	24h					
	3h		0.446	0.224	0.020					
	6h			1.000	0.698					
	12h				0.917					
	24h									

9.8 Pathology scores

Pathology scores at post-mortem - 12 weeks post-M.bovis challenge

Animal ID	No VL LN	Total LN score	No VL Lung lobes	Total lung score	Total path score
C09-5134	2	4	2	7	11
C09-5135	1	3	2	6	9
C09-5136	2	4	1	4	8
C09-5137	1	3	1	4	7
C09-5138	0	0	1	5	5

(VL) – Visible lesions

9.9 Suppliers

Ambion	DNA-Free Kit
Amersham Biosciences	Streptavidin horseradish peroxidase
Applied Biosystems	TaqMan FAST Universal PCR Master Mix ABI Prism 7500 Fast Real-Time PCR System 7500 Fast System SDS Software
Becton Dickinson (BD)	40µm cell strainers FACSFlow FACSAria™ cell sorting system FACS Permeabilisation solution Difco™; BD, Oxford, UK Middlebrook 7H9 culture medium Middlebrook 7H10 agar Middlebrook 7H11 agar FACSCalibur Flow Cytometer
BMG Labtech	FLUOstar Optima Luminometer
Corning Life Sciences	Costar Tissue Culture-Treated Transwells
De Novo Software	FCS Express 3
Eurogentec	Probes (5' FAM and 3' TAMRA)
ICN Biomedicals	3,3'-5,5' tetramethylbenzidine (TMB)
Invitrogen	RPMI SuperScript® III Reverse Transcriptase Alexa Fluor 647 protein labelling kit Vybrant® CFDA-SE Cell Tracer Kit Zenon® labelling kit – IgG2a-FITC Vectors PCR2.1 – TOPO TA vector
Leo Laboratories	Heparin Sulphate
Microsoft	Excel 2003 Software
Miltenyi Biotec	MACS cell separation system Rat Anti-Mouse IgG1 MicroBeads Anti-human-CD14-coupled paramagnetic MACS LS columns
Nalge	24-well tissue culture plates
Nunc	8 well transwell strips 0.2µm

	96 u-well cell culture plates
	96 flat well cell culture plates
	6 well cell culture plates
	0.4mm tissue culture membrane inserts
	F96 Maxisorp Nunc Immunoplate
	96F Maxisorp Black Microwell plate
Perbio	
	SuperSignal Femto Maximum Sensitivity Substrate
Promega	
	GoTaq DNA polymerase
	pGEM-Teasy TA cloning vector
Qiagen	
	Qias shredders
	RNeasy Mini kits
	RNeasy Micro kits
	Qiaquick PCR purification kit
	Qiaprep spin miniprep kit
R&D Systems	
	Bovine IL-2 ELISA kit
Roche	
	Expand High FidelityPlus PCR system
Scientific Laboratory Supplies	
	96-well transparent plates
Sigma-Aldrich	
	Gentamicin
	2-mercaptoethanol (2-ME)
	Non-enzymatic cell dissociation medium
	Histopaque 1083
	Brefeldin A (BFA)
	Paraformaldehyde
	Propidium Iodide
	Concanavalin A (Con A)
	Bovine Serum Albumin (BSA)
	Carbonate/bicarbonate buffer capsules
	Casein
	Tween-20
	1M Sulphuric Acid
	Guanidine Thiocyanate (GTC)
	Ammonium Chloride
	Primers
Terumo Europe	
	18G needles
	Syringes
TCS Cell Works	
	Foetal Calf Serum (FCS)
Thermoscientific	
	NanoDrop 1000 spectrophotometer
Veterinary Laboratories Agency	
	Avian Tuberculin (PPDa)
	Bovine Tuberculin (PPDb)

9.9.1 Recombinant chemokines

Immunotools

Recombinant human CXCL11 (I-TAC)
Recombinant murine CCL5 (RANTES)
Recombinant human CCL2 (MCP-1)
Recombinant human CXCL10 (IP-10)
Recombinant human CCL3 (MIP-1 α)
Recombinant murine CCL3 (MIP-1 α)
Recombinant human CCL19 (ELC)
Recombinant human CCL2 (MCP-1)

Imperial college London – Dr J Pease

Recombinant human CXCL11 (I-TAC)
Recombinant human CXCL9 (MIG)
Recombinant human CXCL10 (IP-10)
Recombinant human CCL17 (TARC)
Recombinant human CCL1 (I-309)
Recombinant human CCL22 (MDC)

Peprotech

Recombinant murine CCL19 (ELC)
Recombinant murine CCL21 (SLC)
Recombinant human CXCL12 (SDF-1a)
Recombinant murine CXCL10 (IP-10)
Recombinant human CX3CL1 (Fractalkine)
Recombinant human CXCL13 (BCA-1)
Recombinant murine CXCL13 (BCA-1)
Recombinant human CXCL16
Recombinant murine CXCL16

R&D Systems

Recombinant human IL-18
Recombinant human CXCL8 (IL-8)

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