Characterisation of tissue homing CD8+ T cells in a murine cytomegalovirus model

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For my father

Sic transit gloria mundi

Abstract

Experimental T cell-inducing vaccines seek to generate rapidly responsive, tissue-homing, antigen-specific T cells. CMV induces an unusual memory population (memory "inflation") of highly differentiated effector memory CD8+ T cells specific for a subset of epitopes and as such is being tested as a vaccine vector. Clearly understanding of the factors involved in memory inflation and tissue-associated CD8+ T cells generated by CMV will aid vaccine design and is of general relevance for tissue protection and pathology.

I sought to examine the IL-18/ IL-18Rα axis in the murine cytomegalovirus model and its potential role in the maintenance of memory inflation or the function of such cells in vivo. IL-18 receptor expression has been linked to tissue-homing IL-17 secreting cells in human and in the final chapter I explored potentially equivalent populations in mice.

MCMV induces increased expression of IL-18rα on tissue-associated antigen specific, (M38+ and m45+), cells in highly significant fashion at both 7 days post infection (dpi) up to 150 dpi ($p < 0.05$ for using paired t-tests). Both IL-18Rα and IL-18 were dispensable in the development of memory inflation. I observed, however, that control of viremia was impaired in mice lacking IL-18Rα but not IL-18.

IL-18Rα+ T cells in mice infected with MCMV can rapidly secrete IFNγ when exposed to IL-12 and IL-18 in the absence of cognate antigen. MCMV infected C57BL/6 mice are protected from infection with *Listeria monocytogenes* and *Mycobacterium tuberculosis* in a time-dependent manner. However this protection is abrogated by removal of IL-18Rα or IL-18. These data are consistent with the idea that non-cognate functioning of T cells is protective against pathogens.

This is the first report showing protection induced by a common pathogen against distinct unrelated pathogens is IL-18/IL-18Rα dependent. My data support a role for induced T cells. Such findings have significant implications in man, both for the pathogenesis of human disease and for vaccine design.

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Publications

Publications resulting from this thesis

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Abbreviations

Ad5	Adenovirus 5
AIDS	Acquired Immune Deficiency Syndrome
Complete RPMI	RPMI media containing 2% Fetal calf serum, penicillin, streptomycin and L-glutamine
CMV	Cytomegalovirus
CON A	Concanavalin A
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic Acid
EBV	Epstein Barr virus
FACS	Fluorescence Activated Cell Sorting
HCV	Hepatitis C virus
HIV	Human Immunodeficiency virus
ICS	Intracellular cytokine staining
IFNγ	Interferon gamma
IL-2	Interleukin 2
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-18	Interleukin 18
LCMV	Lymphocytic choriomeningitis virus
LPL	Lamina propria lymphocytes
LPS	Lipopolysaccharide
MAIT	Mucosal Associated Invariant T cell
MCMV	Murine CMV
MHC	Major histocompatibility Complex
MLN	Mesenteric Lymph nodes
NFKB	Nuclear factor kappa beta
NK	Natural Killer cell
NKT	Natural Killer T cell
PAMP	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PBS/BSA	Phosphate buffered saline with bovine serum albumin
PHA	Phytohaemagglutinin
PMA	Phorbol 12-myristate 13-acetate
RhCMV	Rhesus CMV
SIV	Simian Immunodeficiency Virus

TCR	T cell receptor
TNFα	Tumour necrosis factor alpha
NLR	NOD like receptor
NOD	Nucleotide Oligomerization Domain
RIG 1	retinoic acid-inducible gene 1
RLR	RIG-1-like receptors
RORγt	Retinoic acid receptor-related orphan receptor gamma
RPMI	Roswell Park Memorial Institute 1640 media
TLR	Toll like receptor

Chapter 1

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The *Herpesviridae* are a large family of DNA viruses characterized by their ability to produce persistent infections. Human cytomegalovirus (CMV) is a β-herpesvirus and between 60-80% of the developed world display evidence of infection by the age of 80[1].

Of particular interest to immunologists and vaccinologists is the striking nature of the T cell response to human CMV, driven in part by the complex long-term low-level persistence of the virus. Viral persistence and re-emergence is facilitated by the expression of numerous immune evasion genes encoded within the large viral genome. $CD8^+$ T lymphocytes play an important role in the control of intracellular pathogens such as CMV, typified by the development of large antigen-specific $CD8^+$ T cell populations after primary infection, which contract to form small pools of central memory T cells[2]. Interestingly, comparing human CMV specific responses to other virus-specific T cell responses reveals the striking dominant impact of CMV on the memory T cell compartment; with a median of 9.1% and 10.2% of circulating $CD4^+$ and $CD8^+$ memory T cells being CMV specific, respectively, which may increase even further in the elderly[3,4].

The unusual nature of CMV-specific T cell responses has been predominantly dissected using murine CMV (MCMV). Gradual accumulation of specific $CD8^+$ memory T cell populations was first noted in BALB/c mice and subsequently described in longitudinal studies as memory "inflation"[5,6]. Memory T cell inflation is not restricted to CMVs, and arises also after infection with certain other persistent viruses[7-9].

Overview

Experimental T cell inducing vaccines typically use non-persistent vectors. In the initial phase post vaccination, where antigen is readily available, an effector-memory response develops. However, as the vector is eliminated, the immune response becomes centered on secondary lymphoid tissue and central-memory cells become dominant. It is feasible to theorize that these central memory cells, which require re-exposure to antigen to drive proliferation, differentiation and trafficking, may not be the ideal subpopulation to control rapidly all proliferating pathogens in tissues. Instead, effector-memory T cells respond more rapidly and are located in different tissues including mucosal sites. Thus, placing target antigens in a virus that induces effector-memory T cells could provide the holy grail of vaccinology for certain chronic viral infections and perhaps cancer. The property of CMV to induce such strong effector-memory T cell responses together with the relatively easy transmission (despite pre-existing immunity) and lack of pathogenicity in healthy animals has led to consideration of CMV-based vaccines in both human and animal populations.

Better understanding of the factors driving memory inflation and characterization of atypical lymphocyte subsets present in MCMV will greatly aid both basic science and how this may be harnessed in the design of T cell inducing vaccines.

Human CMV

Prevalence and Incidence

Prevalence data on CMV are widely available in the form of CMV specific IgG and IgM monitoring as part of serological surveys, testing of blood donors and testing of pregnant women.

Data from the U.S.A. shows in 2006 that the prevalence of CMV infection was 58.9% in individuals > 6 years old. CMV seroprevalence increased gradually with age, from 36.3% in 6–11-year-olds to 90.8% in those aged ≥80 years[10].

A further study in 2006 of >14,000 individuals aged 25 – 90 again in the USA showed 66.7% of individuals were seropositive to CMV. Higher mean age, female gender, non-white race/ethnicity, country of origin outside the U.S., lower mean education level, low BMI (<25 kg/m2) and high BMI (≥30 kg/m2) compared to medium BMI (25≤ BMI <30), reported diabetes and high CRP level (≥0.3 mg/dL) were associated with CMV seropositivity[11].

In the developing world seroepidemiological monitoring surveys are often significantly smaller than in the U.S.A and Europe however they suggest higher levels of CMV infection. In Nigeria 97.2% of pregnant women in one survey were anti-CMV IgG positive; in the Sudan 72.2% (2.2% were anti-CMV IgM positive)[12,13]. Blood donors in Southern Brazil exhibited anti-CMV IgG seroprevalence of 96.4% (95% CI: 95.23 - 97.50) and anti-CMV IgM was 2.3% (95% CI: 1.39 - 3.20)[14].

As CMV infection may often be subclinical there is less information available on CMV incidence. In view of the potential devastating effects on the fetus (discussed below) CMV serocoversion rates (from anti-CMV IgG negative to anti-CMV IgG positive) in pregnant women have been examined. Among pregnant women, annual seroconversion rates typically range from 1 to 7% (summary annual rate = 2.3%, 95% CI = 2.1-2.4%). Among day-care

providers, seroconversion rates range from 0 to 12.5% (summary annual rate = 8.5%, 95% CI = 6.1-11.6%). CMV seroconversion in most populations occurs at a relatively low rate[15]. General population rates of CMV seroconversion remain unknown.

Morbidity and mortality

In individuals with a healthy immune system HCMV infections are predominantly asymptomatic but on occasion can cause mononucleosis-like disease similar to that caused by the γ-herpesvirus Epstein-Barr virus. In neonates and immunocompromised individuals, however, CMV infection causes significant clinical problems. Active CMV infection can be characterized into asymptomatic infection (no obvious signs of pathologic symptoms), viral syndrome (fever >38°C for 2 days of unexplained origin and one of leucopenia, myalgia, or arthralgia), and CMV disease (histopathological evidence of CMV, CMV retinitis diagnosed by an ophthalmologist, or CMV in the CSF indicative of CNS disease)[16]. Currently no vaccine is available, and while antiviral therapy exists, these drugs are both toxic and difficult to deliver and some viruses are resistant.

Congenital cytomegalovirus (CMV) infection occurs in 0.6-0.7% of all newborns and is the most prevalent infection-related cause of birth defects and developmental disabilities[17,18]. Developmental disabilities can be severe and include microcephaly, hearing loss, vision loss and cognitive disability. Congenital infection results from in utero transmission from mother-to-fetus, either because the mother has a primary infection during pregnancy or a recurrent infection, defined as either viral reactivation or re-infection with a different strain of CMV[19]. Severe disease can also develop in immunologically immature children after congenital infection or after infection of premature infants[18].

In recipients of solid organ transplants the frequency of CMV disease varies markedly depending on the definition used and the intensity of immunosuppression. Approximately 8% of renal, 29% of liver, 25% of heart, and 39% of lung transplant recipients experience

symptomatic infection[20]. CMV is transmitted to transplant recipients mainly by the donor organ and to a lesser degree by blood products[20]. In view of the lifelong latent infection with CMV the challenge in the transplant recipient is the differentiation of patients with active CMV infection, at risk of tissue invasive CMV disease from those who have subclinical infection and to this end CMV viral load is used to monitor disease[21]. In addition to CMV disease itself there is an increased risk for transplant arteriosclerosis in CMV-seropositive individuals suggesting CMV plays a role in the development of atherosclerosis[22,23].

Seroepidemiological data initially suggested similar rates of cardiovascular disease in anti-CMV IgG positive and negative individuals[24]. However, it is apparent the relationship between anti-CMV IgG and atherosclerosis may be linear in nature with a direct relationship between anti-CMV IgG titre and atherosclerotic plaque size[25]. Data from >14000 individuals collected on CMV serostatus, CRP and mortality revealed even after adjusting for multiple confounders, CMV seropositivity remained statistically significantly associated with all-cause mortality (HR 1.19, 95% CI: 1.01, 1.41)[11].

CMV specific T cells

The development of CMV-related disease in the immunocompromised; e.g. post-transplant and AIDS patients, indicates that T cells are important in controlling CMV[26]. The specific importance of T cells is underlined by the successful usage of adoptive immunotherapy, whereby donor T cells are stimulated *in vitro* using viral lysate or CMV-specific peptides and subsequently are transfused into the patient[27]. CMV replication has been shown to be controlled in the majority of cases in a clinical trial of bone marrow transplant recipients and there is similar, although more limited, evidence for solid organ transplantation[28,29]

In murine models, depletion of multiple cell subsets (including B cells, $CD4^+$ T cells and/or NK cells) is required for full viral reactivation to occur[30]. However, even in otherwise immunocompetent mice there is evidence that inflating memory T cell pools specific for an

epitope from an IE gene product provide a "checkpoint" limiting the stochastic viral reactivation that occurs[5].

Immunosenescence

Definitions, mechanisms and markers are controversial but broadly speaking immunosenescence is used to describe the deleterious phenotypic and functional changes observed in the immune system as it ages. In the mid 1970s it was shown that 80 year olds with anergic T cells had a 2-year mortality rate of 80% as compared with 35% in non-anergic age matched controls[31]. In addition age-associated poor responses to T cell mitogens has been associated with increased mortality[32]. Two major longitudinal studies - OCTO and NONA - focused on groups of healthy elderly volunteers and yielded a set of immune parameters, which were capable of predicting mortality[33-35]. Measurements such as a reduction in the number of B cells, poor T cell proliferative capacity, and increased numbers of $CD8^+$ $CD28^-$ T cells, thus altering the CD4:8 ratio; were part of an "immune risk profile" (IRP) associated with increased all-cause mortality[33,35]. Notably, evidence of CMV infection was part of this IRP cluster[34].

Infection with CMV substantially modifies the distribution of cellular subsets in peripheral blood with higher numbers of $CD8^+$ T cells due to the increase in memory T cells and consequent reduction in size of the naïve T cell pool. These changes are apparent in CMV seropositive individuals at all ages, and this might accelerate immunosenescence[36]

The increasing frequency of CMV-specific cells over our lifetime; especially at extremes of old age suggests immune control of CMV is of paramount importance. Although some studies have associated CMV infection with increased risk of all cause mortality it is as yet unclear on a molecular level why. It has been elegantly shown that CMV-seropositive elderly do not respond as well to influenza vaccine than uninfected age-matched controls; a potential mechanism is the CMV induced accumulation of $CD8^+$ $CD28^-$ cells, which correlate inversely

with responsiveness[37]. However other groups report conflicting findings with vaccine induced immune responses displaying no variation between CMV infected and uninfected cohorts[38]. Moreover, whilst the fraction of CMV-specific $CD8^+$ T cells is very large, other herpesviruses, such as EBV, had a much lesser effect. Indeed CMV seropositivity results in reduced frequency of EBV specific T cells in the elderly suggesting CMV infection may affect responses to other viruses[39]. However, others have found that the magnitude and phenotype of EBV- or influenza-specific $CD8^+$ T cells were not changed by primary CMV infection[40]. The latter finding was confirmed in experimental models in which virus-based vaccines elicited an increase in the size of the memory $CD8^+$ T cell compartment with no impact on other cell populations suggesting that the immune system retains plasticity and can accommodate development of new memory T cells against other pathogens[41]. Overall, it is possible to conclude the accumulation of clonal expansions of $CD8^+$ T cells specific for CMV antigens – excessive "memory inflation" – appears to be a characteristic of immunosenescence, but further work is required to define causal relationship.

CMV as a vaccine vector

A recombinant MCMV expressing foreign viral epitopes either from influenza A virus nucleoprotein (NP366-374) or lymphocytic choriomeningitis virus (LCMV) glycoprotein (GP33-41) induces "inflationary" antigen-specific $CD8^+$ T-cells at a high frequency. These cells protect against subsequent challenge with a recombinant vaccinia virus expressing either NP or GP respectively, as well as LCMV challenge with maintenance of protection up to 200 days post infection[42]. More recently CMV was assessed as a vaccine against simian immunodeficiency virus (SIV), in Rhesus macaques using Rhesus CMV (RhCMV) expressing SIV Gag, Rev-Tat-Nef and Env (RhCMV-SIV). Regardless of RhCMV serostatus macaques could be infected and developed transgene specific $CD4^+$ and $CD8^+$ T cell responses which were predominantly effector-memory in nature and were very long lived – antigen specific responses could be detected up to 4 years post vaccination[43]. A subsequent study compared

the phenotype of SIV-specific T-cell responses in RhCMV seropositive macaques using persistent (RhCMV) vectors and traditional DNA prime /Ad5 boost. Macaques in this study also underwent mucosal SIVMAC239 infection. Vigorous $CD4^+$ and $CD8^+$ T cell responses to all vector-encoded SIV antigens were detectable up to 400 days post vaccination in all groups and although the kinetics of the SIV-specific T cell responses developed differently between groups, the magnitude of the total SIV-specific T-cell response for both $CD4^+$ and $CD8^+$ T cells were similar by day 400. RhCMV as expected induced an effector-memory phenotype (characterized as $CD28^-/CCR7^-$) contrasting with the central-memory phenotype induced by DNA/Ad5. 400 days after initial vaccination animals were challenged with SIVMAC239; macaques immunized with RhCMV-SIV displayed rapid complete control of SIV, and ~50% of the animals were protected for >1 year, which correlated with the magnitude of the peak SIV-specific $CD8^+$ T cell responses in the vaccine phase. In contrast, all of the DNA/Ad5-vaccinated macaques developed progressive infection, with reduced mean plasma viral load compared to controls[44]. The recently reported development of spread-deficient CMV vectors allays some of the safety and bioethical concerns around using a persistent vector with potential for person-to-person transmission which may generate a potentially pathological immune response[45]. Although hurdles still exist, the potential of CMV as a vaccine vector for infectious diseases or cancer is enormous.

MCMV

Immune evasion and Innate Immunity

Mouse cytomegalovirus (MCMV), is a double stranded (ds)DNA virus (230 kb in length) containing approximately 170 genes in the MCMV genome[46]. The virus is cytosine/guanine rich (58.7%) but shows no evidence of CpG suppression along most of its length thus can activate TLR 9 (CpG) and TLR3 (dsDNA)[46,47]. MCMV has broad tissue tropism, and can infect epithelial cells of the host, such as salivary gland tissue, as well as macrophages and lymphoid cells[47].

Cytomegaloviruses (CMVs) are renowned for interfering with the host immune system. By reducing expression of major histocompatibility complex class I (MHC I) molecules MCMV evades antigen presentation and thus cytotoxic CD8+ T cells. Downregulation of MHC-I sensitizes the virus-infected cells to natural killer (NK) cell–mediated killing via the "missing self" axis. MCMV encodes proteins such as m152 and m06 which inhibit surface expression of MHC I molecules[48]. Proteins such as m04, form complexes intracellularly with MHC I and escort them to the cell surface where by engaging NK cell inhibitory Ly49 receptors they prevent NK cell killing[49].

MCMV can compromise NK cell activation via activating receptors such as NKG2D. NKG2D is a potent activating receptor, found on both NK cells and activated CD8+ T cells in mice, which recognizes cellular ligands induced by stress such as infection[50]. MCMV regulates the expression of NKG2D ligands MULT-1, RAE-1 and H60 through the m145, m152 and m155 proteins, respectively[51]. Deletion of either of MCMV genes encoding inhibitors of NKG2D ligands resulted in rescuing the expression of their target ligand and an enhanced sensitivity of mutant viruses to NK cell control in vivo[51].

Different mouse strains display different levels of susceptibility to MCMV with BALB/c mice being susceptible and C57BL/6 mice moderately resistant[52,53]. Genetic analysis identified a

region on the distal end of mouse chromosome 6 - the Cmv1 resistance locus which encodes an activatory NK cell marker named Ly49H or Klr8[54-56]. Ly49H binds to an MCMV-encoded molecule called m157 and thus can recognizes MCMV-infected cells and eliminate them[55,56]. Thus NK cells play a crucial role antigen specific role in the control of MCMV prior to the induction of the adaptive immune response as well as responding to "missing self". The role of NK cells in the chronic CMV infection and prevention of virus reactivation and further disease is poorly defined.

Interplay between innate immune defense and IL-10 can greatly affect the development of the adaptive immune response. In absence of IL-10, NK cells license dendritic cells to prime MCMV-specific CD4 T cells with markedly enhanced priming of MCMV-specific CD4+ T cells occurring in IL-10 deficient mice and thus faster control of viral replication[57]. Furthermore the requirement for CD8+ T cells in early MCMV control inversely correlates with the engagement of Ly49H. Depleting CD8+ T cells in C57BL/6 mice has only a minor effect on the early control of wild-type MCMV. However, in the same strain CD8+ T cells are essential in the control of Δm157 virus; which cannot engage Ly49H supporting the hypothesis that NK cells modulate CD8+ T cell responses down stream[58,59].

Memory Inflation

Infection of C57BL/6 mice with MCMV produces a $CD8^+$ T cell response to at least 24 epitopes derived from 18 viral proteins in the acute phase of infection: however 50 days post infection when viral latency has developed, the immune response is dominated by 5 singular epitopes that elicit inflationary responses (including 2 epitopes derived from the immediate early (IE) gene products)[60]. The MCMV-specific $CD8^+$ T cell responses that dominate during the acute phase are typified by classical expansion, contraction and formation of long-term central-memory pools ($CD27^+$, $CD28^+$, $CD62L^+$, $CD127^+$ and $IL\text{-}2^+$), whereas inflationary responses display an effector-memory phenotype ($CD27^{low}$, $CD28^{low}$, $CD62L^-$, $CD127^-$ and IL-

$2^{+/-}$)[60-62](Figure 1.1). These inflationary T cells are found in diverse hematopoietic and non-hematopoietic organs but their specificity is restricted to a limited number of viral epitopes.

In humans and in primate models of CMV, inflationary CMV specific $CD8^+$ T cells accumulate at high frequencies and have a similar characteristic effector-memory phenotype (e.g. $CD27^-$, $CD28^-$, etc.)[63-65] Inflationary T cells often show restricted TCR usage[66]. Inflation of memory $CD4^+$ T cells has also been observed but, at least in the mouse model of CMV, is less exaggerated[67]. Despite the persistent nature of CMVs, inflationary T cells remain functional and do not show features of exhaustion as observed with certain chronic viral infections[62,64,68,69].

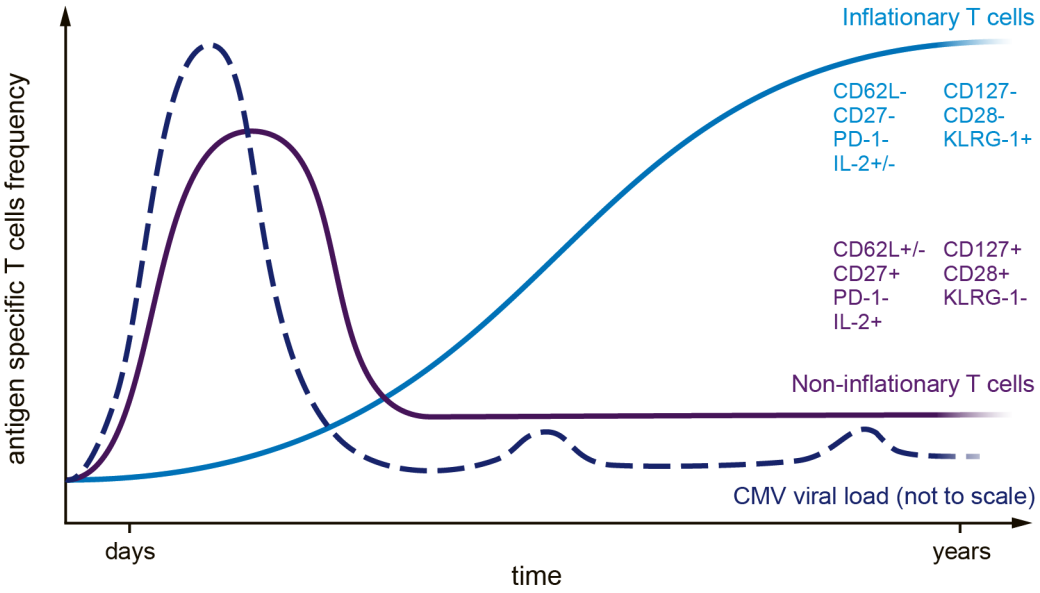

Figure 1.1 Development of CMV-specific memory T cell populations. During CMV infection, non-inflationary T cells undergo expansion, contraction and formation of stable memory pools. By contrast, inflationary T cell pools are characterized by lack of contraction and gradually accumulate in frequency over time aided by low levels of viral reactivation. Characteristic phenotypic features of inflationary versus non-inflationary T cells are shown.

Factors involved in memory T cell inflation

Antigenic stimulation: antigen, tissues and cells

One factor driving this phenomenon must relate to repetitive antigen exposure[70,71]. Support for the hypothesis that antigen persistence and re-exposure is required for memory T cell inflation comes from a series of observations and experiments. Firstly, inflationary memory T cells have phenotypic signs of activation such as low expression of CD62L, CD127, CD27 and CD28[6,61]. Moreover, adoptive transfer of inflationary T cells into naïve recipients results in a failure to divide and survive while the same cells are maintained when transferred into chronically infected mice[62]. Also, when an epitope that in the context of MCMV or HSV-1 infection induces T cell inflation, is expressed in a recombinant vaccinia virus, memory inflation is not elicited[6,70]. This implies that long-term presentation of viral antigen is required and T cell intrinsic factors such as TCR affinity do not drive memory inflation. In fact the functional avidity for peptide-MHC complexes does not predict whether epitopes elicit inflationary or non-inflationary T cell responses nor do they change during the course of a chronic infection[60,72]. Furthermore recently it has been shown that using MCMV rendered unable to spread between cells, by deletion of the essential glycoprotein L, does not impair the development of memory inflation suggesting that once infection is established active viral production is not a prerequisite but merely the presence of the virus in a latent state is sufficient to drive inflation[45].

The genes containing epitopes that induce inflationary responses can be expressed early or late in CMV infection[42,60]. Moreover, both inflationary and non-inflationary epitopes may be found within the same protein, suggesting differential antigen processing events may be involved as well[60]. It is interesting to note that the immunoproteasome, which is constitutively expressed in professional antigen presenting cells and particularly important for processing MHC class I proteins, is less important for memory inflating responses as

compared to non-inflating ones[73]. Thus, epitopes inducing memory inflation might be selected due to this difference in immunoproteasome dependence.

During acute infection, MCMV replicates in diverse tissues such as spleen, liver and lungs whilst during the ensuing persistent phase the salivary glands are the primary site where viral replication is detected[74,75]. However, removal of salivary glands does not influence memory inflation[72], supporting the idea that tissues where the virus becomes latent are important. Although active virus production is not measurable during latency, viral peptide epitopes are most likely present. The ability of CMV to sporadically re-activate in certain tissues such as the lung and thereby produce relevant viral transcripts possibly contributes[5,76-78]. The antiviral $CD8^+$ T cell pool during memory inflation is also not affected by thymectomy, suggesting that the contribution from the naïve pool to "top-up" such large populations is not critical[72].

It is clear that antigen persistence is important for memory inflation, but it remains unclear which cells present the viral antigens and whether both direct and cross-priming are involved. Despite the induction of CMV-encoded immunomodulatory proteins that interfere with MHC class I presentation strong $CD8^+$ T cell responses are elicited suggesting cross-presentation plays a dominant role[79-81]. Expression of MHC class I molecules by infected antigen-presenting cells may not be completely inhibited, thus direct presentation cannot be entirely excluded from contributing to priming. In *Batf3*$^{-/-}$ mice, which have impaired cross-priming due to the lack of $CD8\alpha^+$ and $CD103^+$ DCs, memory inflation still occurs but cross-presentation by other cells in these mice is still possible[82,83]. Using bone marrow chimera models it was shown that inflationary response (to e.g. IE-1 protein) were dependent on antigen-presentation by non-hematopoietic cells[84,85]. However, in these model it was not shown whether direct presentation or cross-presentation (or potentially the recently described "cross-dressing") of MHC-loaded viral antigens (derived from infected non-hematopoietic cells) is important to drive memory inflation[86]. Thus, further evidence is

required to establish whether both direct and cross-presentation during persistent MCMV infection are operational and contribute to memory inflation.

Costimulation

In addition to TCR triggering, costimulatory molecules and inflammatory cytokines provide important signals for programming effector and memory CD8$^+$ T cell responses[87]. The main costimulatory receptor for T cells, CD28 (binding to CD80 or CD86) plays a dominant role during the acute phase, since CD8$^+$ T cell responses in mice lacking this costimulatory interaction are strongly diminished at early timepoints. Remarkably, during the persistent phase the inflationary T cells are able to 'catch up' to wild type levels indicating no CD28 requirement[88]. In contrast, the costimulatory molecules 4-1BB and OX40, which are upregulated on T cells upon TCR triggering, do clearly contribute to the development of inflationary MCMV specific T cell responses since interference with such triggering has a major impact on inflation[89,90].

Cytokines

Using a bone marrow chimeric mouse model with wild-type and *Il2ra*$^{-/-}$ cells, it was shown that an inflationary CMV-specific T cell response is critically dependent on IL-2 while a non-inflationary response was not[91]. In an acute infectious model, it was recently shown that autocrine but not CD4$^+$ T cell or DC-derived IL-2 is important for optimal secondary expansion of memory CD8$^+$ T cell populations[92]. As inflationary CD8$^+$ T cells produce lower levels of IL-2 than non-inflationary T cells, the question is whether autocrine IL-2 is similarly crucial for inflationary CD8$^+$ T cells or if other sources supply this[60,88]. The cytokines IL-7 and IL-15, which are important for homeostatic proliferation of memory CD8$^+$ T cells, have potent proliferative properties for HCMV-specific T cells *in vitro,* but their precise role for memory T cell inflation in an in vivo setting remains to be elucidated[40]. Another important cytokine is IL-10, which clearly restricts inflation of T cells in the chronic phase of CMV infection by unknown mechanisms[93].

CD4+ T cell Help

$CD4^+$ T cells are critical for controlling MCMV replication, especially in the salivary gland during chronic infection, where it was recently shown that these cells operate via direct antiviral actions involving IFNγ[94-96]. $CD4^+$ T cells are generally important for development of memory $CD8^+$ T cell responses, and for inflationary responses even more so[97,98]. The mechanisms for this apparently increased helper-dependence are, however, still unidentified.

Taken together, it appears to maintain the effector-memory-like inflationary T cell populations the presence of latent virus in non-hematopoietic cells is essential and that additional cytokine and costimulatory signals (e.g. IL-2, OX40, 4-1BB) are required to preserve the high frequency of this population. (Figure 1.2)

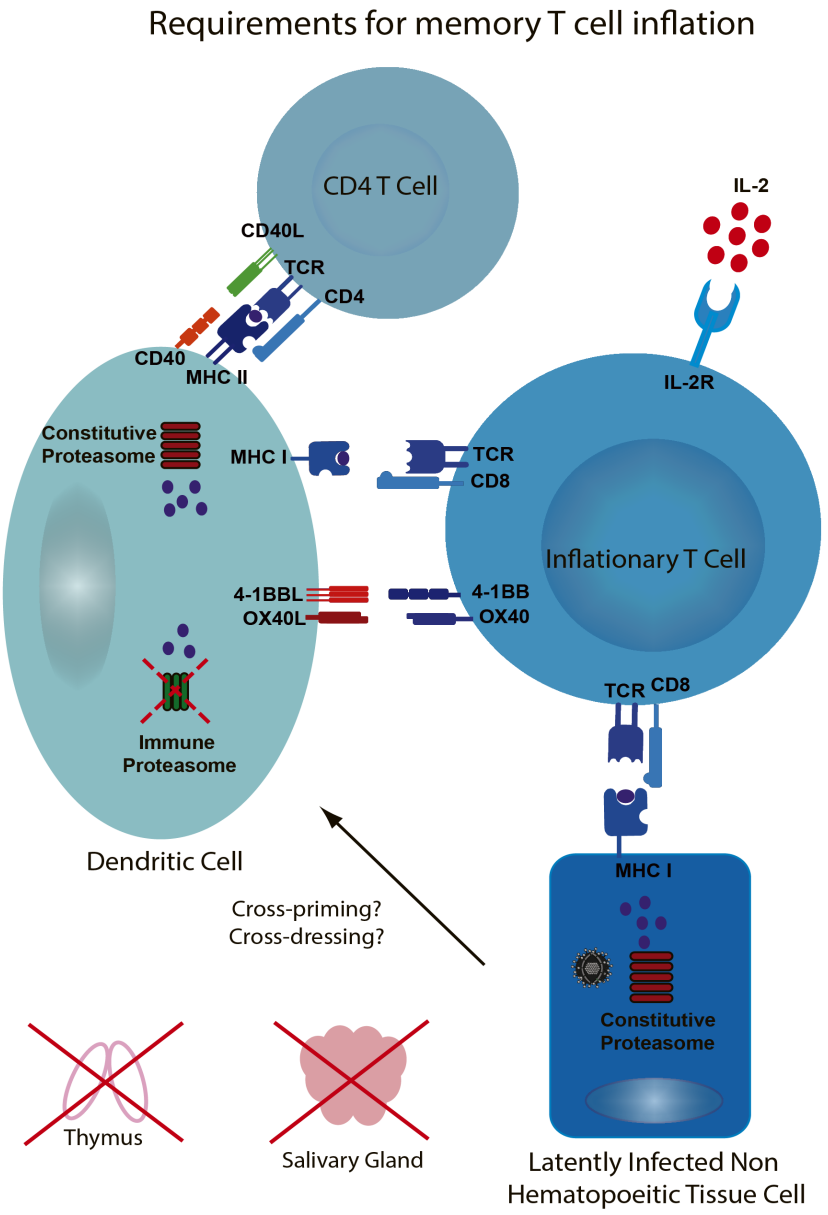

Figure 1.2 - Factors that contribute to memory inflation in CMV infection. In persistent CMV infection, the virus can sporadically reactivate from latency upon which viral proteins are processed by the constitutive proteasome and viral peptides are presented in MHC class I molecules. Viral antigens can either be presented by latently infected non-hematopoietic tissue cells or by cross-primed/cross-dressed dendritic cells. The presentation of the viral peptides persistently re-stimulates the inflationary T cell populations. Additional signals via the co-stimulatory molecules 4-1BB and OX40 contribute to the size of the inflationary T cell pool and IL-2 production is needed for maintenance of the inflationary T cells. CD4+ T cell help is important for memory inflation but new thymic immigrants and viral replication in the salivary glands are not.

MAIT cells

Mucosal-associated invariant T (MAIT) cells are a population of T cells displaying a semi-invariant TCR and restricted by MR1, an evolutionarily conserved major histocompatibility complex related molecule[99]. Exhibiting a predominantly effector phenotype MAIT cells are abundant in human blood and liver representing 1–8% and 20–45% of all T lymphocytes, respectively[100,101]. They follow an atypical pathway of development and preferentially locate to peripheral tissues. Ex vivo human and mouse MAIT cells have been shown to react to bacterially infected cells in an MR1-dependent manner[102].

Development

MAIT cell development occurs in a series of steps with cells developing initially in the thymus and with B cells and commensal flora being required for the subsequent peripheral accumulation[103,104]. Thymuses overexpressing MR1 show an increase in TCRs utilizing Vβ6 and Vβ8 segments, (preferentially used by MAIT cells)[103], compared to MR1-deficient thymuses. MAIT cell development is dependent on expression of the selecting molecule, MR1, but both B cells and thymic tissue are dispensable and the MR-1 expressing cell type is as yet unknown. However, expansion of peripheral populations of MAIT cells is dependent on B cells expressing MR1[103,104]. Notably MAIT cells fail to develop in mice reared in germ free environments indicating commensal flora is required for their expansion in the periphery[104].

In humans the TCR consists of a Vα7.2-Jα33 chain, which is found preferentially associated with a limited number of TCRβ chains (Vβ2 and Vβ13)[105]. The invariant TCR in mice is characterized by expression of Vα19-Jα33 preferentially linked to Vβ6 and Vβ8 chains[105].

MAIT cells display a unique ontogeny compared to both conventional αβ T cells and NK T cells although much is still to be discovered – such as the transcription factor driving this phenomenon and the relative paucity of such cells in mice.

Characteristics

In humans MAIT cells exhibit high expression of the C-type lectin CD161 and IL-18Rα and have been described to be CD8αβ, CD8αα, or double-negative (DN)[106]. In addition MAIT-cells exhibit similar characteristics to Th17 cells, including cytokine expression (IL-17A and IL-22), transcription factors (RORγt and RUNX2), chemokine receptors (CCR6 and CCR2), cytokine receptors (IL23R and IL-18Rα) as well as CD161[102]. In the blood of healthy adults MAIT cells exhibit an effector (CD95hi CD62Llo) memory (CD27+ CD45R0+ CD45RAlo CD122+) phenotype[102]. In umbilical cord blood they expressed markers of naïve cells (CD45RA+CD45RO−)[106]. In view of the absence of MAIT cells in germ free mice, their ability to reconstitute when mice are colonized and the postnatal acquisition of memory markers by human MAIT cells suggests MAIT cells are probably triggered by commensal flora colonization[104,106,107].

MAIT cells can also be characterized by the surface expression of the transporter ABCB1 (MDR)[102]. ABCB1 protein prevents stem cell apoptosis and differentiation, and rapidly effluxes toxins and chemotherapeutic agents suggesting MAIT cells are long lived and self renewing 108,109.

Role in disease

The distribution of MAIT cells in the intestine and liver coupled with their expansion in the face of commensal bacteria suggests a close relationship with microbes. In vitro MAIT cells react, by secreting IFNγ or upregulating CD69, with MR-1 expressing cells in the presence of a diverse group of organisms - including Gram-negatives (e.g. *Escherichia coli*), Gram-positives (e.g. *Bacillus* species and *Staphylococcus aureus*) as well as Mycobacteriaceae (e.g. *Mycobacterium tuberculosis*) and yeasts (e.g. *Candida* or *Saccharomyces*)[107,110]. Conversely virally infected APC's, toll like receptor ligands and certain bacteria, such as *Listeria monocytogenes* and *Streptococcus spp.* are appear unable to trigger MAIT cells to produce IFNγ[107,110]. Taken together this pattern of reactivity suggests MAIT cells react with an as yet undetermined ligand derived from bacteria.

Accurate modeling of the role of MAIT cells in disease is difficult in view of the putative lack of a murine model. Data in mouse models requires usage of mice transgenic for the invariant α chain of the TCR and such mutations may grossly affect the development of conventional T cell subsets[111]. Surrogate indicators suggest MAIT cells play an important role in vivo during pulmonary bacterial infections where MAIT cell numbers in the blood decrease significantly are increased at the site of infection[107,110]. It is important to view these findings carefully as numbers of samples analyzed are relatively small.

Of particular interest is the finding in hepatitis C infection that over 50% of HCV specific liver resident CD8+ T cells express the marker CD161 with approximately 15% of HCV-specific cells in the peripheral blood also expressing CD161[112]. Further examination of these CD161 bright cells reveals expression of the same pattern of molecules consistent with type 17 differentiation, seen in MAIT cells (IL-17, IL-22, RORγt, IL-23R, IL-18 receptor, CCR6 and CXCR6)[113]. CD161 expression when coupled with IL-18Rα on virus specific CD8+ T cells defines an ABCB1+ self renewing chemotherapy resistant subset of T cells[108].

MAIT cells however are defined by an invariant TCR and inability to respond to virally infected cells[107]. Current analysis of Tc17 cells indicates that although the majority, like MAIT cells, are Vα7.2+, these cells do not completely encompass the CD161high population[106]. In an influenza infection model IL-17-secreting CD4+ T and CD8+ T localize to the lung following infection and depletion of IL-17 increases morbidity and mortality[114].

Clearly further work is required to accurately delineate these novel T cell subsets and define their role in vivo – this is hard to achieve in human studies. The interplay between murine homologs of CD161, IL-18Rα expression and type 17 function and the impact of CMV infection will therefore be examined further in Chapter 5.

IL-18

IL-18 was initially described as an IFNγ-inducing factor circulating in in mice infected with *Propionibacterium acnes* and subsequently challenged with lipopolysaccharide[115].

A member of the IL-1 superfamily IL-18 is initially synthesized as an inactive precursor (24 kDa) and requires caspase-1 cleavage for processing into a mature molecule of 18 kDa[116]. Although notably, unlike IL-1β which is synthesized in response to inflammatory stimuli, relatively large reserves of pro IL-18 are consistently maintained which means caspase-1 mediated cleavage is the main source of biologically active IL-18[116].

Structure and function

The pattern recognition receptors of the innate immune system consist of toll like receptors (TLRs), nucleotide binding and oligomerisation domain-like receptors (NLRs) and retinoid acid–inducible gene–1 (RIG-1)-like receptors (RLRs)[117]. These receptors recognize pathogen-associated molecular patterns (PAMPs) from bacterial cell walls and nucleic acids. NOD-like receptors located in the cytosol NLRs form part of vital molecular platforms that organize signaling complexes such as inflammasomes which result in the cleavage of caspase-1 and production of active IL-18 underlining its importance in the early immune response[118]. Pro-IL-18 has been located in cells of both haemopoietic and nonhaemopoietic lineages including macrophages, dendritic cells, Kupffer cells, keratinocytes, chondrocytes, synovial fibroblasts, and osteoblasts[119].

At the receptor level, the activity of IL-18 is via a heterodimeric complex, the IL-18 receptor (IL-18R) complex, consisting of IL-18Rα and non-binding signaling chain IL-18Rβ. Following binding of IL-18 to the IL-18Rα, IL-18Rβ then binds to form a high affinity complex with the ligand which recruits IL-1R activating kinase (IRAK), and subsequently activates NFκB. IL-18Rα is widely expressed and can be found on both naïve and memory T cells, NK cells and

numerous others including macrophages, B cells, neutrophils, basophils, mast cells, endothelial cells, smooth muscle cells, synovial fibroblasts, chondrocytes, and epithelial cells[119].

Binding of IL-18 to the IL-18 receptor complex on lymphocytes has a wide range of effects in addition to inducing production of IFNγ; enhancing T and NK cell maturation, cytokine production (including granulocyte-macrophage colony-stimulating factor (GM-CSF), TNF-α, IL-1β) chemokines, and cytotoxicity[120]. IL-18 also increases FasL on NK cells and consequent Fas-FasL-mediated cytotoxicity.[121,122]. On non T cell populations IL-18 exhibits wide ranging effects[120].

Role in disease

Broadly, IL-18 is produced by antigen presenting cells such as macrophages and dendritic cells in the presence of different microbial components and whilst playing an important role in innate immunity it is apparent IL-18 has wide ranging effects on cells in the adaptive immune system.

In multiple sclerosis high levels of IL-18 are found in the serum and CSF of patients compared to healthy individuals[123,124]. IL-18Rα is highly expressed by macrophages and synovial lymphocytes in both rheumatoid and osteoarthritis couple with high levels of IL-18 in synovial fluid[125]. In inflammatory bowel disease IL-18, IL-18Rα, and caspase-1 levels are increased in chronically inflamed mucosa as compared with early lesions or normal tissue[126,127]. Furthermore IL-18 deficient mice are resistant to experimental colitis and mice over expressing IL-18 develop significantly worse pathology in these models[127]. The AIM2 inflammasome recognises cytosolic dsDNA and subsequently activates caspase-1. In vivo, Aim2- and Asc-deficient mice infected with MCMV have reduced IL-18 concentrations in the serum, and severely attenuated IFN-γ production by NK cells. IL-18 is readily detectable in the sera of wild-type C57BL/6 mice as early as 36 hours after infection with MCMV[128].

IL-18 and its receptor complex are clearly implicated in numerous diseases. Their role in the immunobiology of CMV will be examined further in Chapter 4.

Aims of this thesis

In view of the increased interest in the use of herpesviruses a potential vectors for T cell inducing vaccines and the clear importance of understanding how to generate a long lived effector memory T cell response I seek to understand further the mechanisms involved in generating memory inflating T cells and the quality of these cells.

Using MCMV as a model infection I aim to

- Examine whether IL-18 and the IL-18Rα are involved in the generation and maintenance of memory inflating cells (Chapter 3)
- Examine the functional role of IL18R expression during CMV infection (Chapter 4)
- Examine murine equivalents of human CD161+/IL-18Rα+ T cell subsets and the impact of MCMV on their frequency and function (Chapter 5).

Chapter 2

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Mice

C57BL/6 mice were bred in an SPF animal facility at the University of Oxford, UK or obtained from Harlan UK. B6.129P2-Il18tm1Aki/J (IL-18 knockouts) and B6.129P2-Il18r1tm1Aki/J (IL-18Rα knockouts) were obtained from The Jackson Laboratory. Experiments were performed using age matched control mice with the permission of the Home Office according to the Animals Procedures Act of 1986.

Viruses

MCMV strain (Strain Smith, ATCC: VR194) was provided by Professor U.H. Koszinoswki, Department of Virology, Max von Pettenkofer Institute, Munich Germany. Viruses were stored at -80°c.

Production of MHC Class I Tetramers

Recombinant heavy chains (H2-Db and Kb) and ß2-microgolbulin were produced in Escherichia Coli (stain BL21), purified from inclusion bodies and refolded in the presence of human β2-microgolbulin and the relevant peptide shown below. Refolded complexes were enzymatically biotinylated, purified by FPLC and tetramerized.

Table 2.1 – Amino acid sequences for MCMV peptides used in this thesis

Peptide Name	Sequence	Amino Acid	MHC restriction
M38b	SSPPMFRV	316–323	Kb
M45	HGIRNASFI	985-993	Db

The amount of Phycoerythin (PE) and Allophycocyanin (APC) Streptavidin to add was performed by calculating the volume of streptavidin conjugate that must be added to give a 1:1 ratio of biotinylated MHC to biotin binding sites. Tetramerized complexes were stored at 4°c.

NKR-P1d antibody and hybridoma

This was kindly supplied by Professor Wayne Yokoyama, (Washington University in St. Louis School of Medicine).

Hybridoma cells, (stored in liquid nitrogen) were retrieved and thawed in a 37°c water bath. Once thawed cells were added to 50mL R10 media and centrifuged at 500g for 5 minutes. Viability was checked using trypan blue. Cells were then seeded into T-185 flask, incubated at 37°C and examined daily. Once confluent cells were harvested, centrifuged at 1000rpm for 5 minutes and resuspended in fresh R10. Cells were counted and concentrations altered to ensure a final density of at least 2.5 x 10^5 cells/ml. in 300–400mL media. At this stage cells were decanted into roller bottles and placed on rollers at 37°c for two days. After 48 hours density of cells was checked using trypan blue if exceeding 5 x 10^5 cells/mL R10 was added to increase the culture volume to 1–1.2 L. After 7-10 day / when media orange the roller bottles are decanted in to smaller flasks and centrifuged for 10 minutes at 2500g. Supernatants were then filtered through a steritop 0.22μM filter system and stored at -80°c until required for purification.

Antibody purification

Supernatants were defrosted and 1/100 - 1/50 volume 1.0M Tris pH 8.0 added. Protein A or G columns (Invitrogen) containing 1–3 mL of beads were set up at 4°c and washed sequentially with 10 volumes of 8M urea, 1 volume milliQ water, and 10 volumes of .02M NaOH. 5-10 column volumes of the wash solution was run over the column to produce a

baseline on the monitor printout. Antibody solution was then passed over the column followed by another 5-10 column volumes of the wash solution. Antibody was then eluted from the beads using an elution solution and 1/10 volume 1M Tris pH 8.0 immediately added to neutralize the collected antibody. Finally the collected solution is dialysed in 4 L of 1X PBS for 3 changes at 4°C. A 280 nm absorbency reading on the OD spectrophotometer. (1 OD = .75 mg/ml) is taken and then antibody concentrated using a Millipore Centriplus (YM-30, 50 or 100) in a fixed angle centrifuge at 4 C at a 3,000g max is required. Samples were taken for endotoxin testing. Once desired concentration was reached (usually 1-2 mg/ml), the antibody was frozen for future use in a -80 C freezer.

Cell Preparation

Spleens were harvested in PBS and disintegrated using 70μm plastic net strainer. The resulting cell suspension was washed and pelleted by centrifugation. The pellet was resuspended in ACK lysis buffer for red cell lysis. The pellets were resuspended in RPMI-5%.

To isolate lymphocytes from the lung tissue; lungs were harvested in PBS, dissected into small 1mm cubes and digested with collagenase and DNAse for 1 hour at 37°c. Organs were filtered through 70μm nylon filter and washed in PBS then resuspended in FACS buffer (PBS, 1% BSA and 0.1% Sodium Azide).

For isolating cells from the liver, tissue was perfused via the hepatic artery with ice cold PBS and organs harvested into PBS. Livers were disintegrated using a 70μm filter and pelleted by centrifugation. The pellet was resuspended in 5ml 30% Percoll (Amersham Biosciences) and layered on a 70% Percoll solution in a 15ml Falcon tube. Samples were centrifuged (2000rpm) for 25min without brakes and the leukocyte population at the interface recovered, washed in PBS and resuspended in FACS buffer.

Blood was harvested into Eppendorfs containing 200µl of 0.5mM EDTA either by bleeding from the tail vein or aspiration from the left ventricle post mortem.

Sorting Cells By MACS

CD8+ cells were isolated from spleens. Single cell suspensions were obtained. Cells were resuspended and filtered in FACS buffer (PBS, 0.5% FCS, 2mM EDTA) at a concentration of 90µl/10^7 cells. Cells were purified by positive selection using CD8+ T cell selection kit (Miltenyi) adding the appropriate magnetic beads and incubating at 4°c for 10 minutes. Cells were washed and resuspended in 2mL FACS buffer and sorted using the AutoMACS (Miltenyi) or MS columns (Miltenyi).

Tetramer and Antibody Staining

Tetramer staining was performed on 50µL of whole blood or 1x10^6 cells and various fluorescently labelled antibodies were used (listed below). Tetramers were added then cells incubated at 37°C for 20 minutes. Plates were immediately centrifuged at 1500 rpm for 3 minutes then other antibodies added. Cells were then incubated at 4°c, washed with FACS buffer. Samples were acquired on a LSRII and analysed with Flowjo (Tree star).

Intracellular Cytokine Staining

Lymphocytes were isolated as described above then 1 x 10^6 cells were seeded per well in a 96 well plate. Cells were restimulated for 2 hours at 37°C with either 10-6µM M38 or m45 peptide or 200ng/mL PMA together with 500ng/mL ionomycin. After 2 hours GolgiPlug (BD biosciences) was added to each well and cells incubated for a further 4 hours at 37°c. Plates were centrifuged at 1500 rpm for 3 minutes. Staining antibodies were specific for mouse and purchased from a variety of suppliers as at the end of this chapter. After blocking Fc receptors with anti-CD16/CD32, cells were surface stained for 30 minutes at 4°c. Cells were

permeabilized in Cytofix/Cytoperm solution as per manufacturer's instructions (BD Biosciences) stained for intracellular cytokines. Samples were analyzed using a BD LSR II flow cytometer (BD Biosciences) and FlowJo version 9.2 (Tree Star, Inc.; USA). Background responses in unstimulated control cells were subtracted from the stimulated response.

Tc17 Polarisation

Spleens were harvested from C57BL/6 mice. CD8+ T cells (CD8+) were obtained via magnetic bead separation according to the manufacturer's protocol (Miltenyi Biotec). CD3/CD28 Dynalbeads (Invitrogen) were used for polyclonal activation. Culture medium used was IMDM (Invitrogen) supplemented with 1.0mM sodium pyruvate (Sigma-Aldrich), 0.1mM nonessential amino acids (Invitrogen), 100IU/ml penicillin, 100µg/ml streptomycin, 50µM 2-ME (Sigma-Aldrich), and 5% heat-inactivated FCS Skewing conditions were as follows: Tc17 (20ng/ml IL-6, 5ng/ml TGF-β, 20ng/ml IL-1β, 20ng/ml IL-23, 10µg/ml anti-IFNγ, and 10µg/ml anti-IL-4). Cells were cultured for 2 days and then rested for 3 days before restimulation with PMA (200ng/ml) and ionomycin (500 ng/ml) before analysis or sorting. Where indicate IL-21 (50ng/mL) or IL-22 (50 ng/ml) were added. All cytokines were from Peprotec except for IL-23 (R&D Systems). Anti-IFNγ and anti-IL-4 were from eBioscience.

DNA extraction

Tissues were homogenized using a MagNA Lyser instrument (Roche Diagnostics). Whole DNA was isolated using the High Pure PCR Template Preparation Kit (Roche Diagnostics).

Quantification of MCMV genome copy numbers in tissue

Real-time quantitative PCR was performed using a LightCycler (Roche Diagnostics) and the LightCycler FastStart DNA MasterPLUS HybProbe reaction mix (Roche Diagnostics)

according to the manufacturer's protocol. Data analysis was performed with LightCycler Software 3 (Roche Diagnostics). Oligonucleotides were purchased from Microsynth (Balgach, Switzerland). The following oligonucleotides from MCMV glycoprotein B sequences were used as primers for real-time quantitative PCR: 5'-AGGGCTTGGAGAGGACCTACA-3' and 5'-GCCCGTCGGCAGTCTAGTC-3'. The following oligonucleotides were used as probes: 5' GCTCTATTGATACTCCGCGCGTTA 3' and 5' AACAGCTAGACGACAGCCAACGCACCG 3'.

Mycobacterium tuberculosis challenges

Mycobacterium tuberculosis challenges were kindly carried out by Dr Elma Tchilian.

Mtb stock

Mycobacterium tuberculosis, Erdman strain was provided by Dr. Amy Yang, (FDA Centre for Biologics Evaluation and Research, Bethesda, USA) and was stored frozen at -80oC.

Aerosol challenge

Aerosol challenges were performed using a modified Henderson apparatus[129]. Briefly, mice were restrained and placed in a sealed chamber. The chamber was then filled with an aerosol of a diluted Mtb mixture for 20 minutes, before the chamber was flushed with air for a further 10 minutes. Mtb deposition in the lungs was measured at 24 hours post-challenge.

Mtb colony forming unit (CFU) quantification

Tissue homogenisation

At the desired time point post-challenge, mice were culled and the lungs and spleen removed. The tissues were then disrupted in order to release any Mtb. Each individual tissue was placed in a conical vial containing borosilicate beads (Sigma Aldrich, UK) and 1ml PBS and beaten for 1 minute using a Mini Beadbeater (Stratech Scientific Ltd, UK).

Plating of homogenate

Ten-fold serial dilutions of the individual tissue homogenates were made and a known volume plated on 7H11 Middlebrook agar plates (E&O laboratories, UK). The plates were incubated for approximately 3 weeks at 37oC and 5% CO2, after which the number of colonies was counted.

Listeria monocytogenes challenges

L. monocytogenes stock

Listeria monocytogenes DPL 1942 OVA (LM-OVA), an attenuated strain expressing ovalbumin was supplied by Professor Phillip Ashton Rickardt (Imperial College, London). Listeria monocytogenes DPL 1942 is act A deficient. The attenuated actA mutant of Listeria monocytogenes can enter and multiply in the cytosol of the infected host cell, but is deficient in actin-dependent cell-to-cell spread. The LD50 is estimated to be ~5 x 10^5 in WT C57BL/6 mice.

L. monocytogenes was grown on a brain heart infusion media (BHI) (Sigma) supplemented with 5μg/mL erythromycin (Sigma). A single colony was selected and inoculated into 10mL tryptic soy broth (TSB) (Sigma) containing 5μg/mL erythromycin and incubated overnight in a shaker (37 °C, 240 rpm), until the optical density 600 was 0.1 (equivalent of 1 x 10^9 cfu per mL). 1mL aliquots were taken without glycerol to produce injection stocks.

L. monocytogenes infections

For infection a vial was thawed and inoculated into 9mL TSB erythromycin in a shaker (37 °C, 240 rpm) until an OD600 of 0.06 – 0.1 was reached (between 10^8 and 10^9 cfu/mL). Appropriate amounts as required were spun at 14000 rpm for 2 min in 1.5mL Eppendorfs, then resuspended in PBS to give a final concentration 2.5 x 106 cfu/mL .

For all primary bacterial infections WT C/57B6 mice, IL-18Rα−/− mice, and IL-18−/− mice were given 5 × 10^5 CFU of actA-deficient LM-OVA intravenously. Injection stocks were retained and plated immediately to ensure accurate challenge.

Listeria monocytogenes colony forming unit (CFU) quantification

Tissue homogenisation

At the desired time point post-challenge, mice were culled and the spleen, liver and lungs removed. Total organs were weighed and then a sample taken, weighed and added to a vial containing borosilicate beads and 1 mL 0.1% Igepal in PBS. The tissues were then disrupted in order to release any *L.monocytogenes*. Tissues were homogenized using a MagNA Lyser instrument (Roche Diagnostics).

Plating of homogenate

Ten-fold serial dilutions of the individual tissue homogenates were made and a known volume plated on Brain Heart Infusion agar plates containing 5μg/mL erythromycin The plates were incubated for approximately 48 hours on a bench top after which the number of colonies was counted.

Flow cytometry antibodies used in this thesis

Marker	Fluorochrome	Working Dilution	Supplier	Catalogue Number
CCR7	Alexa Fluor 700	1/100	eBioscience	56-1971
CD19	Alexa Fluor 700	2/100	eBioscience	56-0193-82
CD62L	Alexa Fluor 700	1/50	BD	553150
CD8	Alexa Fluor 700	1/100	eBioscience	56-0081
CD3	Alexa Fluor 700	1/100	eBioscience	56-0032-82
IL-12 (p35 subunit)	Alexa Fluor 647	1/50	eBioscience	51-7352-80
IL-17A	Alexa Fluor 647	1/100	eBioscience	51-7172-80
CCR6	APC	1/10	R&D	FAB590A
CD11c	APC	1/100	BD	550261
CD14	APC	1/50	eBioscience	17-0141-81
CD4	APC	1/100	eBioscience	17004082
CD44	APC	1/200	eBioscience	17-0441-82
CD62L	APC	1/100	BioLegend	104412
CD8	APC	1/200	eBioscience	17-0081
CXCR6	APC	1/150	RD	FAB2145A
IFNg	APC	1/100	eBioscience	17-7311-82
IFNg	APC	1/50	BD	554413
IL-2	APC	1/50	BD	554429
NK.1.1 (CD49b)	APC	1/100	eBioscience	17-5941

TNFa	APC	1/400	eBioscience	17-7321-81
CD3	eFluor 450	1/200	eBioscience	48-0032-82
CD44	eFluor 450	1/400	eBioscience	48-0441
CD8	eFluor 450	1/200	eBioscience	48-0081-82
IFNg	Efluor 450	1/100	eBioscience	48-7311-80
KLRG1	eFluor 450	1/100	eBioscience	48-5893-80
CD4	eFluor 605NC	1/100	eBioscience	93-0041-42
CD8	eFluor 605NC	1/400	eBioscience	93-0081-73
CD11c	FITC	1/400	BD	557400
CD19	FITC	1/200	BD	553785
CD3	FITC	1/200	eBioscience	11-0031
CD44	FITC	1/200	eBioscience	11-0621
CD62L	FITC	1/200	eBioscience	11-0621-82
CD8	FITC	1/100	eBioscience	11-0081
CD8	FITC	1/50	BD	553031
IFNg	FITC	1/50	eBioscience	11-7311-82
IFNg	FITC	1/100	Caltag	RM9001
IL-17A	FITC	1/100	eBioscience	11-7177-81
IL-18Ra	FITC	1/10	R&D	FAB1216F
NKG2A/C/E	FITC	1/100	BD	550520
NKG2D (CD314)	FITC	1/50	BioLegend	115711
TNF a	FITC	1/100	eBioscience	11-732-182

Vb2	FITC	1/50	BD	553280
Vb5.1 / 5.2	FITC	1/50	BD	553189
Vb6	FITC	1/200	BD	553193
Vb7	FITC	1/50	BD	553215
CCR6	PE	1/100	R&D	FAB590P
CCR7	PE	1 / 100	eBioscience	12-1971-82
CD4	PE	1/100	eBioscience	12-0041-83
CD8	PE	1/100	BD	553033
CD8	PE	1/100	eBioscience	12-0081
CXCR6	PE	3/50	R&D	FAB2145A
IL-17A	PE	1/100	eBioscience	12-7177-81
IL-17F	PE	1/100	eBioscience	12-7471
IL-2	PE	1/100	BD	554428
IL-22	PE	1/50	eBioscience	12-7221-80
Vb8	PE	1/100	BD	553862
CCR6	PE-Cy7	1/100	BioLegend	29-2L17
CD127 (IL7R)	PE-Cy7	1/50	eBioscience	25-1271-82
CD3a	PE-Cy7	1/100	eBioscience	25-0031-81
CD4	PE-Cy7	1/200	eBioscience	25-0042-82
CD44	PE-Cy7	1/100	BioLegend	103030
CD62L	PE-Cy7	1/200	eBioscience	553150
CD8	PE-Cy7	1/200	eBioscience	25-0081-81

IFNg	PE-Cy7	1/200	eBioscience	25-7311
IL-2	PE-Cy7	1/100	eBioscience	25-7021-82
KLRG-1	PE-Cy7	1/100	eBioscience	48-5893
NK1.1 (CD49b)	PE-Cy7	1/100	eBioscience	25-5971-82
NKG2D (CD314)	PE-Cy7	1/100	eBioscience	25-5882-81
CD11b	PerCp-Cy5.5	1/100	eBioscience	45-0112-82
CD27	PerCp-Cy5.5	1/100	BioLegend	124214
CD28	PerCp-Cy5.5	1/100	BioLegend	102114
CD28	PerCp-Cy5.5	1/100	eBioscience	45-0281-80
CD8	PerCp-Cy5.5	1/200	eBioscience	45-0081-82
IFNg	PerCp-Cy5.5	1/50	eBioscience	45-7311-80
IL-17A	PerCPCy5.5	1/100	eBioscience	45-7177-80

Antibodies were titrated individually prior to use on splenocytes from naïve and or mice 7 days post infection with MCMV either as surface staining or in PMA/ionomycin stimulated cells. Suitable titrations were obtained by visually examining plots and comparing the geometric mean fluorescence intensities of marker negative and marker positive populations.

Chapter 3

The role of IL-18 in memory inflation

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Introduction

Infection of C57BL/6 mice with MCMV produces a characteristic $CD8^+$ T cell response in the acute phase of infection to at least 24 epitopes derived from 18 viral proteins. However, 50 days post infection when viral latency has developed, the immune response is dominated by five epitopes only[60]. During latent infection T cells specific for these few epitopes accumulate at high frequency and this phenomenon is termed "memory inflation". The dominant MCMV-specific $CD8^+$ T cell responses during the acute phase are typified by classical expansion, contraction and formation of long-term central-memory pools ($CD27^+$, $CD28^+$, $CD62L^+$, $CD44^+$ and $IL\text{-}2^+$), whereas inflationary responses display an effector-memory phenotype ($CD27^-$, $CD28^-$, $CD62L^-$, $CD44^+$ and $IL\text{-}2^{+/-}$)[6,45,60,61]. Using MHC-Class I tetramers it is possible to track MCMV specific CD8+ T cells through the course of infection and examine phenotypic and functional characteristics at multiple sites. The underlying mechanisms of memory T cell inflation are beginning to be understood - antigen persistence and re-exposure, co-stimulation of CD8+ T cells and CD4+ T cell help – but as yet it is unclear how MCMV can induce populations of antigen-specific, tissue-resident, functional T-cells[130]. Improved understanding of the factors required to generate a memory inflating population would enable better design of T cell inducing vaccines.

Interleukin-18, (IL-18, IL-1F4 or IFNγ-inducing factor), is a member of the IL-1 family of cytokines. Like other members of the IL-1 family, IL-18 is synthesized as a 24-k-Da precursor protein requiring cleavage by activated caspase-1 prior to secretion as active 18-k-Da IL-18[119]. With IL-12 it participates in the induction of IFNγ production and thus contributes to Th1 immune responses. There is evidence that under certain conditions i.e. in the absence of IL-12, it can induce a Th2 response (IL-4)[119]. IL-18 signals through the IL-18 receptor, which consists of IL-18Rα (IL-18R1 or IL1R-related protein), the major ligand binding protein and IL-18Rβ required for downstream signaling (also known as IL-1R accessory protein-like (IL-1RAcP))[131]. Signaling through the IL-18 receptor complex involves a cascade of downstream

molecules (overlapping significantly with TLRs) such as MyD88, IRAK with eventual activation of NF- KB and JNK[119].

During infection with *Listeria monocytogenes* expressing ovalbumin (LmOVA), IL-18 related genes (namely IL-18Rα, IL-18Rβ and IL-18) are significantly up regulated in OT-1 ova-specific CD8+ T- cells. However OT-1 CD8+ T- cells develop with the identical dynamics and function in both IL-18Rα$^{-/-}$ and IL-18$^{-/-}$ mice infected with LmOVA[132]. In the LCMV-Armstrong model, expression of MyD88 in T cells is required for optimal T cell expansion initially, but expression is not necessary for the development and maintenance of a stable memory T cell population[133].

Thus IL-18 and IL-18Rα are not required for the development of classical memory. It is not yet known whether expression of IL-18 and IL-18Rα are induced by MCMV or if they are required for the development of memory inflation.

Aims

I aimed to examine

- Is IL-18Rα expression increased in MCMV infection?
- What is the phenotype and function of IL-18Rα+ CD8+ T cells in MCMV?
- Do M38 and M45-specific CD8+ T cells express IL-18Rα?
- Do M38 and M45-specific CD8+ T cells develop differently in IL-18$^{-/-}$ and IL-18Rα$^{-/-}$ mice infected with MCMV?
- Are the phenotype and function of M38+ and M45+specific CD8+ T cells in IL-18$^{-/-}$ and IL-18Rα$^{-/-}$ altered?

Results

IL-18Rα expression in MCMV infection

To assess whether MCMV infection induces expression of IL-18Rα C57BL/6 mice were infected intravenously with 1 x 10^6 pfu MCMV and sacrificed at different days post infection (dpi).

Expression of IL-18Rα was detectable on CD3- cells and both CD4+ and CD8+ T cells from blood, liver, lung and spleen in naïve mice. IL-18Rα expression significantly increased on CD8+ T cells after MCMV infection (figure 3.1 A and B) compared to baseline (day 0) (Table 3.1).

Table 3.1 – IL-18Rα expression upon CD8+ T -cells is significantly increased after infection with MCMV. CD8+ T cells from the spleens of naïve C57BL/6 mice or C57BL/6 infected with 1 x 10^6 pfu MCMV at the time points indicated were examined for IL-18Rα expression. Data shown is mean percentage +/- S.E.M. Data represent 3 experiments per time point minimum n=4 per experiment.

ORGAN	D0	D7	D50	D100	D200
Spleen	6.27 ±0.61	20.95 ±1.11	29.31 ±1.07	16.71 ±3.00	17.19 ±1.7
Liver	4.38 ±0.3	21.61 ±1.9	32.15 ±3.4	29.52 ±3.5	26.52 ±2.7
Lung	3.55 ±0.11	18.40 ±2.1	37.72 ±3.6	36.57 ±1.4	26.52 ±2.6
Blood	3.95 ±0.3	19.30 ±1.9	39.71 ±1.97	34.42 ±2.69	28.01 ±2.00

Increased IL-18Rα expression was detectable upon CD8+ T cells in all tissues. It peaked at 50 dpi then began to decline, although 200 dpi it remained elevated above baseline. Compared

to CD4+ T cells from uninfected mice, IL-18Rα expression was detected on a higher frequency of CD4+ T cells in liver, lungs and blood in a similar pattern to CD8+ T cells but at a lower magnitude. In splenic tissue IL-18Rα expression on CD4+ T cells did not vary. CD3- cells displayed an undulating kinetic of IL-18Rα expression but this did not vary significantly from baseline.

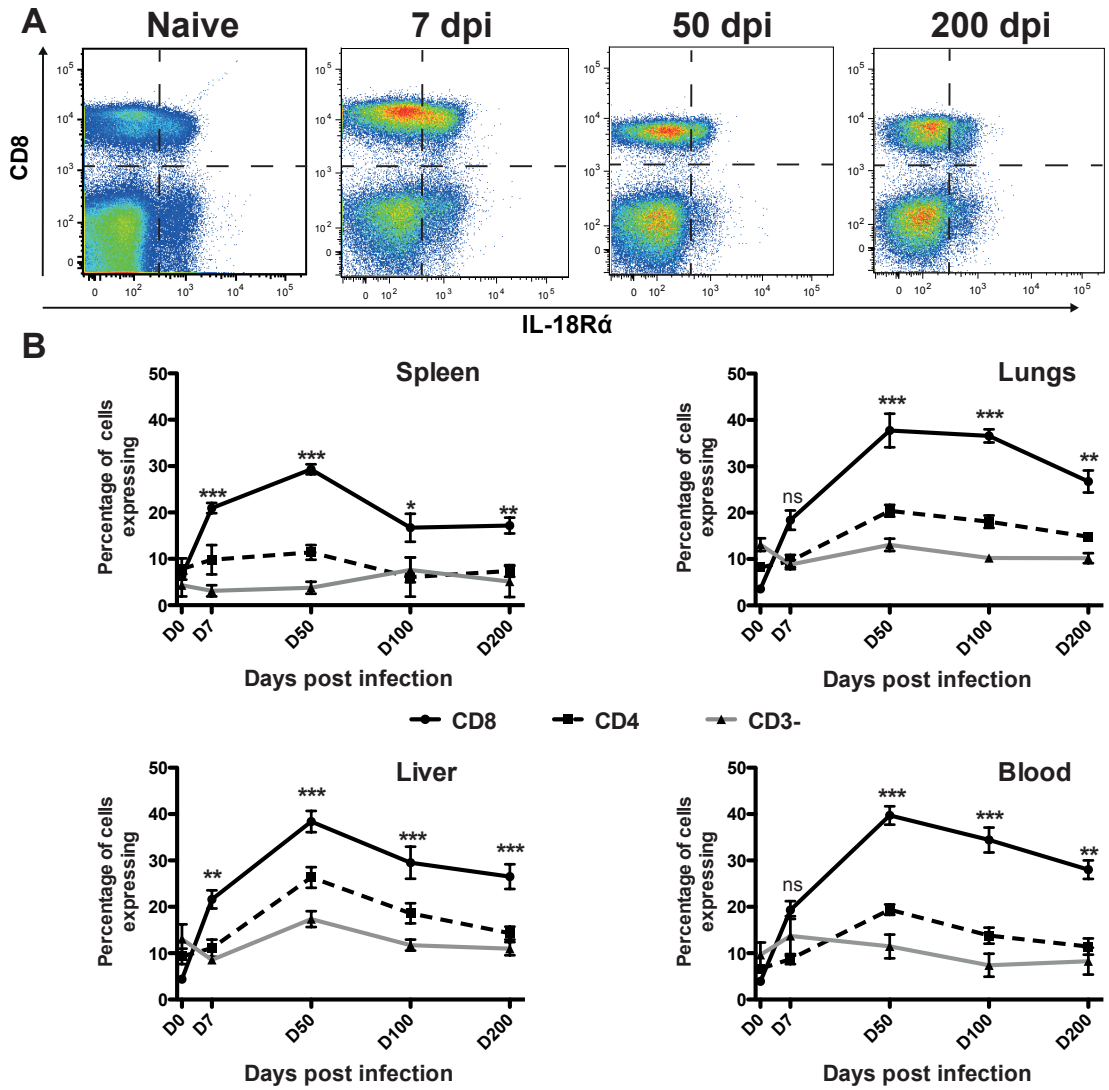

Figure 3.1 - IL-18Rα expression in MCMV infection. C57BL/6 mice were infected with 1 x 10^6 pfu MCMV and organs harvested at indicated time points. **A** Flow cytometry plots displaying expression of IL18Rα on splenic CD8+ T-cells in naïve mice and mice infected with MCMV (gated on live, lymphocytes, CD3+). **B** Time course expression of IL-18Rα on CD3- (grey) CD3+CD4+ (dotted) and CD3+CD8+ (solid) cells in different tissues after MCMV infection (mean +/- S.E.M.). * = p<0.05, ** = p<0.005, *** = p<0.0005 by Kruskal Wallis indicating comparison of IL-18Rα expression on CD8+ T cells at that time point compare to day 0. Data represent 3 experiments per time point minimum n=4 per experiment.

Tetramers specific for inflating M38+ (M38-specific) cells and non–inflating M45+ (M45-specific) CD8+ T cells allow clear identification of antigen specific cells. I showed that IL-18Rα is highly expressed (40 – 70% MCMV-specific cells express IL-18Rα) upon both inflating and non-inflating T-cells in all tissues (figure 3.2 A and B, table 3.2) with expression peaking 50 - 100 days post MCMV infection then beginning to decline. Compared to tetramer negative cells (shaded grey figure 3.1 B) both M38+ and M45+ cells express significantly higher levels of IL18Rα.

Expression of IL-18Rα is induced by MCMV infection most notably on CD8+ rather than CD4+ T cells. Amongst CD8+ T cells MCMV-specific cells display high levels of IL-18Rα. However this is not specifically related to inflation as it was observed on both M38-specific and M45-specific CD8+ T cells. Using MHC-I tetramers for other inflating or non-inflating epitopes would enable clear delineation of whether this is a bystander phenomenon on CD8+ T cells in MCMV or truly antigen-specific.

Table 3.2 – IL-18Rα expression upon M38+ and M45+ CD8+ T cells. M38+ and M45+ CD8+ T cells from the spleens of C57BL/6 mice infected with 1 x 10^6 pfu MCMV at the time points indicated were examined for IL-18Rα expression. Data shown is mean percentage +/- S.E.M. Data represents 3 experiments per time point minimum n=4 per experiment.

ORGAN		D7	D50	D100	D200
Spleen	**M38**	40.78 ±6.5	57.50 ±3.3	53.13 ±1.7	44.00 ±2.8
Liver		55.95 ±4.2	56.65 ±2.8	66.23 ±2.3	51.76 ±7.8
Lung		39.64 ±3.8	65.30 ±3.3	44.79 ±3.2	36.65 ±1.8
Blood		40.33 ±5.3	55.73 ±3.7	41.02 ±3.1	42.58 ±1.8
Spleen	**M45**	45.33 ±3.9	66.34 ±4.7	69.44 ±5.2	46.97 ±3.3
Liver		55.84 ±4.1	73.85 ±2.8	65.88 ±4.6	45.12 ±2.8
Lung		37.73 ±3.4	67.70 ±5.5	61.55 ±4.8	48.97 ±8.7
Blood		36.11 ±5.9	63.38 ±2.8	57.66 ±4.6	35.36 ±2.7

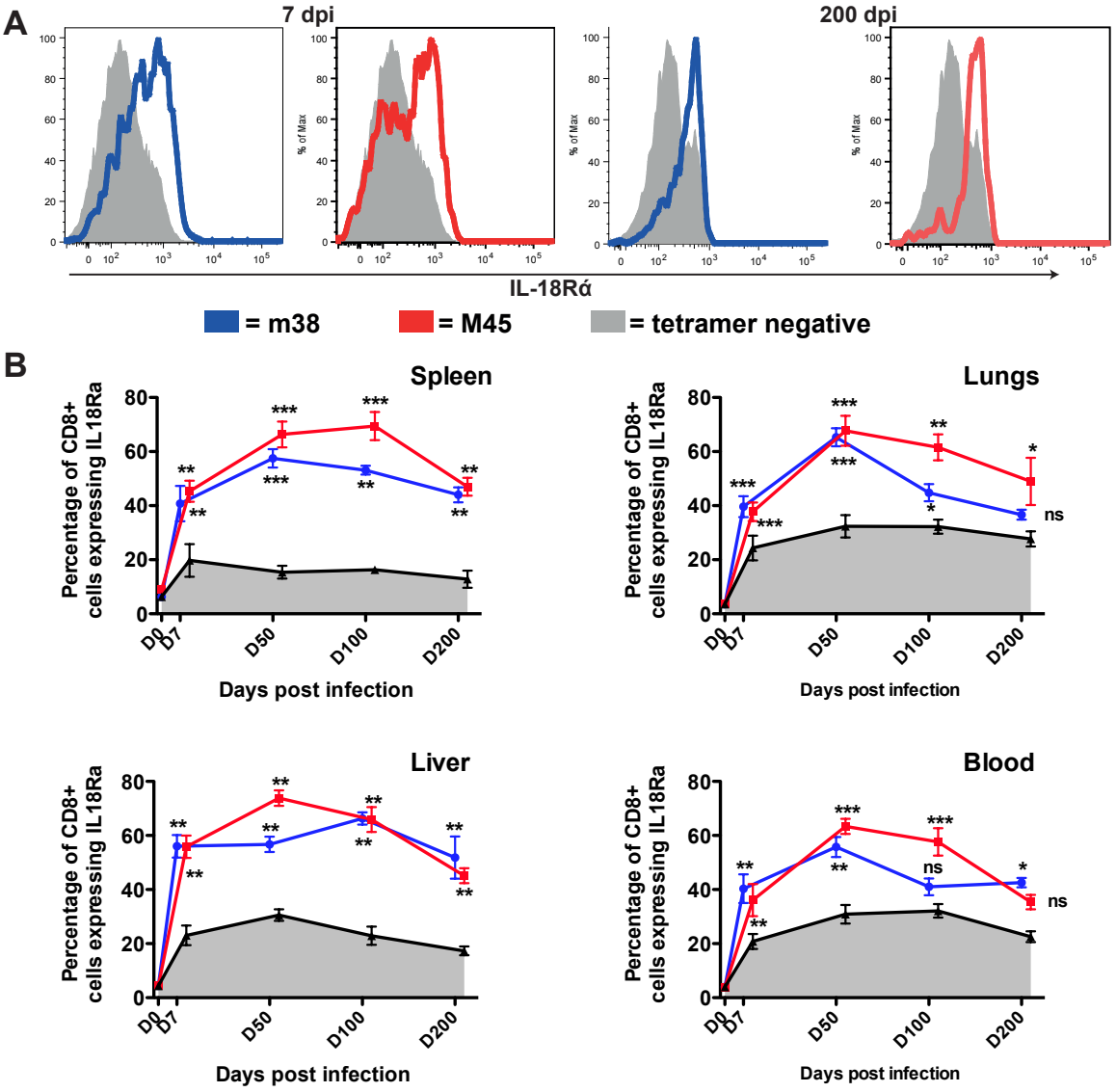

Figure 3.2 - IL-18Rα expression on antigen specific cells in MCMV infection. C57BL/6 mice were infected with 1 x 10^6 pfu MCMV and organs harvested at indicated time points. **A** Overlaid histograms displaying IL-18Rα expression in tetramer negative (grey shading) or M38 (blue) or M45 (red) CD8+ T cells fro the spleen 7 or 200 days post infection (dpi). **B** Frequency of IL-18Rα expression on M38+ or M45+ cells compared with bulk CD8+ (mean +/- S.E.M). * = p<0.05, ** = p<0.005, *** = p<0.0005 by Mann Whitney U test. Data represents 3 experiments per time point minimum n=4 per experiment.

Phenotype and function of IL-18Rα expressing CD8+ T cells in MCMV

In order to better understand the phenotype of CD8+ T cells in naïve and MCMV infected mice expressing IL-18Rα I sought to delineate the memory phenotype of these cells using CD44 and CD62L. A recent theory postulates a small portion of CD8+ T cells specific for inflating epitopes maintain a central memory phenotype during acute infection – and it is these that will proliferate and subsequently inflate[134]. I wished to elucidate if IL-18Rα expression, or lack of, would delineate such a subset. Using intracellular cytokine staining I aimed to elicit if IL-18Rα+ CD8+ T cells were functionally different in terms of IFNγ secretion.

Splenocytes were harvested from naïve mice, and mice 7 or 100 days post infection with 1 x 10^6 pfu MCMV. Naïve mice have a population of CD8+IL-18Rα+ T cells and analysis reveals the majority are CD44+ (69.2+/-5.2% splenic CD8+ T-cells [mean +/- S.E.M]) and CD62L+ (mean 85.7+/-0.7% [mean +/- S.E.M]), broadly typifying a central memory population (figure 3.3 A). During the acute phase of MCMV infection CD8+IL-18Rα+ T cells display a CD44+CD62L- phenotype but at 100 dpi many re-express CD62L (figure 3.3 A).

CD44

CD44 is a cell adhesion molecule widely expressed on immune cells where it mediates adhesion to an extracellular matrix glycosaminoglycan - hyaluronan. Once T cells are activated by antigen they induce CD44 to bind to hyaluronan expressed by endothelial cells. This causes a rolling interaction, which together with chemokine signaling, up-regulates integrin-mediated adhesion and leads to subsequent migration to inflammatory sites. As such, CD44 can be seen as a surrogate marker of antigen exposure, and typically in MCMV both M38 and M45 cells express high levels of CD44[135].

On examination of IL-18Rα+ antigen-specific cells in acute (7dpi) and chronic (100 dpi) MCMV nearly all $M38^+$ (96.2+/-1.1% 7 dpi, 96.9 +/- 0.9% 100 dpi [mean+/- S.E.M]) and $M45^+$ (96.97+/- 1.01% at 7 dpi 96.4+/-1.91% 100 dpi [mean+/- S.E.M]) CD8+ T cells express CD44

(figure 3.3 B). This pattern is consistent on M38- and M45-specific CD8+ T cells in liver, lung and blood (data not shown). Notably, by 100 dpi IL-18Rα- M45-specific CD8+ T cells display significantly less CD44 compared to IL-18Rα+ M45-specific CD8+ T cells (35.8+/-10.2%[mean+/- S.E.M]) and a similar pattern can be observed in liver lung and spleen although it does not reach statistical significance.

CD62L

Lymphocytes expressing CD62L can enter secondary lymphoid tissues by binding GlyCAM-1 on the surface of high endothelial venules. This slows lymphocyte trafficking through the blood, and facilitates entry into secondary lymphoid organs. Naïve T-cells need to enter secondary lymph nodes to encounter cognate antigen. Central memory T-cells re-express L-selectin to localize in secondary lymphoid organs where they reside ready to proliferate upon re-encountering antigen[136]. Effector memory T-cells cleave CD62L from their surface to allow peripheral circulation[137]. CD8+ T cells specific for the non-inflating M45 epitope re-express CD62L as part of their central memory phenotype. Conversely M38+ inflationary T cells retain an effector memory surface phenotype expressing low levels of CD62L[60].

As expected by the current memory inflation dichotomy whilst few antigen-specific cells express CD62L 7 dpi, by 100 dpi M45+IL-18Rα+ CD8+ T cells begin to re-express CD62L (2.4+/-0.4% in the spleen at 7 dpi expanded to 44.92+/-7.2% [mean+/-S.E.M] figure 3.3 B), suggesting a significant proportion of M45+ cells maintain an effector phenotype. However this phenomenon is not exclusively identified by expression of IL-18Rα and indeed may be a partial reflection of the fact we did not examine peripheral lymph nodes where central memory T cells reside. MCMV-specific CD8+ T cells from liver lung and spleen displayed identical patterns of CD62L expression.

Additionally I assessed whether IL-18Rα would identify a central memory (CD44+CD62L+) subset of M38+ CD8 T cells during acute infection – the putative candidates for inflation.

Very few M38-specific CD8+ T cells displayed a central memory phenotype at day 7 and those located displayed similar levels of IL-18Rα expression (data not shown).

Interferon gamma

Interferon gamma, (IFNγ), is a cytokine critical for fully functioning innate and adaptive immunity. It has a myriad of functions including direct antiviral effects, promotion of NK cell activity, increase of antigen presentation and lysosome activity of macrophages, and promotion Th1 differentiation[60]. The importance of IFNγ in MCMV is underlined by the fact mice deficient in IFNγ are far more susceptible to MCMV and when recombinant IFNγ was administered to susceptible mice prior to infection there was a significantly reduced mortality, lower MCMV titers and fewer histopathologic lesions[138,139].

In response to PMA/ionomycin stimulation, CD8+ T cells secrete IFNγ and examination of IFNγ producing splenic CD8+ T cells in naïve mice revealed high levels of IL-18Rα expression – however at 7 and 100 dpi with MCMV this was significantly reduced ($p < 0.0005$ by Kruskal Wallis ANOVA; figure 3.3C). It is perhaps possible that during MCMV infection the large proportion of effector memory T cells (i.e. M38-specific and other unmeasured inflating epitopes) expressing IL-18Rα are more susceptible to activation induced cell death than central memory IL-18Rα+ CD8+ T cells in naïve mice and my observations merely reflect the potency of PMA/ionomycin stimulation.

High levels of IL-18Rα are detected upon both splenic M45- and M38 specific CD8+ T cells (at day 7 45.3+/-3.9% and 40.8+/-6.5% respectively [mean+/-S.E.M]) increasing to >50% by 50 dpi. In view of this it is unsurprising that approximately 50% of CD8+IFNγ+ cells responding to cognate peptide are IL-18Rα+ at both acute and chronic time points. IL-18Rα expression does not clearly delineate a responding or non-responding subset of antigen specific cells.

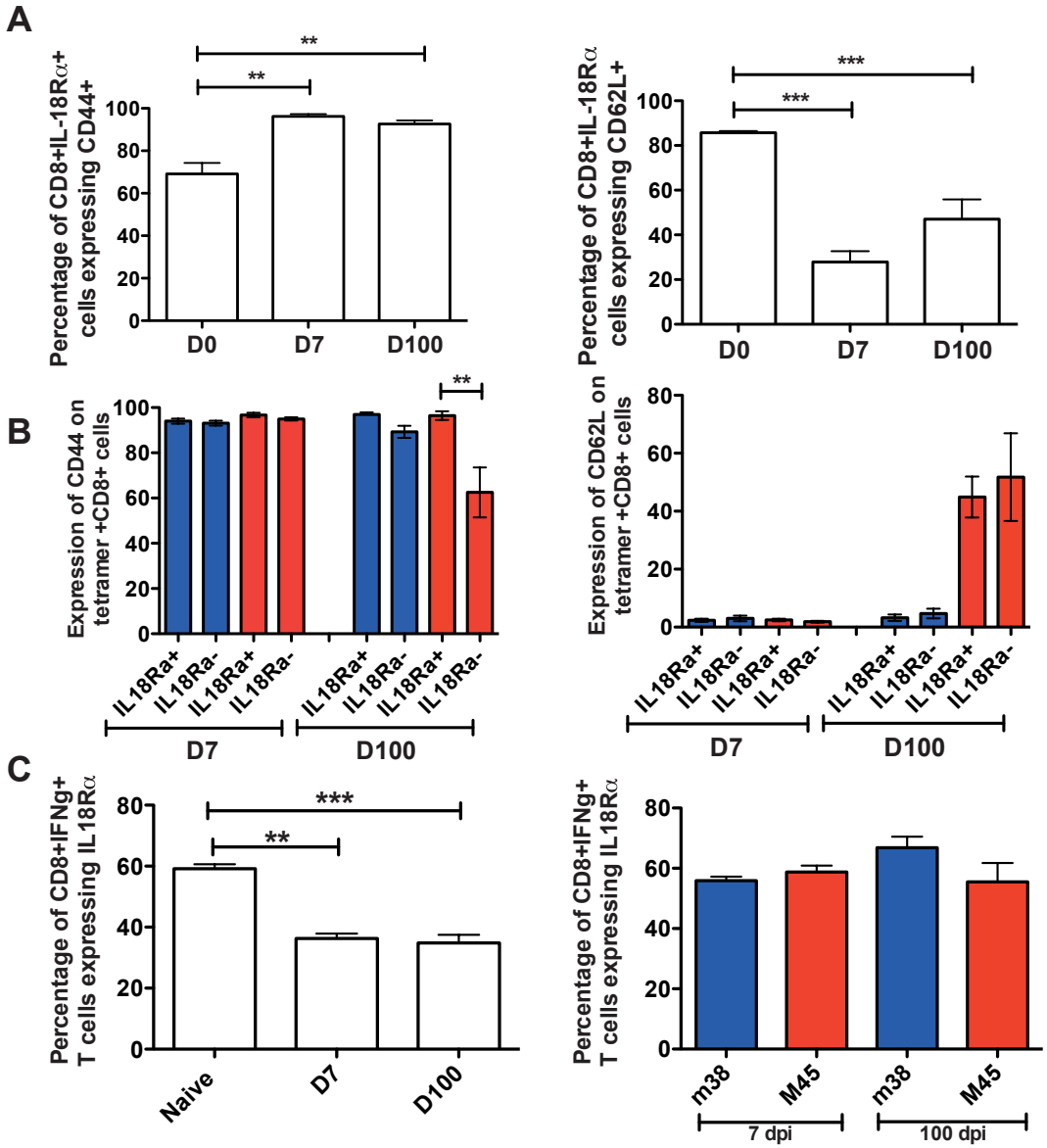

Figure 3.3 - Phenotype and function of IL-18Rα expressing CD8+ T cells in MCMV. C57BL/6 mice were infected with 1 x 10^6 pfu MCMV and splenocytes isolated at indicated time points. **A** Expression of CD44 and CD62L on CD8+IL-18Rα+ splenic T-cells in naïve mice and mice 7 or 100 dpi with 1x 10^6 pfu MCMV. **B** Comparison of CD44 and CD62L expression on M38+ (blue) and M45+ (red) IL18Rα+ or IL-18Rα MCMV-specific cells in the spleens of mice 7 or 100 dpi. **C** IL-18Rα expression on IFNγ secreting CD8+ T cells in responding to PMA/ionomycin or peptide as indicated. Data shown (mean +/- S.E.M) represents 4 experiments n= 5 per experiment. * = $p<0.05$, ** = $p<0.005$, *** = $p<0.0005$ by Kruskal Wallis ANOVA (**A** and **C**) or Mann Whitney U test (B).

Role of IL-18Rα in memory inflation

Despite relatively high levels of IL-18Rα expression on antigen-specific cells I was unable to define a clear phenotype in terms of CD44 or CD62L expression or IFNγ secretion. To examine whether IL-18 or IL-18Rα played a role in the development of memory inflation I infected IL-18Rα$^{-/-}$ (B6.129P2-Il18r1tm1Aki/J) or IL-18$^{-/-}$ (B6.129P2-Il18tm1Aki/J) mice intravenously with 1 x 10^6 pfu MCMV and examined the frequency, phenotype, and function of M38 and M45 specific CD8+ T cells that developed.

Frequency of MCMV specific cells

Mice were infected with MCMV and culled 7, 50, 100 and 200 dpi. Lymphocytes from blood, liver, lungs and spleen were examined. For comparison a similar number of C57BL/6 mice were concurrently infected and examined (the data plotted as a shaded gray area in figure 3.4 A, B, C, D). As expected, from previous work examining the role of IL-18Rα or MyD88 in classical memory[132,133], the kinetic of M45-specific cells was unchanged in either IL-18Rα$^{-/-}$ or IL-18$^{-/-}$ mice compared with C57BL/6 in any organ or time point. Notably the frequency of M38-specific cells did not display any significant changes from the kinetic seen in C57BL/6 mice (figure 3.4 A, B, C, D).

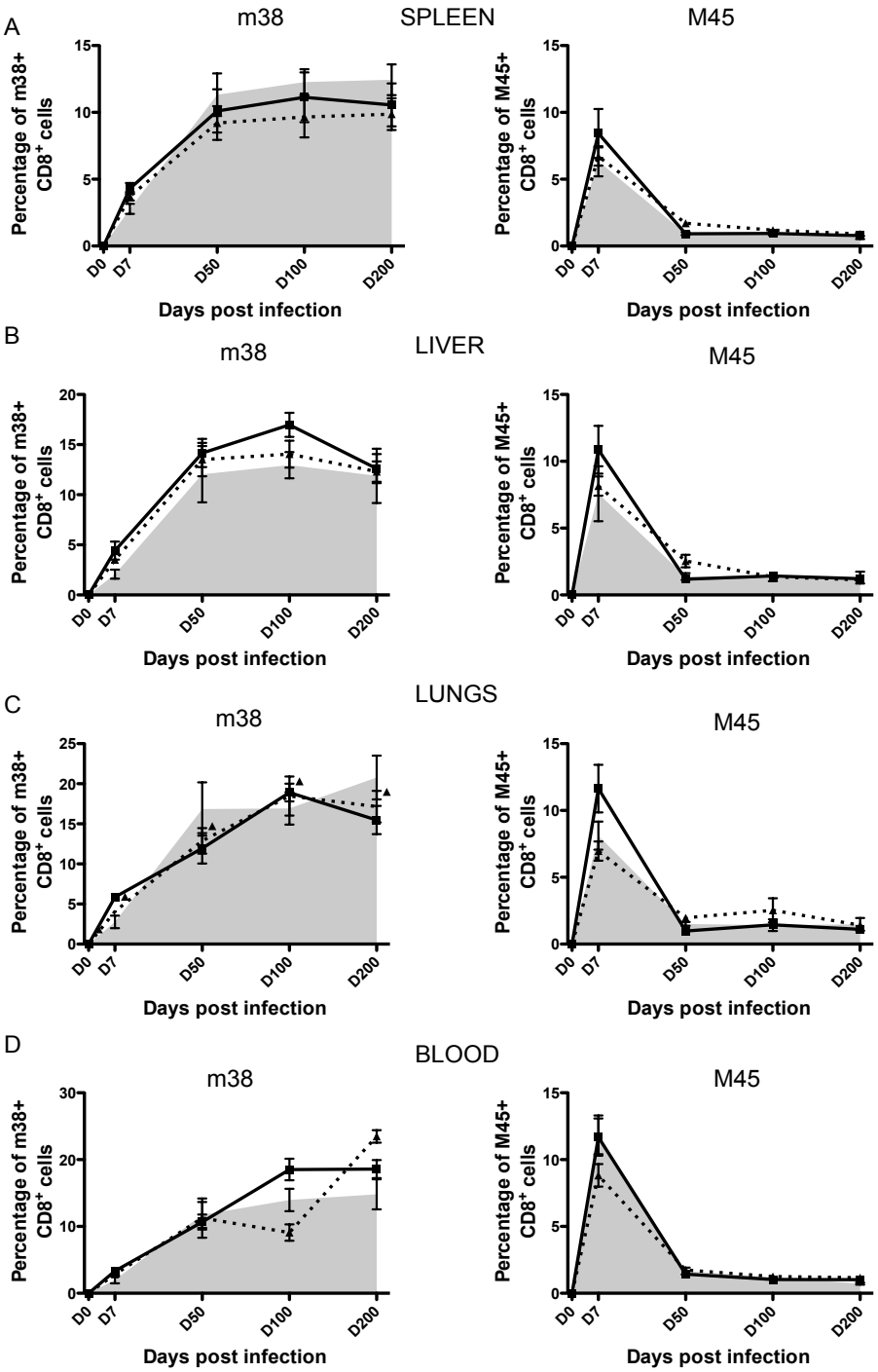

Figure 3.4 - Role of IL-18 and IL-18Rα in memory inflation. IL-18Rα$^{-/-}$ and IL-18$^{-/-}$ mice were infected with 1 x 10^6 pfu MCMV and lymphocytes isolated at indicated time points from different organs. Frequency of M38+ and M45+ CD8+ T-cells from **A** Spleen **B** Liver **C** Lung **D** Blood (mean +/- S.E.M). IL-18Rα$^{-/-}$ dotted line, IL-18$^{-/-}$ solid line. Shaded grey area C57BL/6 mice for comparison. Data represents 1 set experiments per time point n = 5 per group.

Phenotype of MCMV-specific cells

Memory inflating CD8+ T cells display a characteristic set of surface markers. Despite the paucity of difference in the magnitude of both inflating and non-inflating CD8+ T cells in either IL-18Rα$^{-/-}$ or IL-18$^{-/-}$ mice compared with C57BL/6 mice there remained the possibility that the frequency of MCMV specific cells was unchanged but the phenotype of these cells was altered. A panel of phenotypic markers typically used to differentiate between inflating and non-inflating MCMV-specific cells was used to assess whether the M38– and M45-specific CD8+ T cells developing in IL-18Rα$^{-/-}$ or IL-18$^{-/-}$ mice maintained their characteristic phenotype.

CD44

In C57BL/6 mice and IL-18Rα$^{-/-}$ or IL-18$^{-/-}$ knockouts the pattern of CD44 expression on tetramer positive cells is nearly identical (figure 3.5). In the lungs of C57BL/6 and IL-18Rα$^{-/-}$ mice 50 dpi levels of CD44 found on both M38+ and M45+ cells are lower than at any other time point – a phenomenon not described in literature. By 100 dpi CD44 expression on pulmonary MCMV specific cells has returned to the high levels seen 7 dpi and this is maintained at 200 dpi suggesting these data represent anomalous readings. M45+ cells in the spleen of IL-18$^{-/-}$ mice express significantly lower levels of CD44 100 and 200 dpi. This likely represents anomalous measurements.

CD62L

Inflationary M38+ cells in both IL-18Rα$^{-/-}$ and IL-18$^{-/-}$ displayed a nearly identical pattern of CD62L expression (compared to C57BL/6 mice, figure 3.6). M45+ cells displayed similar kinetics of CD62L expression in IL-18Rα$^{-/-}$ with a significant increase at day 200. Notably in IL-18$^{-/-}$ mice CD62L was re-expressed by M45+ cells at a statistically significant higher frequency in blood, liver and lungs compared to C57BL/6 mice and IL-18Rα$^{-/-}$ mice. Rapid re-expression of CD62L suggests that M45 antigen may be less available in the peripheral tissues of IL-18$^{-/-}$ mice and/or for less time i.e. viral load may be better controlled.

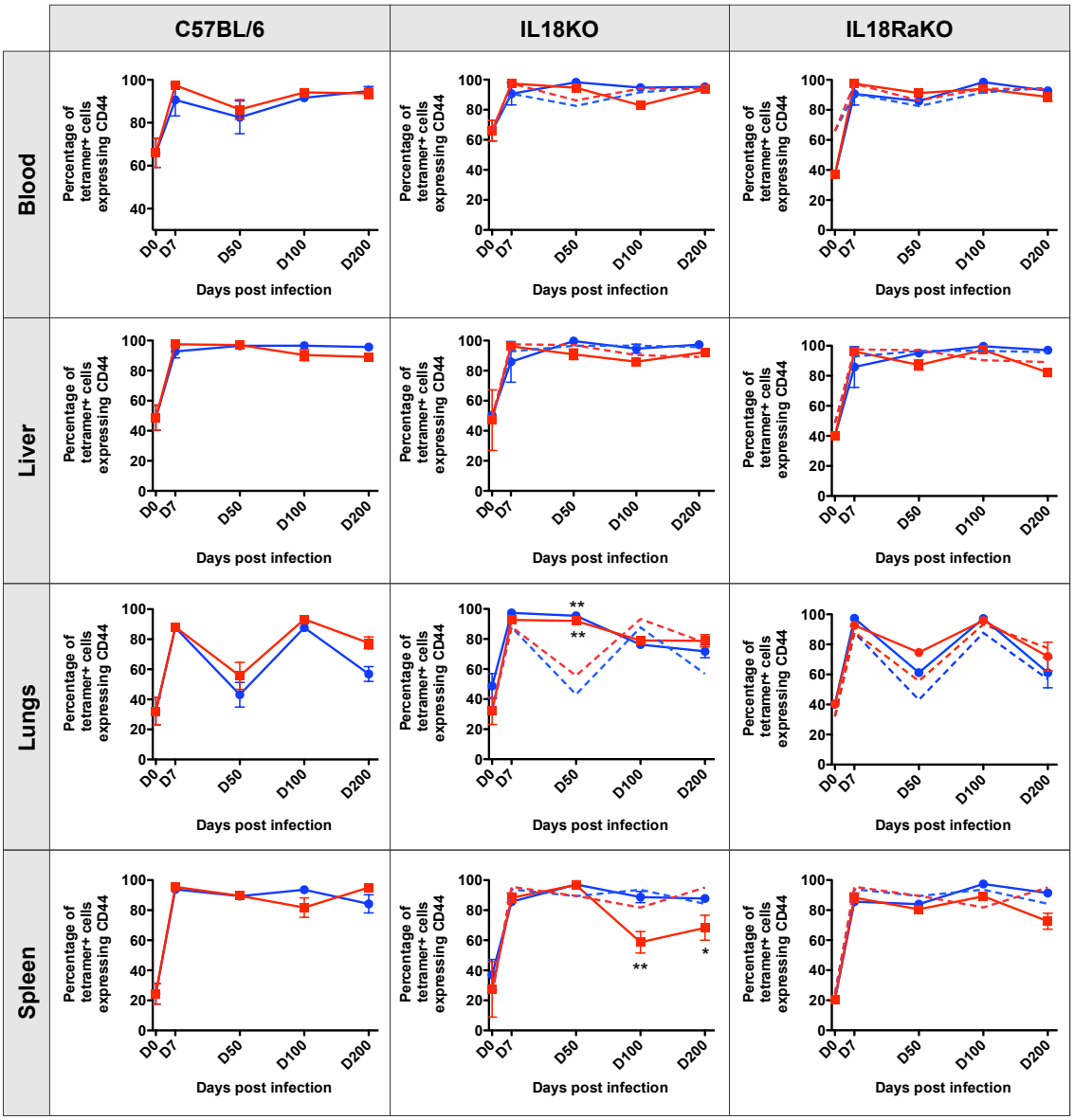

Figure 3.5 - CD44 expression on antigen specific cells in C57BL/6, IL-18$^{-/-}$ and IL-18Rα$^{-/-}$ mice. IL-18Rα$^{-/-}$ or IL-18$^{-/-}$ mice were infected with 1 x 10^6 pfu MCMV and lymphocytes isolated at indicated time points from different organs. Frequency of CD44+ M38+ (blue) or M45+ (red) CD8+ T cells. Superimposed on graphs from IL-18Rα$^{-/-}$ or IL-18$^{-/-}$ mice are results from C57BL/6 mice (dotted lines). Data shown (mean +/- S.E.M) represents per time point 2 experiments n= 5 C57BL/6 and 1 experiment n=5 in IL-18Rα$^{-/-}$ or IL-18$^{-/-}$. * = p<0.05, ** = p<0.005, *** = p<0.0005 by Mann-Whitney U test.

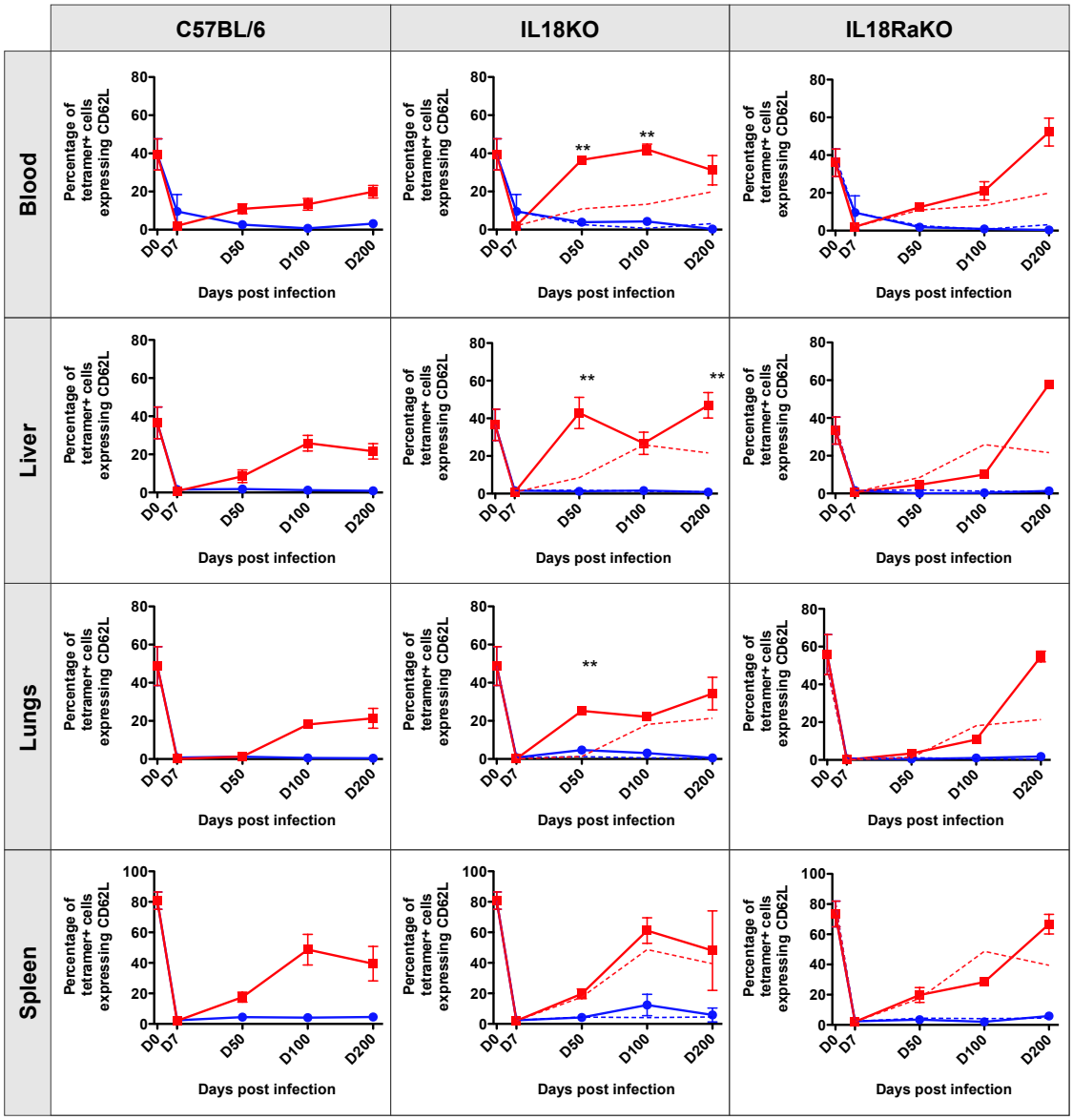

Figure 3.6 - CD62L expression on antigen specific cells in C57BL/6, IL-18Rα$^{-/-}$ and IL-18$^{-/-}$ mice. IL-18Rα$^{-/-}$ and IL-18$^{-/-}$ mice were infected with 1 x 10^6 pfu MCMV and lymphocytes isolated at indicated time points from different organs. Frequency of CD62L+ M38+ (blue) or M45+ (red) CD8+ T cells is shown. Superimposed on graphs from IL-18Rα$^{-/-}$ or IL-18$^{-/-}$ mice are results from C57BL/6 mice (dotted lines). Data shown (mean +/- S.E.M) represents per time point 2 experiments n= 5 C57BL/6 and 1 experiment n=5 in IL-18Rα$^{-/-}$ or IL-18$^{-/-}$. * = $p<0.05$, ** = $p<0.005$, *** = $p<0.0005$ by Mann-Whitney U test.

CD27

CD27, a member of the TNF/NGF-R family, is expressed on the majority of naïve lymphocytes and is one of the myriad of molecules responsible, together with its ligand CD70, for co-stimulation and thus optimal T cell responses[140]. T cells that have encountered antigen maintain expression of CD27. However repeated antigenic stimulation leads to loss of CD27 expression. Loss of proliferative capacity coupled with enhanced cytotoxicity is suggestive of a strongly effector phenotype[2,141]. M45 specific T-cells maintain higher levels of expression of CD27 compared to M38+ CD8+ T cells[60,61].

In IL-18Rα$^{-/-}$ and in IL-18$^{-/-}$ mice the pattern of CD27 expression on inflating and non-inflating MCMV specific cells was broadly the same as the pattern observed in C57BL/6 mice (figure 3.7).

CD127 (IL-7R)

The maintenance of pools of memory T cells is regulated by homeostatic mechanisms independent of TCR signaling or antigen. It instead involves cytokines from the common γ chain family of cytokines – IL-7 and IL-15[142]. A CD8+ T cell response to a pathogen has three consistent stages: expansion of an effector cell population and differentiation; contraction of this effector pool; and subsequent stabilisation and maintenance of the memory cell pool. IL-7R or CD127 is expressed on T cells at almost all stages of development, with evidence that CD127 expression is reduced upon T cell activation; only a small proportion of effector cells which retain CD127 expression then survive contraction via apoptosis and form long-term memory cells[143]. In MCMV during latent infection CD127 is expressed on non-inflating M45+ cells. Inflating M38-specific CD8+ T cells down-regulate CD127 early in MCMV infection and do not re-express it[61].

In IL-18Rα$^{-/-}$ and in IL-18$^{-/-}$ mice lymphocytes were examined and the pattern of CD127 expression on inflating and non-inflating MCMV specific cells was broadly the same as the pattern observed in C57BL/6 mice (figure 3.8). However in both IL-18Rα$^{-/-}$ and in IL-18$^{-/-}$

mice tetramer specific cells showed lower levels of CD127 at days 50 and 100. Although this resolved by 200 dpi it suggests the kinetics for viral control may be altered in mice lacking IL-18 or its receptor with antigen persisting for longer in mice deficient for IL-18 and IL-18Rα.

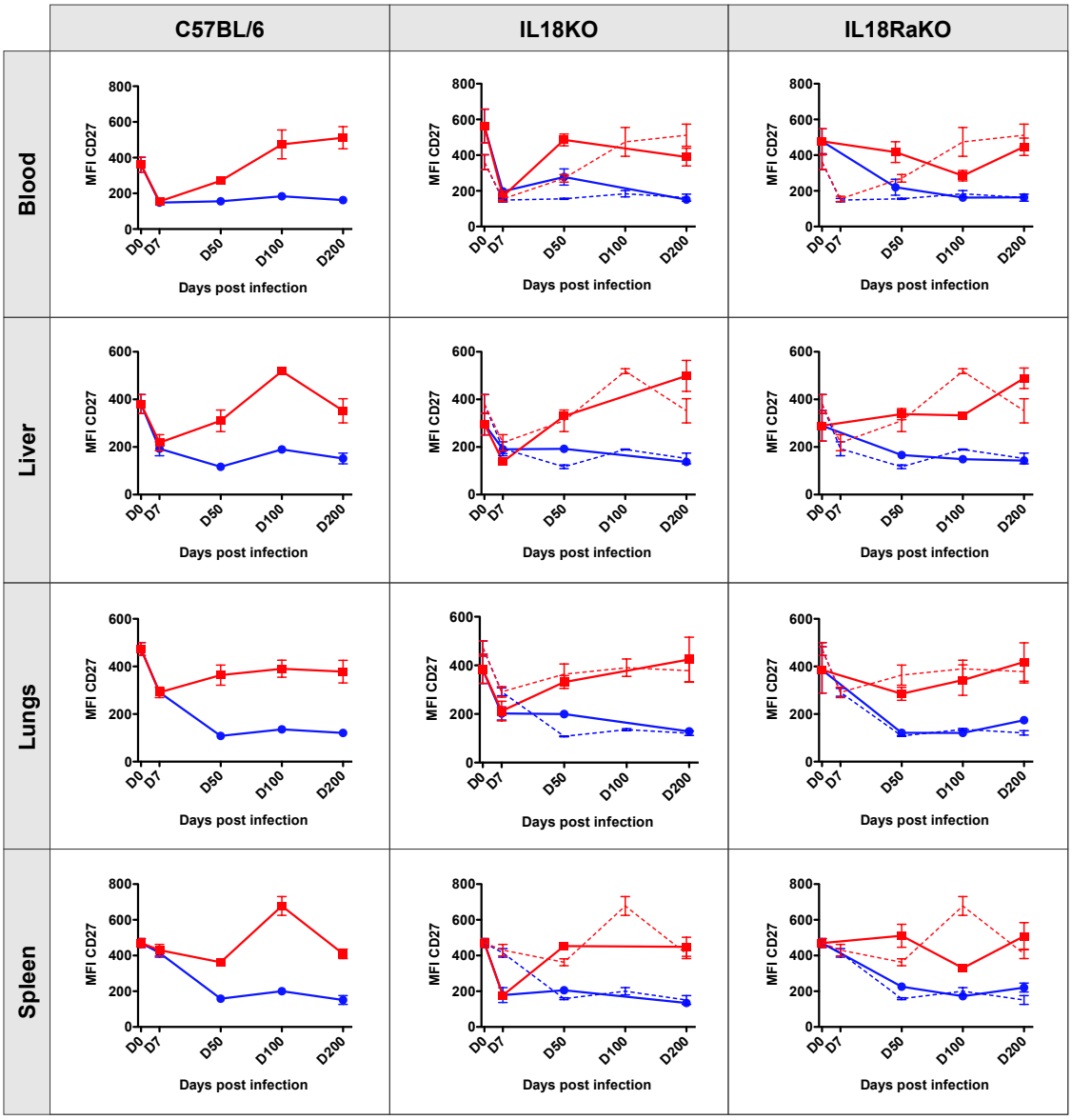

Figure 3.7 - CD27 expression on antigen specific cells in C57BL/6, IL-18Rα$^{-/-}$ and IL-18$^{-/-}$ mice. C57BL/6, IL-18Rα$^{-/-}$ or IL-18$^{-/-}$ mice were infected with 1 x 10^6 pfu MCMV and lymphocytes isolated at indicated time points from different organs. Median fluorescence intensities (MFI) of CD27 on M38+ (blue) or M45+ (red) CD8+ T cells are shown. Superimposed on graphs from IL-18Rα$^{-/-}$ or IL-18$^{-/-}$mice are results from C57BL/6 mice (dotted lines). Data shown (geometric mean +/- S.E.M) represents per time point 2 experiments n= 5 C57BL/6 and 1 experiment n=5 in IL-18Rα$^{-/-}$ or IL-18$^{-/-}$. * = p<0.05, ** = p<0.005, *** = p<0.0005 by Mann-Whitney U test.

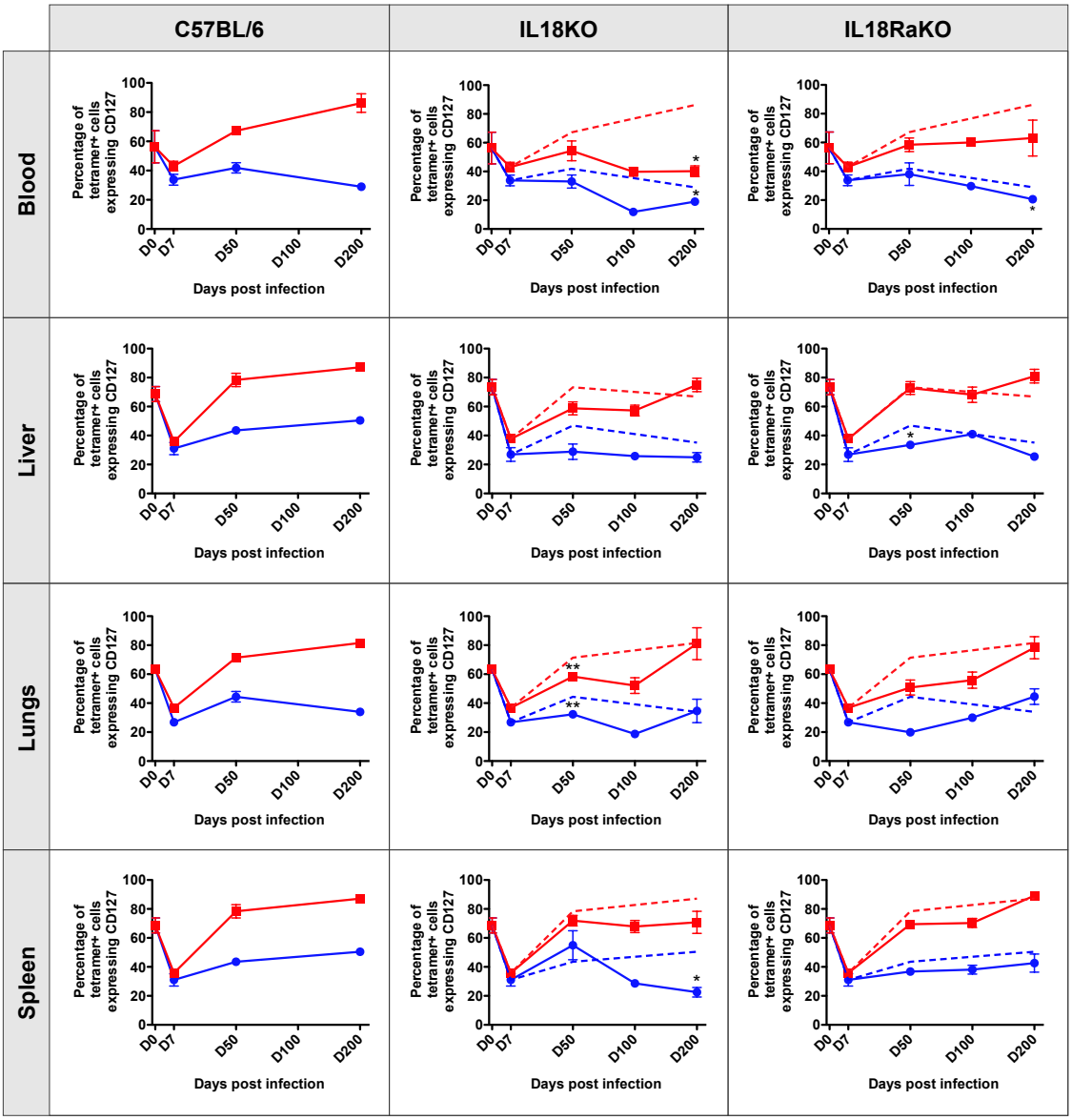

Figure 3.8 - CD127 expression on antigen specific cells in C57BL/6, IL-18Rα$^{-/-}$ and IL-18$^{-/-}$ mice. C57BL/6, IL-18Rα$^{-/-}$ or IL-18$^{-/-}$mice were infected with 1 x 10^6 pfu MCMV and lymphocytes isolated at indicated time points from different organs. Frequency of CD127+ M38+ (blue) or M45+ (red) CD8+ T cells is shown. Superimposed on graphs from IL-18Rα$^{-/-}$ and IL-18$^{-/-}$ mice are results from C57BL/6 mice (dotted lines). Data shown (mean +/- S.E.M) represents per time point 2 experiments n= 5 C57BL/6 and 1 experiment n=5 in IL-18Rα$^{-/-}$ or IL-18$^{-/-}$. * = p<0.05, ** = p<0.005, *** = p<0.0005 by Mann-Whitney U test.

NKG2A, C, E

NKG2A, NKG2C and NKG2E are transmembrane proteins with extracellular C-type lectin domains. These proteins form heterodimers with CD94 recognizing the non-classical MHC molecule Qa1 as ligand. The NKG2A/CD94 complex delivers an inhibitory signal, however NKG2C/CD94 and NKG2E/CD94 provide activating signals[144]. These complexes are expressed on NK cells and on a subset of T cells, where expression is significantly increased following exposure to antigen and maintained during the memory phase[144]. The actual role of NKG2/CD94 complex is unclear – partly linked to the varying specificities of currently commercially available antibodies. Both M45+ and M38+ express NKG2A, C, E at higher levels than CD8+ T cells in naïve mice (9.6+/-2.4% of splenic CD8+ T cells in naïve mice vs. 44.1+/-2.7% (M38) and 46.4+/- 4.9% (M45) 7 dpi [mean+/-S.E.M.]. However inflating M38+ cells express NKG2A, C, E at even higher levels during latency (66.3+/-3% M38-specific cells express NKG2A, C, E at 50 dpi compared with 45+/-6.4% M45-specific CD8+ T cells [mean+/-S.E.M]). There is no observable difference between NKG2A, C, E expression between C57BL/6 mice and knockouts (figure 3.9).

NKG2D

NKG2D is distantly related to the NKG2 family above, but significantly does not form a heterodimer with CD94, instead it is expressed as a homodimer and provides activatory signals by recruiting either DAP-10 or DAP-12[145]. NKG2D is expressed upon activated CD8+ T cells where it acts as a co-stimulatory receptor.

MCMV encodes several genes which downregulate the cellular ligands MULT-1, RAE-1 and H60 which bind NKG2D[51]. Indeed recombinant mouse CMV expressing the high-affinity NKG2D ligand RAE-1 is profoundly attenuated in vivo with lower viral DNA loads and can be controlled by immunodeficient mice[146].

In MCMV infected C57BL/6 mice both inflating (M38) and non-inflating (M45) cells increase expression of NKG2D following infection and MCMV specific cells gradually express higher

levels of NKG2D over time with the highest levels expressed on inflating cells (peaking at D100 where 52+/-7.6% M38-specific splenic CD8+ T cells express NKG2D compared to 23.6+/- 1.2% M45+CD8+ T cells [mean+/-S.E.M]) (figure 3.10). In IL-18Rα$^{-/-}$ and in IL-18$^{-/-}$ knockout mice the basic kinetic observed in C57BL/6 mice is repeated, however, in IL-18$^{-/-}$ the magnitude is once more reduced in both M38+ and M45+ cells resident in lung liver and spleen during latency where for example only 31.2 +/-1.1% M38- and 13.4+/-5.2% M45-specific CD8+ T cells express NKG2D at 50 dpi [mean+/- S.E.M.].

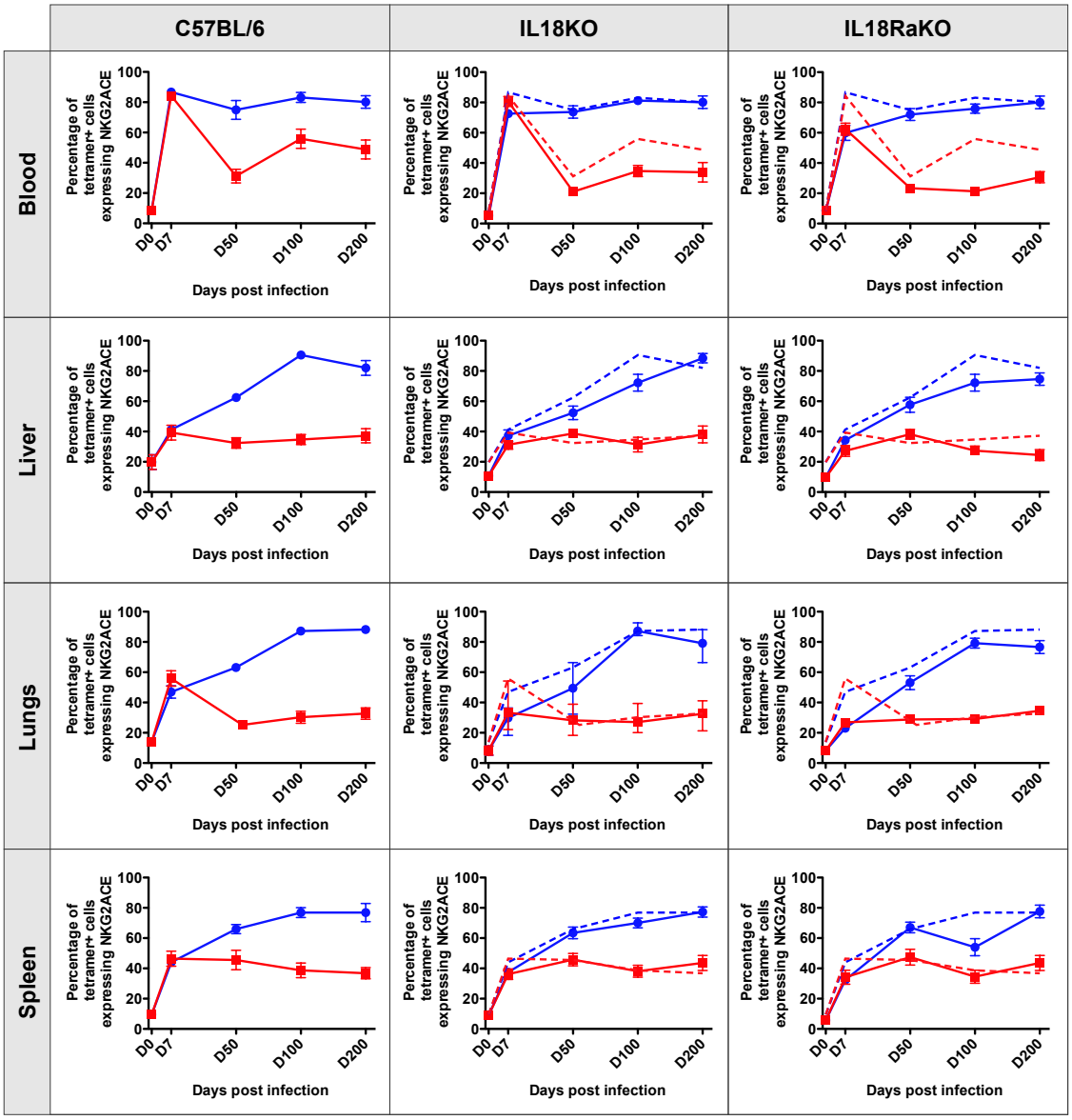

Figure 3.9 - NKG2A, C, E expression on antigen specific cells in C57BL/6, IL-18Rα$^{-/-}$ and IL-18$^{-/-}$ mice. C57BL/6, IL-18Rα$^{-/-}$ and IL-18$^{-/-}$ mice were infected with 1 x 10^6 pfu MCMV and lymphocytes isolated at indicated time points from different organs. Frequency of NKG2A, C, E+ M38+ (blue) or M45+ (red) CD8+ T cells is shown. Superimposed on graphs from IL-18Rα$^{-/-}$ and IL-18$^{-/-}$ mice are results from C57BL/6 mice (dotted lines). Data shown (mean +/- S.E.M) represents per time point 2 experiments n= 5 C57BL/6 and 1 experiment n=5 in IL-18Rα$^{-/-}$ and IL-18$^{-/-}$. * = $p<0.05$, ** = $p<0.005$, *** = $p<0.0005$ by Mann-Whitney U test.

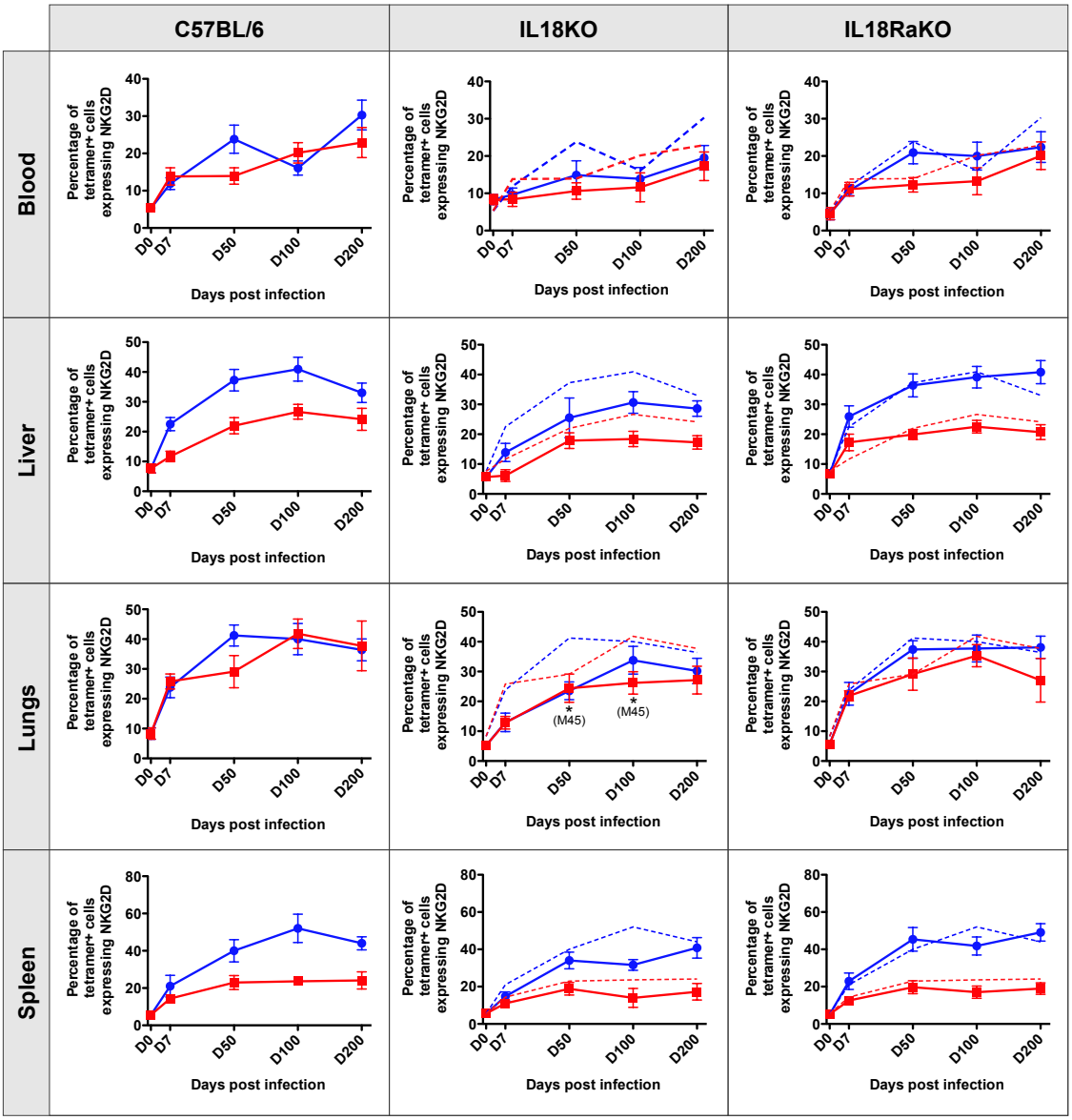

Figure 3.10 - NKG2D expression on antigen specific cells in C57BL/6, IL-18Rα$^{-/-}$ and IL-18$^{-/-}$ mice. C57BL/6, IL-18Rα$^{-/-}$ and IL-18$^{-/-}$ mice were infected with 1 x 10^6 pfu MCMV and lymphocytes isolated at indicated time points from different organs. Frequency of NKG2D+ M38+ (blue) or M45+ (red) CD8+ T cells is shown. Superimposed on graphs from IL-18Rα$^{-/-}$ and IL-18$^{-/-}$ mice are results from C57BL/6 mice (dotted lines). Data shown (mean +/- S.E.M) represents per time point 2 experiments n= 5 C57BL/6 and 1 experiment n=5 in IL-18Rα$^{-/-}$ and IL-18$^{-/-}$. * = p<0.05, ** = p<0.005, *** = p<0.0005 by Mann-Whitney U test.

KLRG1

A member of the C-type lectin-like family, killer cell lectin-like receptor G1 (KLRG1) is an inhibitory receptor expressed on certain subsets of NK and T cells[147]. Generally used as a marker of terminally differentiated effector-memory T cells, it has been shown deficiency in KLRG-1 does not affect development and function of either NK or CD8+ T cells after viral infection[148].

In MCMV infection inflating cells such as M38+ cells express high levels of KLRG-1 whereas M45+ cells express significantly lower levels[45].

Expression of KLRG-1 on MCMV-specific cells in IL-18Rα$^{-/-}$ and IL-18$^{-/-}$ knockout mice follows a similar kinetic to that seen in C57BL/6 mice. Again, the trend is towards lower levels of expression on both inflating and non-inflating cells. This is statistically significant at 50 dpi in both IL-18Rα$^{-/-}$ and in IL-18$^{-/-}$ mice in all organs suggesting a less differentiated phenotype (figure 3.11).

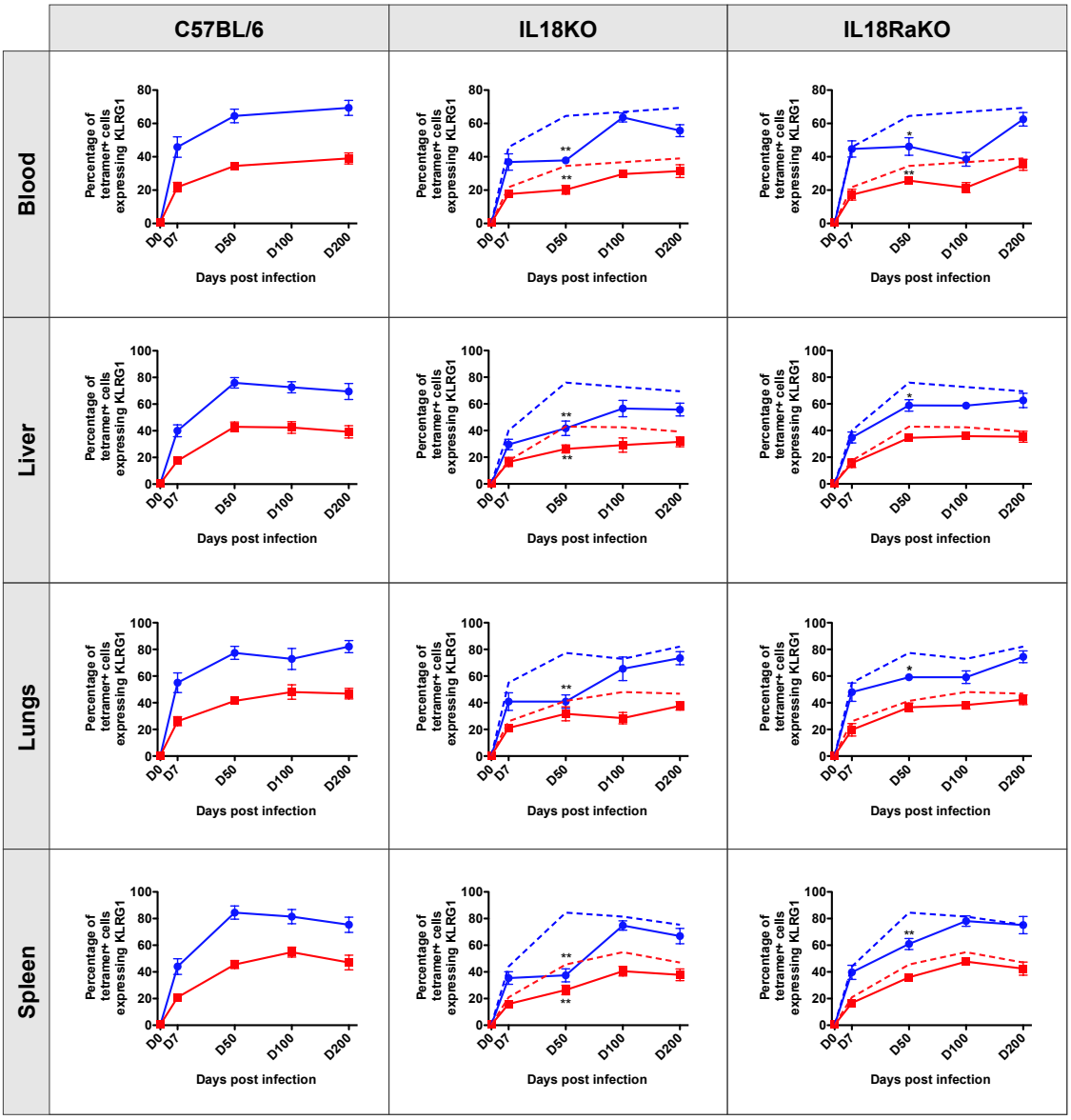

Figure 3.11 - KLRG1 expression on antigen specific cells in C57BL/6, IL-18-/- and IL-18Rα-/- mice. C57BL/6, IL-18Rα$^{-/-}$ and IL-18$^{-/-}$ mice were infected with 1 x 10^{6} pfu MCMV and lymphocytes isolated at indicated time points from different organs. Frequency of KLRG1+ M38+ (blue) or M45+ (red) CD8+ T cells is shown. Superimposed on graphs from IL-18Rα$^{-/-}$ and IL-18$^{-/-}$ mice are results from C57BL/6 mice (dotted lines). Data shown (mean +/- S.E.M) represents per time point 2 experiments n= 5 C57BL/6 and 1 experiment n=5 in IL-18Rα$^{-/-}$ and IL-18$^{-/-}$. * = p<0.05, ** = p<0.005, *** = p<0.0005 by Mann-Whitney U test.

Function of MCMV specific cells

To examine whether absence of IL-18 or IL-18Rα affected the function of MCMV-specific cells splenocytes were harvested from mice infected with 1 x 10^6 pfu MCMV acutely (7 dpi), and during latent infection (50, 100 and 200 dpi). Following 6 hours stimulation with PMA/ionomycin, M38 or M45 peptide, IFNγ production was assessed. Production of IL-2 and TNFα was examined in naïve, 7 dpi and 50 dpi mice.

Interferon gamma

There was no significant difference in IFNγ secretion detectable in naïve or knockout mice in response to PMA/ionomycin or peptide stimulation (figure 3.12 A).

IL-2

IL-2 is necessary for the growth and function of T cells – in its absence adaptive immunity is profoundly dysregulated[149]. In MCMV infection both inflating and non-inflating CD8+ T cells can produce IL-2, albeit at low levels.

In naïve and acutely infected mice IL-2 production following PMA/ionomycin stimulation was similar. However 50 days post MCMV infection both IL-18Rα$^{-/-}$ and in IL-18$^{-/-}$ knockout mice produced significantly more IL-2 in response to PMA/ionomycin. On examination of peptide-specific responses in acute infection there is no difference in IL-2 secretion by M38 responsive cells. However M45 responsive cells from mice lacking IL-18Rα or IL-18 produce more IL-2, although this is not statistically significant. By 50 dpi both M38- and M45-responsive cells produce more IL-2 in response to peptide, although again this is not statistically significant (figure 3.12 B).

TNFα

TNFα is a cytokine involved in inflammation and the acute phase response. Produced by a wide range of cells, particularly macrophages, it has diverse and wide ranging effects from acting on the hypothalamus to provoke fever to working in an autocrine fashion, enhancing

phagocytosis by macrophages[20,149]. Neutralization of endogenous TNFα in MCMV infected mice abolishes the antiviral activity of CD4+ T cells and reduces the antiviral effect of CD8+ T cells[150]. PMA/ionomycin stimulation showed no difference in TNFα secretion between splenocytes from C57BL/6 mice or either IL-18Rα$^{-/-}$ or IL-18$^{-/-}$ mice. In acute infection peptide specific TNFα secretion is of similar magnitude, however after 50 days MCMV infection both M38- and M45-specific responses are higher in IL-18Rα$^{-/-}$ and IL-18$^{-/-}$ mice, although this does not attain significance (figure 3.12 C).

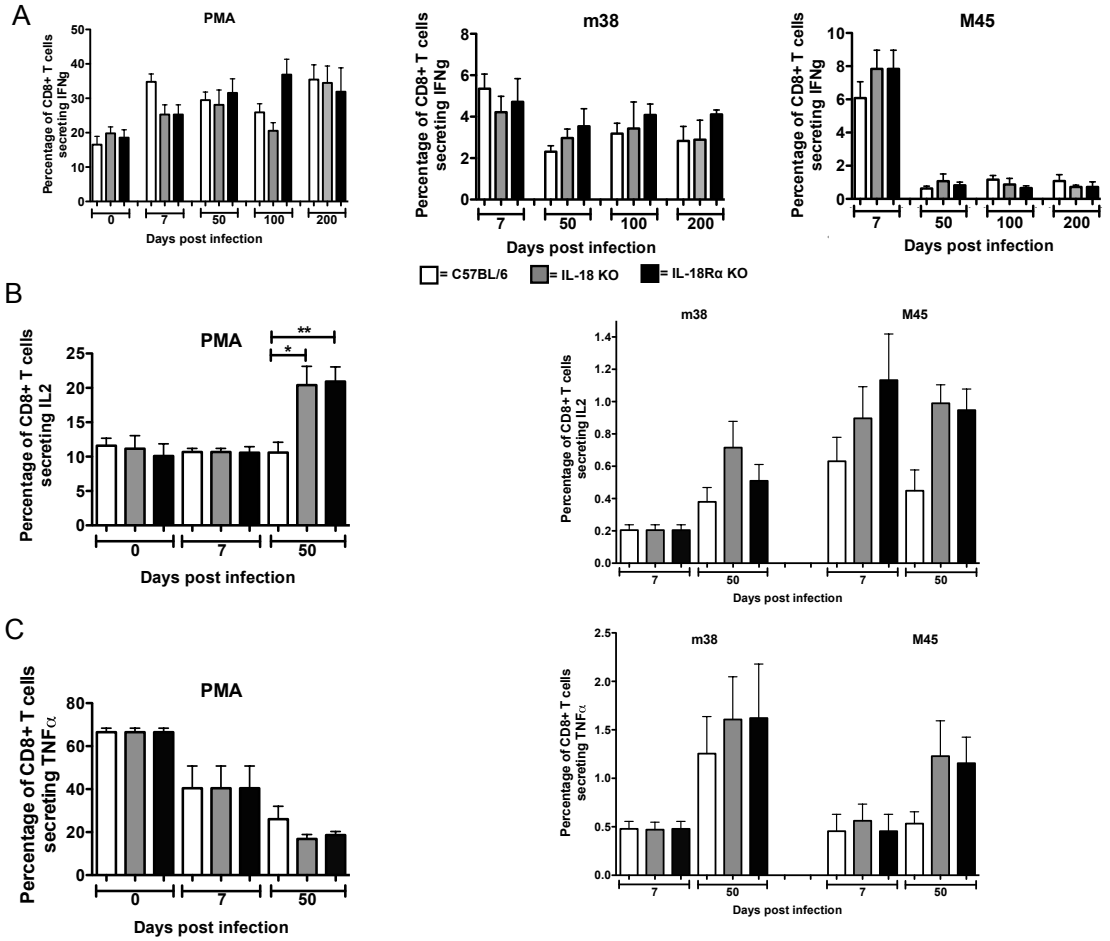

Figure 3.12 - Cytokine secretion in C57BL/6, IL-18$^{-/-}$ and IL-18Rα$^{-/-}$ mice following MCMV. C57BL/6, IL-18Rα$^{-/-}$ and IL-18$^{-/-}$ mice were infected with 1 x 10^6 pfu MCMV and splenocytes isolated at indicated time. Splenocytes were incubated with either PMA/ionomycin or M38 or M45 peptide. Frequency of CD8+ T-cells secreting **A** IFNγ **B** IL-2 **C** TNFα in response to PMA or peptide (as indicated). Data shown (mean +/- S.E.M) represents per time point 2 experiments n= 5 C57BL/6 and 1 experiment n=5 in IL-18Rα$^{-/-}$ and IL-18$^{-/-}$. * = p<0.05, ** = p<0.005, *** = p<0.0005 by Mann-Whitney U test.

Absence of IL-18 or IL-18Rα alters cell count

A striking difference was observed in cell count when processing tissues. Mice were housed in the same facility with identical diets and infected with the same production lot of MCMV. At 7 dpi leucocyte counts in the tissues of both IL-18Rα$^{-/-}$ and IL-18$^{-/-}$ mice were significantly higher than leucocyte counts from C57BL/6 mice. The mean number of leucocytes in the spleen of IL-18Rα$^{-/-}$ mice was approximately 4 times higher than cell counts in C57BL/6 mice (279.0 x 10^6 +/- 6.49 compared to 68.14 x 10^6 +/- 28.91 [mean+/-S.E.M]. This pattern, also observed in livers and lungs during acute infection, was absent 50 days post MCMV infection (figure 3.13 A). No difference in cell count was observed in naïve C57BL/6 mice or IL-18Rα$^{-/-}$ and IL-18$^{-/-}$ mice (data not shown).

To further explore this phenomenon a panel of surface markers was developed to elicit which leucocyte subsets, if any, were expanded in IL-18Rα$^{-/-}$ and IL-18$^{-/-}$ mice. Surprisingly no particular group – T cells, granulocytes, macrophages, NK cells or dendritic cells - occurred at a significantly higher frequency (figure 3.13 B). Obviously various unmeasured subsets such as gamma delta T cells or NK T-cells may be the contributing element. Our findings are disparate to other groups examining the role of IL-18 or IL-18Rα in infectious challenge models where cells counts during acute infection are not altered in IL-18 or IL-18Rα mice infected with either *Mycobacterium tuberculosis*, *Listeria monocytogenes* or *Salmonella typhimurium*[132,151,152].

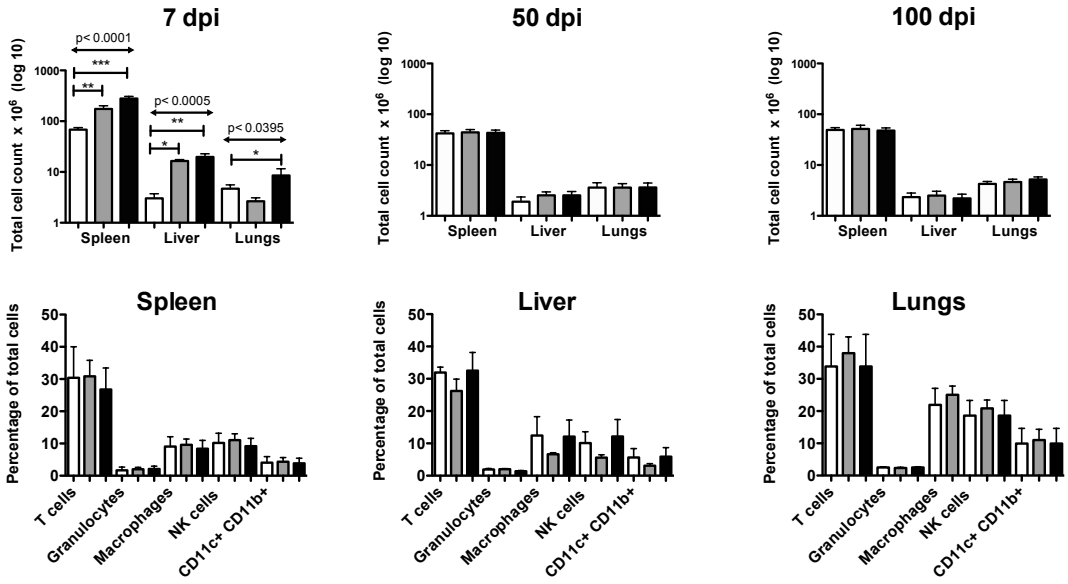

Figure 3.13 - Cell counts in C57BL/6, IL-18$^{-/-}$ and IL-18Rα$^{-/-}$ mice following MCMV infection. C57BL/6, IL-18Rα$^{-/-}$ and IL-18$^{-/-}$ mice were infected with 1 x 10^6 pfu MCMV. **A** After processing leucocyte counts from organs were estimated using either trypan blue and a haemocytometer or a Countess® Automated Cell Counter at time points indicated. Data shown (mean +/- S.E.M) represents (per time point) 6 experiments n= 5 C57BL/6 and 2 experiments n=5 IL-18Rα$^{-/-}$ and IL-18$^{-/-}$. **B** 7 days after MCMV infection organs were harvested and cellular subsets (T cells, granulocytes, macrophages, NK cells and dendritic cells) examined using flow cytometry. Data shown represents 1 experiment n= 6.

IL-18Rα deficient mice do not control MCMV viremia

In order to establish whether viral genome is controlled in mice lacking IL-18 or IL-18Rα, we performed quantitative realtime PCR. MCMV DNA copy numbers per microgram total DNA were assessed in spleen, liver, and lung at different time points after infection with MCMV.

IL-18Rα$^{-/-}$ mice fail to control MCMV viremia, with viral load in the spleen increasing 7 dpi from an average of 17 700 (+/- 3667) copies per μg DNA to over 400 000 (+/- 60 000) copies 50 dpi and remain elevated at 100 dpi. In both the liver and lung of IL-18Rα$^{-/-}$ mice viral load also increases from 7 dpi in striking comparison to IL-18$^{-/-}$ where MCMV viral load kinetic mirrors those seen in C57BL/6 mice (figure 3.14 A). IL-18Rα$^{-/-}$ mice did not display any mortality or indeed any signs or symptoms of illness.

It is also important to note viral loads are quantified using per μg of DNA however at day 7 the vastly different cell counts in both IL-18Rα$^{-/-}$ and IL-18$^{-/-}$ were not taken into account in this analysis.

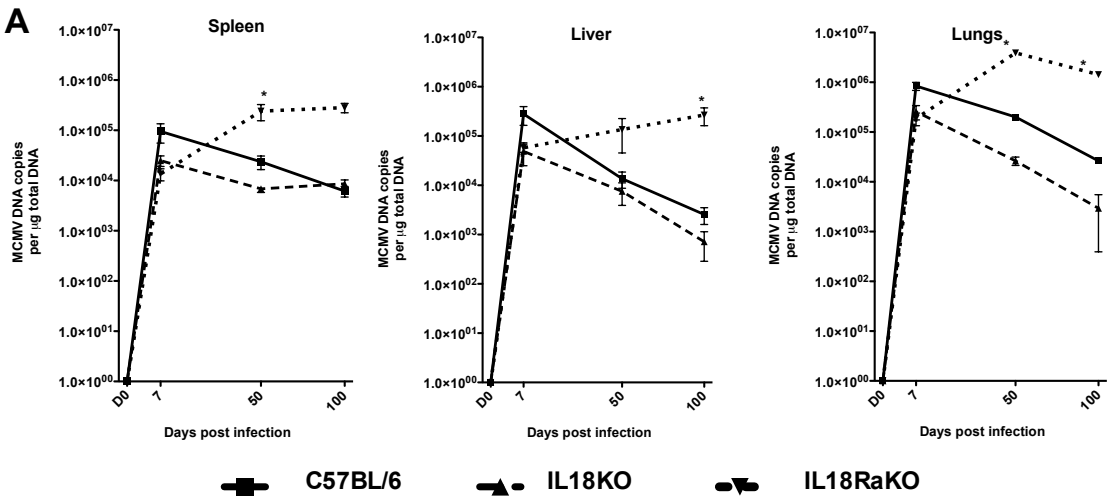

Figure 3.14 - MCMV viral loads in C57BL/6, IL-18$^{-/-}$ and IL-18Rα$^{-/-}$ mice. C57BL/6, IL-18Rα-/- and IL-18-/- mice were infected with 1 x 10^6 pfu MCMV. **A** Viral genome distribution after infection with MCMV. MCMV DNA copy numbers per µg total DNA were determined with quantitative realtime PCR at time points indicated after immunization in liver, lung and spleen. Data shown represents 3 mice per time point for each mouse strain. * = $p<0.05$, ** = $p<0.005$, *** = $p<0.0005$ by Mann-Whitney U test.

IL-18Rα expression on systemic CD8+ T cells is not influenced by the introduction of gut flora

The colon is home to an extremely dense bacterial microflora that can reach densities of up to 10^{12} bacteria per gram. Colonization of this rich habitat with a commensal bacterial microbiota starts immediately at birth and continues through childhood until a stable commensal flora is established[153]. Intestinal bacteria are beneficial to their host, providing colonization resistance for pathogens and stimulating the mucosal and systemic immune systems to mature[154]. The concept of manipulating gut flora began in the mid-1960s when researchers began to colonize germ free (GF) mice with selected microbes from "standard" (pre SPF) mice[155]. Bacteria included in these first studies were selected on their ease of growth and non-pathogenicity. In 1978 this "cocktail" was improved and strengthened and was given the name Altered Schaedler Flora (ASF), consisting of Lactobacillus acidophilus, Lactobacillus salivarius, Bacteroides distasonis, a spiral-shaped bacterium, and three extremely oxygen-sensitive (EOS) fusiform bacterium[156]. In GF mice, invariant natural killer T cells accumulate in the colonic lamina propria and lung, resulting in increased morbidity in models of inflammatory bowel disease and allergic asthma as compared with that of specific pathogen-free mice. Colonization of neonatal GF mice with conventional microbiota protected the animals from mucosal iNKT accumulation and subsequent pathology illustrating that exposure to gut flora can drive systemic immune effects[157].

In view of the presence of IL-18Rα expressing CD8+ T cells in naïve mice we hypothesized that IL-18Rα on T cells is a marker of antigen experience. As such we explored whether exposure to gut flora could drive this phenomenon. In a germ free mouse facility three separate groups of mice were examined; (i) raised entirely in a germ free environment (Germ Free or GF), (ii) raised in a germ free environment until 6 weeks then colonized with Altered Schaedlers Flora (ASF colonised), and (iii) colonized with ASF from birth (ASF birth).

Mice under went tail bleeds at day 0, day 21 and on day 42 mice were sacrificed and cells extracted from peripheral blood, lungs and spleen. Samples were examined for expression of IL-18Rα together with a panel of markers examining phenotype (CD44, CD62L, Ki67 and KLRG-1).

Table 3.3 Frequency of IL-18Rα on CD4 and CD8 T cells originating in mice raised in an SPF environment (Oxford) or a germ free environment (Switzerland). Data shown is arithmetic mean +/- standard error of the mean. Represents 5 experiments (n=25 Oxford) and 1 (n=4 Switzerland). p values derived using Mann Whitney U test.

Organ	SPF mice (Oxford)		Germ Free Mice (Switzerland)			
	CD4+	CD8+	CD4+	CD8+	CD4+	CD8+
Blood	6.63 ±0.67	3.95 ±0.31	6.49 ±1.89	6.69 ±1.3	ns	ns
Lungs	8.17 ±0.41	3.55 ±0.11	13.9 ±1.3	18.93 ±1.60	p = 0.0294	p = 0.004
Spleen	7.53 ±0.8	6.27 ±0.61	7.36 ±0.76	8.38 ±0.86	ns	ns

Similarly to naïve C57BL/6 mice in our facility, both CD4+ and CD8+ T cells in germ free mice express IL-18Rα. There are no significant differences detectable in IL18Rα expression in blood and spleen. In the lung tissue of germ free mice both CD4+ and CD8+ T cells display significantly more IL-18Rα than naïve mice housed in the specified pathogen free (SPF) conditions provided at Oxford suggesting perhaps mucosal immune surfaces are more affected by changes in gut flora.

Colonizing mice with ASF bacteria results in increased expression of IL-18Rα on CD8+ T-cells in blood. By 42 days post colonization CD8+ T cells in ASF mice expressed significantly higher levels of IL-18Rα than mice raised with ASF since birth ($p < 0.05$ for by Kruskal

Wallis) (Figures 3.15 A and B). Levels of IL-18Rα on CD4+ T-cells augmented in a similar fashion but this was not significant.

On examination of CD8+ T cells in the lung, there is a non-significant trend to higher IL-18Rα expression in the ASF birth group. In the lungs of germ free and ASF colonized mice CD8+ T cells express similar level of IL-18Rα. CD4+ T-cells in the lungs and spleens of ASF colonized and ASF birth mice express higher levels of IL-18Rα than naïve mice (figure 3.15 A). Overall exposure to gut flora appears to augment IL-18Rα expression upon CD4+ and CD8+ T cells most markedly in the lung, although results in the ASF colonized group were variable, suggesting 42 days may be too early to see clear differences on T cells.

To examine whether IL-18Rα+ cells were proliferating effector cells CD8+IL-18Rα+ T cells were analyzed for expression of the surface markers CD44, CD62L and KLRG-1. In addition cells were stained intracellularly for the proliferation marker Ki67. IL-18Rα+ CD8+ T cells were a mixed central memory (CD44+CD62L+) and naïve (CD44- CD62L+) phenotype and did not vary when ASF were introduced; this is similar to naïve CD8+IL18Rα+ from SPF mice (figure 3.15 C, figure 3.3 A). Exposure to ASF did not cause proliferation of CD8+ T cells as measured by an increase in Ki67 staining. ASF colonized mice did display an increase in KLRG-1 expression on CD8+IL-18Rα+ in spleen, lung and blood suggesting they may have a more differentiated effector phenotype (figure 3.15 C and D).

Broadly colonization with gut bacteria did not induce a clear proliferating effector memory subset of IL-18Rα+CD8+ T cells. However it may be that by 42 days after colonization cells exposed to gut flora have lost their effector phenotype, whilst increased KLRG-1 expression indicates a more differentiated phenotype.

At day 42 splenocytes were stimulated with PMA/ionomycin to assess production of the cytokines IFNγ, IL-2 and TNFα. Surprisingly I found CD8+ T cells from germ free mice produced significantly less of all three cytokines than CD8+ T-cells from ASF colonized or

ASF birth mice (figure 3.16 A). In all conditions i.e. germ free, ASF colonized or ASF birth approximately 1/3 of IFNγ secreting cells expressed IL-18Rα. Notably CD8+IL-2+ T-cells in ASF colonized and ASF born mice expressed higher levels of IL-18Rα (reaching statistical significance in ASF colonized mice $p < 0.05$ by Kruskal Wallis). A similar pattern was observed in TNFα secreting CD8+ cells (figure 3.16 B).

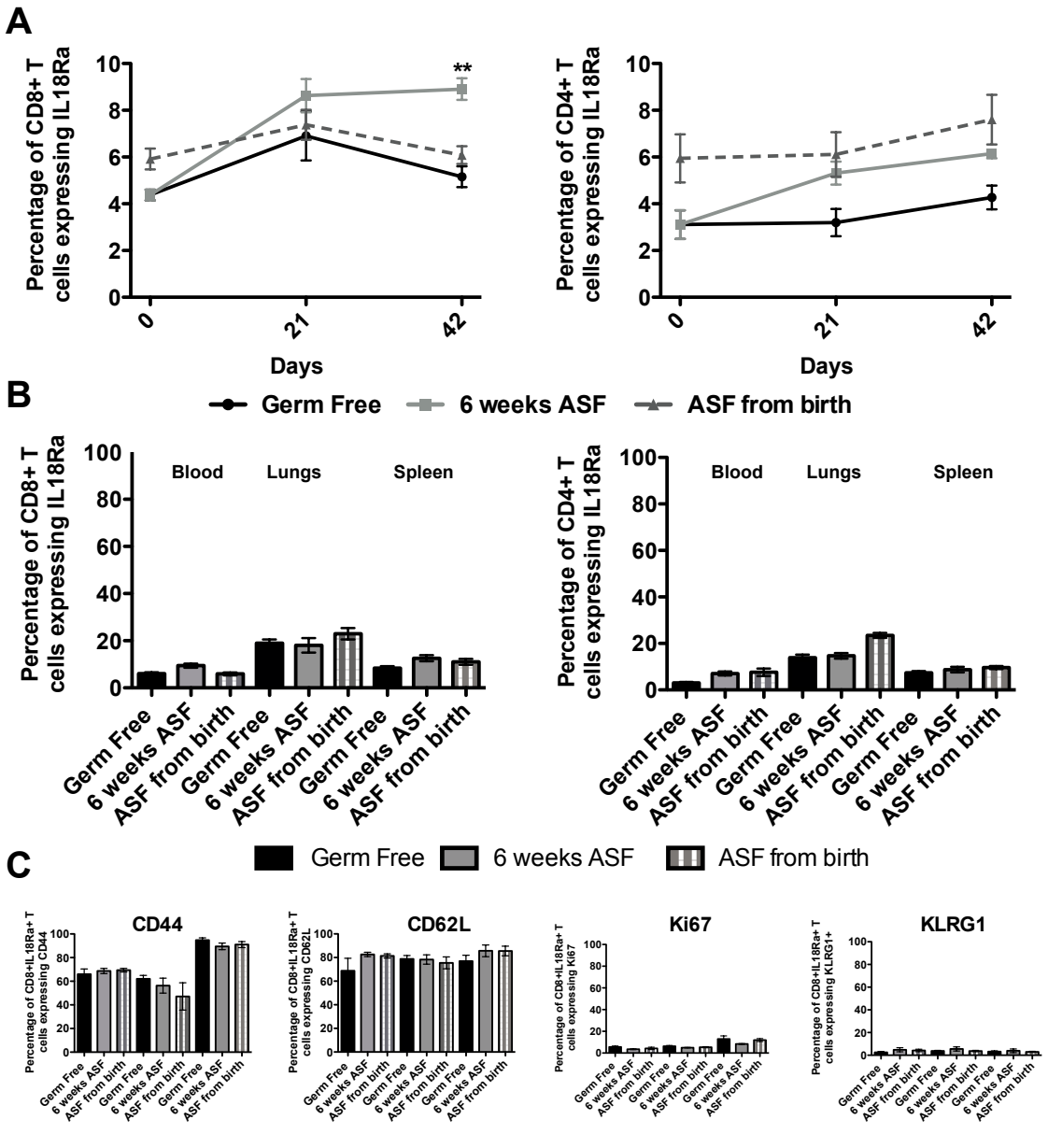

Figure 3.15 - IL18Rα expression on systemic CD8+ T cells is not influenced by the introduction of gut flora. C57BL/6 mice aged 4 weeks (n=4 per group) were raised in an entirely germ free facility, exposed to Altered Schadlers Flora for 6 weeks or exposed from birth with ASF. **A** Serial tail bleed were performed to assess IL-18Rα expression on CD8+ or CD4+ T-cells. **B** After 6 weeks exposure to ASF CD8+ and CD4+ T-cells in organs were examined for levels of IL-18Rα. **C** CD8+IL-18Rα+ T cells in organs were examined for levels of CD44, CD62L, KLRG1 and Ki67. Data shown (mean +/- S.E.M) represents 1 experiment n= 4 per group.

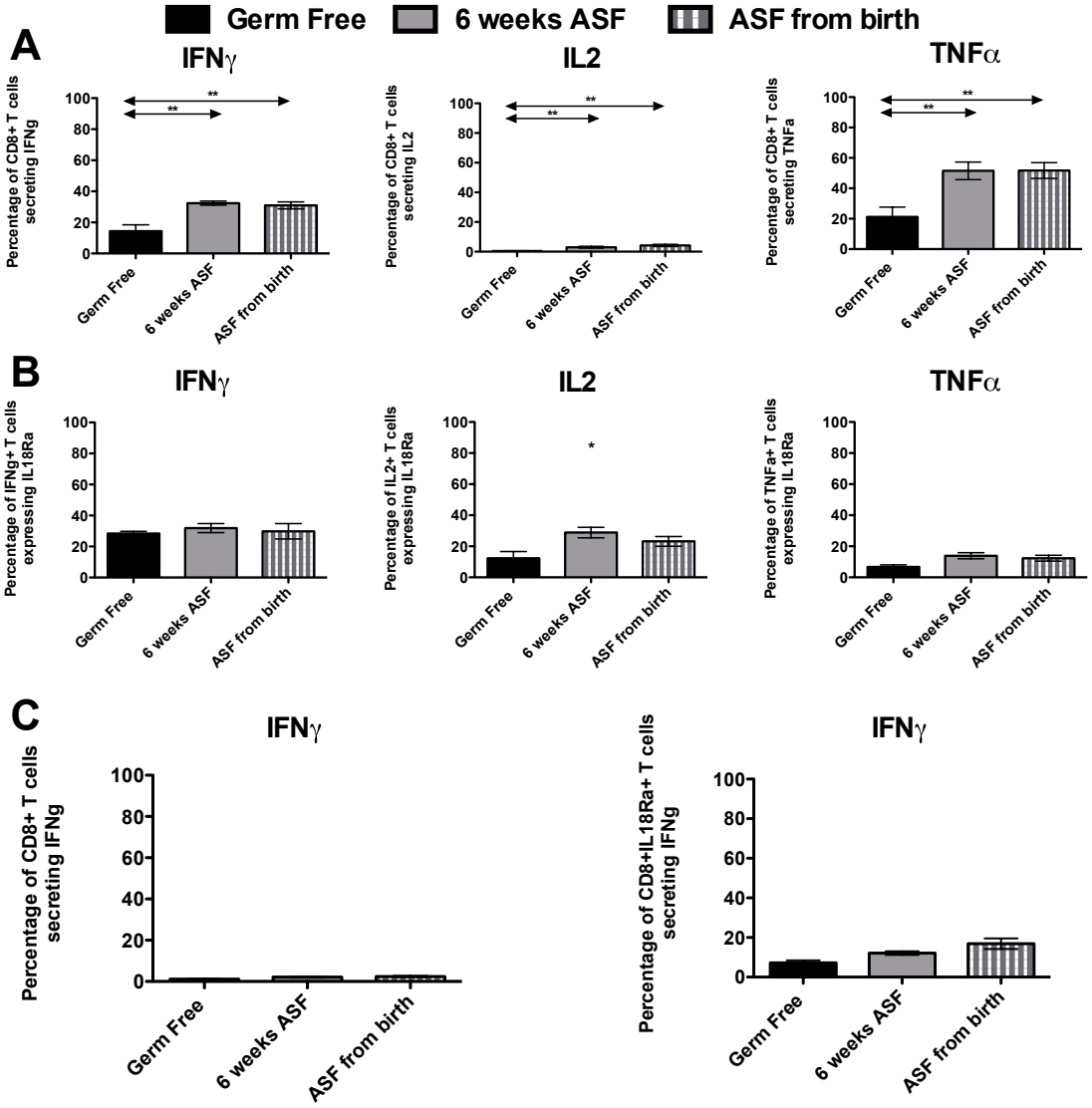

Figure 3.16 - Secretion of cytokines is increased by the introduction of gut flora. C57BL/6 mice aged 4 weeks (n=4 per group) were raised in an entirely germ free facility, exposed to Altered Schadlers Flora for 6 weeks or exposed from birth with ASF. After 6 weeks splenocytes were harvested and stimulated with PMA/ionomycin and **A** the frequency of IFNγ, IL-2 and TNFα producing CD8+ cells compared. **B** Cytokine+ cells were examined for IL-18Rα expression. Data shown (mean +/- S.E.M) represents 1 experiment n= 4 per group. * = p<0.05, ** = p<0.005, *** = p<0.0005 by Kruskal Wallis.

Discussion

In this chapter it is obvious that IL-18 is dispensable for the development of memory inflating T cells in MCMV. M38+ CD8+T cells in both IL-18$^{-/-}$ and IL-18Rα$^{-/-}$ develop at an indistinguishable frequency to M38+ T-cells in C57BL/6 mice. Previously published work examining the role of IL-18 in infectious models where classical memory develops show IL-18 and IL-18Rα are dispensable for classical memory formation and our data on M45+ CD8+ T-cells supports this[132,133]. Notably IL-18Rα-/- mice fail to control MCMV viremia although they do not display any morbidity or mortality as a result. Currently published data in the *Salmonella typhimurium* model suggests IL18$^{-/-}$ knockout mice display higher bacterial counts than IL-18Rα$^{-/-}$ (although IL-18Rα$^{-/-}$ display higher bacterial counts than C57BL/6 mice). Similarly in a *Haemophilus influenza*e infection model IL18$^{-/-}$ deficient mice display higher lung pulmonary cfu compared to C57BL/6 mice[152,158]. No murine studies have been carried out examining the role of IL-18 or IL-18Rα in persistent infections.

Differences in control of MCMV viral load

A possible explanation for such a marked discrepancy between IL-18$^{-/-}$ and IL-18Rα$^{-/-}$ mice is the potential promiscuity of the IL-18Rα receptor. Discrepancies are reported in the behavior IL-18 and IL-18Rα deficient mice and cells. IL-18 deficient mice are susceptible to experimental autoimmune encephalomyelitis (EAE), a Th-17-mediated mouse model of multiple sclerosis, whereas IL-18Rα deficient mice are resistant, suggesting involvement of an IL-18Rα ligand other than IL-18[159]. Conversely in a mouse model of diabetes IL-18 deficient pancreatic islet cells survived longer and gave better glycemic control than IL-18Rα deficient mice[160]. Data from our work supports the notion that IL-18Rα may respond to another member of the IL-1 family of cytokines.

NK cells play a key role in the early immune response to MCMV in C57BL/6 mice where the Ly49H on NK cells receptor is activated by binding the MCMV-encoded ligand m157 and the

magnitude of CD8+ T-cell response required is directly affected by the amount of Ly49H on NK cells[58,161]. Absence of IL-18Rα on NK cells may affect their functional capacity.

IL-18 and IL-18Rα in memory inflation

Memory inflating cells have a typical phenotype showing signs of activation, such as low expression of CD62L, CD127, and CD27 and CD28, together with expression of terminal differentiation, such as expression of high levels of KLRG1, NKG2D and NKG2A, C, E and reduced secretion of IL-2[42,60,61]. Broadly speaking inflating M38+ and non-inflating M45+ cells in IL-18 and IL-18Rα deficient mice expressed the typical phenotypic marker of inflating cells. There are some subtle differences and occasionally these appear statistically significant but in view of the volume of data this is more likely to represent a type I error. However both IL-18 and IL-18Rα deficient mice showed slower acquisition of KLRG1 on both M45 and M38 positive cells – this is of uncertain significance as KLRG1 has been shown to be dispensable in varicella zoster virus (VZV), LCMV and MCMV[148].

In MCMV both IL-18 and IL-18Rα are dispensable for the generation of both primary and memory inflating immune responses. However the magnitude of primary immune responses – as calculated by the elevated total leucocyte count in all compartments - has not been reported previously. In LCMV, MyD88 (and as such IL-18 and IL-18Rα) are not required to generate effective secondary immune responses.

Rapid proliferation in response to cognate antigen is a definitive characteristic of memory CD8+ T cells and ensures rapid clearance of reinfecting pathogens. I did not assess the protective quality of antigen-specific memory in MCMV infected IL-18 or IL-18Rα deficient mice so whilst appearing indistinguishable from MCMV-specific cells in C57BL/6 mice there remains a possibility at secondary challenge that differences may occur. Recently it has been shown in vivo that memory T cells encountering cognate antigen presented by DC's show an accumulation of IL-18 at the DC:T cell synapse and mice deficient for IL-18 showed delayed

expansion of memory CD8+ T cells in vivo[162]. Use of M38 tetramers conjugated to the ribosome-inhibiting protein saporin which causes death of M38-specific cells when internalised has elucidated that latent MCMV in C57BL/6 mice is partly controlled by M38-specific cells (Sims et al unpublished observation). It can be postulated that MCMV viral loads in IL-18$^{-/-}$ mice would not have been as well controlled if such a DC:T cell interaction was key.

IL-18 is recognised to induce the production of IFNγ and to enhance Th1 responses[163]. In chronically MCMV infected IL-18$^{-/-}$ and IL-18Rα$^{-/-}$ mice I do not observe any differences in IFNγ secretion but do see significantly higher levels of IL-2 produced in response to PMA/ionomycin stimulation. Also, peptide-specific responses generated higher levels of IL-2, although this was not significant. In IL-18Rα$^{-/-}$ knockouts higher levels of virus and subsequent proliferation of T-cells may underlie increased levels of IL-2 production. However IL-18$^{-/-}$ mice display similar IL-2 production and control MCMV.

During acute infection total cell counts for both IL18$^{-/-}$ and IL-18Rα$^{-/-}$ are significantly raised although I was unable to attribute this to expansion of a specific subset. This has not been reported before and is worthy of consideration.

Gut flora does not drive acquisition of IL-18Rα on CD8+ T cells

Germ free mice display levels of IL-18Rα expression on T cells indistinguishable to C57BL/6 mice from our SPF facility suggesting either exposure to self or food may be sufficient to drive acquisition of IL-18Rα. High expression of CD44 suggests these cells are antigen experienced rather than naïve. We observed a small increase in IL-18Rα expression on T cells. This may be linked to the fact that dendritic cells loaded with commensal bacteria do not penetrate further than the draining mesenteric lymph nodes, thus protective mucosal immunity to commensal organisms is maintained separately from systemic immunity[154]. In

this scenario gut flora drives a local expansion of IL-18Rα+ T cells and examination of either the lamina propria or mesenteric lymph nodes would be more appropriate.

Conclusions

- A subset of antigen experienced CD8+ T cells in naïve mice express IL-18Rα.
- MCMV infection induces significantly high levels of IL-18Rα expression on MCMV-specific CD8+ T cells.
- Neither IL-18 nor IL-18Rα appear to be required for successful generation of memory inflating T cells.
- The phenotype and function of MCMV specific cells in IL-18 and IL-18Rα deficient mice is broadly the same as in C57BL/6 mice.
- In IL-18 and IL-18Rα deficient mice cell counts in all organs are significantly higher than in C57BL/6 mice during acute infection.
- IL-18Rα deficient mice fail to control MCMV infection with no deleterious side effects. The observation that IL-18 deficient mice control infection normally supports the hypothesis that IL-18Rα is a promiscuous receptor, although the mechanism of the failure of control requires further analysis.
- Gut flora may partially drive IL-18Rα expression upon CD8+ T cells although more work with varying lengths of exposure is required.

Chapter 4

Non-cognate functions of MCMV-specific T cells

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Introduction

The innate immune system consists of the cells and mechanisms that defend the host from infection by other organisms in a non-specific manner. This means that the cells of the innate system recognize and respond to pathogens in a generic way, but unlike the adaptive immune system, it does not confer long-lasting or protective immunity to the host. Innate immunity includes as an important component cytokines such as IL-12 and IL-18, which recruit cells from the innate and adaptive immune system to sites of infection and inflammation as well as directing their function. Exposure to a wide variety of pathogens (viruses, bacteria, parasites, and fungi) can trigger IL-12 and IL-18 production by antigen presenting cells such as macrophages and dendritic cells[164-167]. Indeed active microbial infection is not necessary to initiate cytokine synthesis with microbial products such as lipopolysaccharide, peptidoglycan, unmethylated CpG DNA, or Leishmania major LeIF protein also capable of inducing IL-12 and IL-18 production in vitro.

For many intracellular pathogens (such as *Listeria monocytogenes*, *Salmonella typhimurium*, *Toxoplasma gondii*, *Yersinia enterocolitica*, *Mycobacterium leprae*, and *Cryptococcus neoformans*,) the rapid production of IFNγ by NK cells plays an important role in early host resistance to infection[152,168-171]. IFNγ production by NK cells can be triggered by combinations of cytokines including IL-12 and IL-18 and in many bacterial infection models this is clearly dependent on IL-12 and IL-18[172]. The importance of IL-12 and IL-18 in the immune response is underlined by the fact human herpesvirus 6 selectively reduces IL-12 production and both poxviruses and papillomaviruses encode IL-18-binding proteins that greatly suppress IL-18 activity[173-175].

Splenocytes from naïve mice incubated in vitro with either *Burkholderia pseudomallei* or *Listeria monocytogenes* (L.M) secrete IFNγ and examination of IFNγ+ cells reveals a significant proportion of these cells are CD8+CD44+ T cells (5% IFNγ+ cells are CD8+ compared with 12% expressing NK1.1)[176,177]. Similarly CD8+ T cells isolated from the

spleens of naïve C57BL/6 mice respond when cocultured in vitro with *Listeria monocytogenes* infected macrophages by secreting IFNγ, this response is abrogated when IL-12 and IL-18 are blocked and responding T cells are identified as CD44+[177]. Taken together these data suggest CD44+CD8+ T cells are capable of secreting IFNγ in response to cytokines without TCR engagement. Further work in the *Listeria monocytogenes* model adoptively transferring memory OT-1 ova-specific CD8+ T cells (these cells express a TCR specific for the SIINFEKL peptide of ovalbumin) into IFNγ$^{-/-}$ mice shows these cells can mediate protection against challenge with *Listeria monocytogenes* expressing ovalbumin[178].

In BALB/c mice 8 days post LCMV Armstrong infection 70–80% of NP118 specific CD8+ T cells produce IFNγ after stimulation with IL-12 and IL-18. Cytokine mediated IFNγ secretion continues be exhibited by NP118+ CD8+ T cells 500 days after infection although it is of lower magnitude (74.4% at 8 dpi, 20% NP-118 at 500 dpi). At the peak of infection NP118 specific cells can produce IFNγ in response to IL-12 and IL-18 individually but by 500 dpi memory NP118-specific T cells cannot respond to IL-18 alone, even at high cytokine concentrations instead requiring IL-12 to decrease the threshold of T cell activation[179]. Similarly to MCMV-specific cells NP118-specific CD8+ T cells express high levels of IL-18Rα.

Recent work has shown a direct link between flagellin-dependent NLRC4 inflammasome activation and protective IFNγ production by noncognate memory CD8+ T cells. Flagellin derived from *Salmonella typhimurium* activated NLRC4 inflammasomes in CD8α+ DCs. Inflammasome activation resulted in IL-18 secretion and IFNγ secretion by memory T cells[180]. Similarly NLRC4 activation by flagellin offers protection against *Legionella pneumophila*[181].

Murine gammaherpesvirus 68 (γHV-68) is a member of the γ herpesvirus family, a large family of DNA viruses characterized by their ability to produce persistent infections[182]. Intranasal infection of mice with γHV-68 causes an acute respiratory infection that is rapidly resolved, followed by the establishment of a lifelong latent infection in memory B cells,

macrophages and dendritic cells[183,184]. Unlike the β herpesvirus MCMV ϒHV-68 does not induce an inflating population of CD8+ T cells and it appears CD4+ T cells play a dominant role in long term virus control[183,184].

Mice infected with the herpesvirus murine gammaherpes virus 68 (γHV-68) are transiently resistant to infection with the bacterial pathogens *Listeria monocytogenes* and *Yersinia pestis*. Acutely (7 days post infection) no protection is observed but after 28 days mice challenged with either *Listeria* or *Yersinia* have significantly better survival than uninfected C57BL/6 mice. However by 84 days after γHV-68 infection this protection has waned[185,186].

The sera of mice infected with γHV-68 exhibit significantly higher levels of both IFNγ and TNFα than uninfected mice at 28 and 41 days post infection i.e. during latency. The authors theorize that increased levels of IFNγ in latent γHV-68 infection causes prolonged activation of macrophages as illustrated by cytoplasmic vacuolization, membrane ruffling, increased size and up regulation of surface major histocompatibility complex (MHC) class II molecules and thus enhanced bacterial clearance[185]. Of note in γHV68 latently infected mice depletion of CD4 and CD8+ T cells does not abrogate protection from bacterial challenge. During acute infection IFNγ levels would be expected to be high but macrophages in acute γHV-68 infection do not appear to be protective despite being activated.

Mice 28 days post infection with MCMV show reduced bacterial counts when challenged with *Listeria monocytogenes* but no protection is seen in mice infected with herpes simplex virus-1 (HSV-1) suggesting persistent viral infection is necessary but one which is anatomically localized such as HSV-1 in neural ganglia is insufficient[185].

It is clear memory CD8+ T cells induced by bacterial and viral infections are capable of responding to cytokines by secreting IFNγ and this phenomenon is entirely TCR independent[176,177,179]. Cytokine responsive memory CD8+ T cells can mediate protection against infection[178]. γ and β herpesviruses can provide short term protection against

bacterial challenge and this is believed to be linked to heightened immune activation due to latent viral infections[187]. I hypothesised MCMV induced memory T cells are responsive to IL-12 and IL-18 and can mediate protection against bacterial challenge.

Aims

I aimed to examine

- Does MCMV induces memory T cells responsive to IL-12 and IL-18?
- What is the phenotype of IL-12 and IL-18 responsive cells?
- Do IL-12 and IL-18 induce production of cytokines other than IFNγ?
- Is cytokine responsiveness is MCMV-specific?
- Does exposure to IL-18 and IL-12 cause up regulation of IL18Rα?
- How rapidly CD8+ T cells respond to IL-12 and IL-18?
- If MCMV induces cytokine responsive cells, could these be responsible for protection against bacterial challenge?

Results

IL-12 and IL-18 together trigger production of IFNγ

Initially I sought to elicit whether in addition to responding to peptide antigen, CD8+ T cells in MCMV maintain an innate capacity to be activated and produce IFNγ in response to cytokines elicited during secondary infection[57,188]. In acute LCMV Armstrong infection NP118-specific cells secrete IFNγ in response to stimulation with IL-12 and IL-18 individually and when admixed; by 100 dpi IFNγ production in response to IL-18 alone is absent but remains present (although at lower magnitude) to IL-12 alone and IL-12/IL-18 mixture[188].

Splenocytes from naïve mice and mice 50 dpi with 1 x 10^6 pfu MCMV were stimulated with 5, 50, 500 and 1000ng/mL IL-12 and IL-18 and a mixture of IL-12 and IL-18 at 10ng/mL for 6 hours and IFNγ production assessed. In naïve C57BL/6 mice CD4+ and CD8+ T cells and CD3- cells produced IFNγ in response to IL-12/IL-18 at 10ng/mL (1.5+/-0.3%, 1.8+/-0.7% and 3.9%+/-0.4% [mean+/-S.E.M] respectively). The frequency of cells responding to IL-18 alone was uniformly low and only a low frequency of CD4+ and CD8+ T cells responded to IL-12 alone, however CD3- cells notably responded to IL-12 in a dose dependent fashion (figure 4.1 A and B).

50 days after MCMV infection frequencies of CD3- cells and CD4+ T cells responding to IL-12 and IL-18 10ng/mL remained similar to those found in naïve mice (3.2+/-0.6% and 2.2+/-0.3% [mean +/- S.E.M] respectively). The frequency of CD8+ T cells responding to IL-12 and IL-18 mixture (10ng/mL) is notably higher in MCMV-infected mice compared to naïve mice (7.8 +/-1.4%) and indeed higher than the frequency of M38 and M45 peptide responsive cells in the same experiments. In MCMV infected mice responses to single cytokine stimulation are of a similarly low magnitude to naïve mice.

Overall therefore the impact of MCMV was an increase in IL-12/IL-18 responsiveness of CD8+ T cells in spleen of approximately 4-5 fold (7.8% vs. 1.8% $p < 0.005$ Mann Whitney U test). At the same time there was no change in the cytokine responsiveness of CD3- populations (3.3% vs 3.1%, p=n.s, Mann Whitney U test).

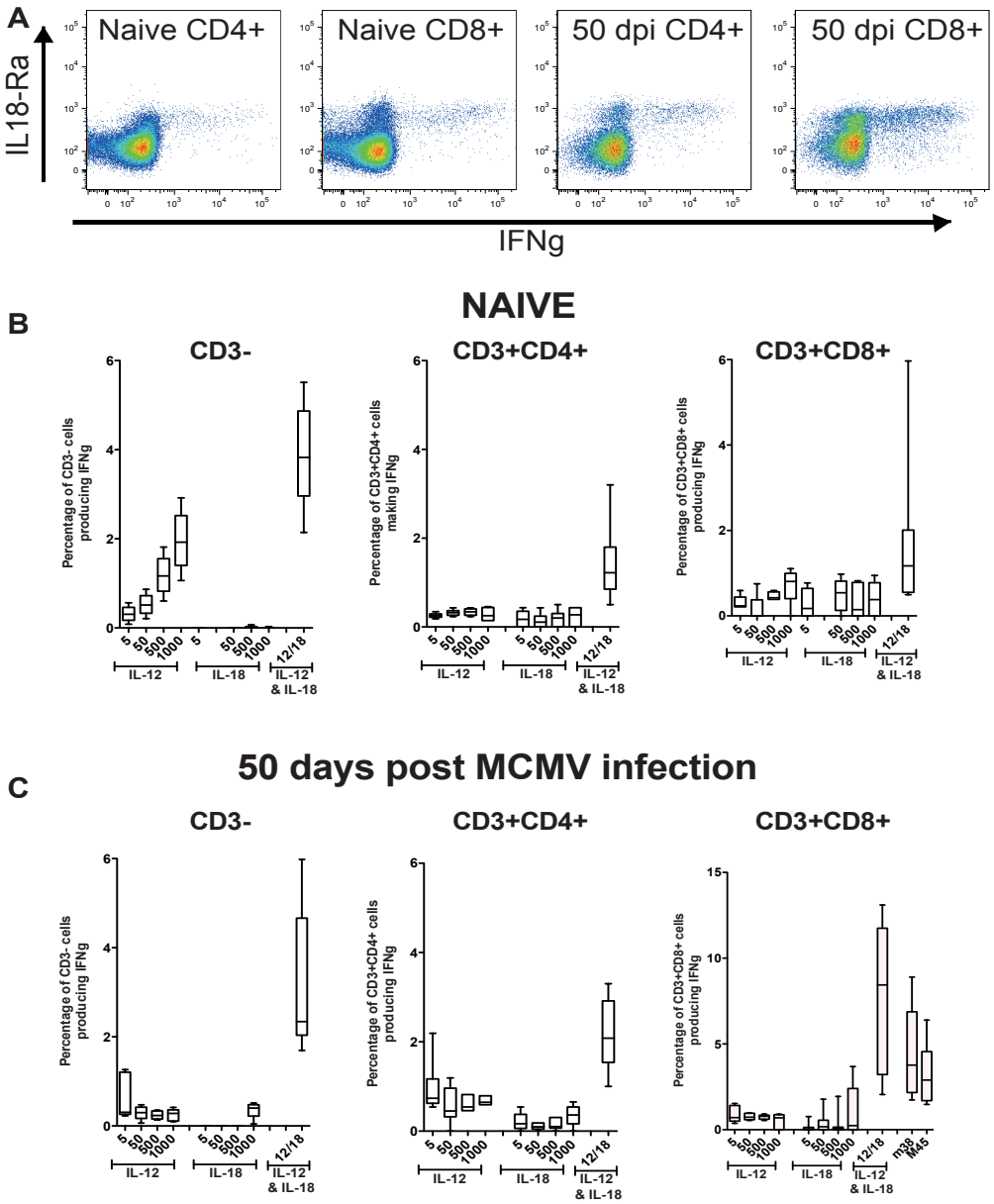

Figure 4.1 – CD8+ T cells respond to cytokine stimulation by making interferon gamma (IFNγ). Lymphocytes were isolated from spleens and stimulated with different concentrations of IL-12 and IL-18 alone or together for 6 hours (Brefeldin A was added after 2 hours). IFNγ production was measured. **A** Flow cytometry plots displaying IFNϒ production by CD4+ and CD8+ T cells from the spleens of naïve mice or mice 50 dpi 1 x 10^6 pfu MCMV. Percentage of cells producing IFNγ in response to 5, 50, 500 or 1000 ng/mL of IL-12 alone, IL-18 alone or 10ng/mL IL-18 and IL-12 together (background subtracted). Shown are CD3- cells, CD3+CD4+ and CD3+CD8+ **B** from naïve spleens **C** spleens 50 dpi MCMV. Box and whisker plots showing mean and 10 – 90th percentiles. Data shown in **B** and **C** represents 1 experiment n=5 per group.

MCMV infection significantly increases the frequency of cytokine responsive T cells

In order to examine the kinetics of IL-12 and IL-18 responsive cells in MCMV infection C57BL/6 mice were infected intravenously with 1 x 10^6 pfu MCMV. Mice were euthanized 7, 50, 100 and 200 days post infection and splenocytes incubated with 10ng/mL IL-12 and IL-18.

After infection with MCMV there was a significant increase in the frequency of CD8+ T cells secreting IFNγ in response to stimulation with IL-12/IL-18. At day 7 9.4+/-1.5% [mean +/-S.E.M] of CD8+ T cells produced IFNγ in response to IL-12/IL-18 compared to 6.9+/-0.5% M45-peptide responsive cells. IL-12/IL-18 responsive CD8+ T cells were maintained at a significantly higher frequency than found in naïve mice until 200 days post MCMV infection (Figure 4.2 A).

CD4+ T cells respond to IL-12/IL-18 with a similar kinetic to CD8+ T cells although at a consistently lower frequency, only at day 7 was the frequency of responsive cells significantly increased over baseline (1.5+/-0.2% at day 0 to 6.8+/-1.4% 7 dpi [mean+/-S.E.M.]). The frequency of cytokine responsive CD3- cells, which encompass NK cells, is not altered by MCMV infection.

Our data shows the frequency of CD8+ T cells responding to IL-12/IL-18 are significantly enriched above baseline by MCMV infection and this is apparent even 200 days post infection although the magnitude of the response declines over time and the statistical significance decreases. Cytokine responsive CD4+ T cells peak 7 days post infection then return to near baseline levels and CD3- cytokine responsive cells do not vary in frequency. CD3- cells encompass NK cells and B cells and it is possible amongst CD3- cells a particular subset may show increased responsiveness to IL-12/IL-18 but are not visible within the overall population.

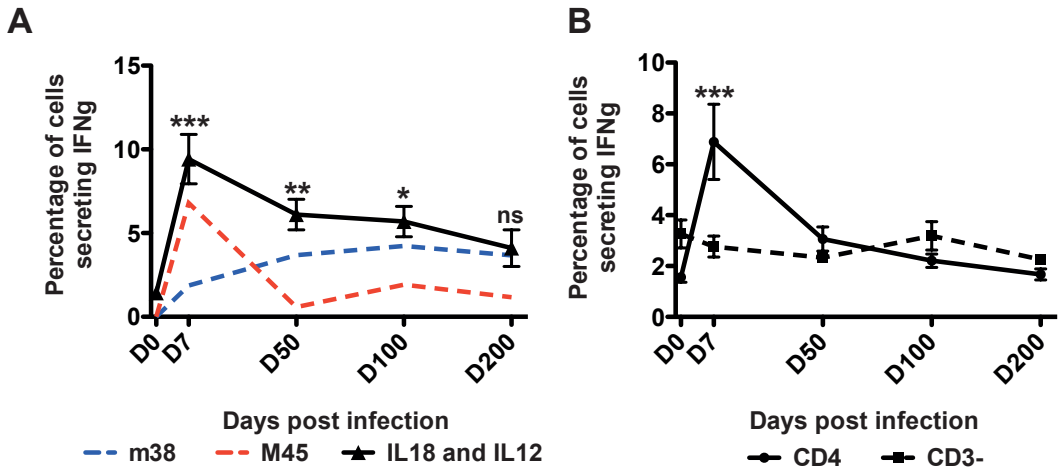

Figure 4.2 - Time course of IFNγ production in response to 10 ng/mL IL-12 and IL-18. C57BL/6 mice were infected intravenously with 1 x 10^6 pfu MCMV and tissues harvested from naïve mice and mice 7, 50, 100, and 200 dpi. Lymphocytes were isolated from spleens and stimulated with different concentrations of IL-12 and IL-18 alone or together for 6 hours (Brefeldin A was added after 2 hours). IFNγ production was measured. Percentage of cells producing IFNγ in response to 10 ng/mL IL-12 and IL-18 (background subtracted) from **A** CD3+CD8+ and **B** CD3-, CD3+CD4+ cells. Shown in blue and red are responses to M38 and M45 peptide respectively. * $p<0.05$, **$p<0.005$, $p<0.0005$ timepoint compared to result at D0 by Kruskal Wallis ANOVA with Dunn's correction. Data shown is mean +/- S.E.M. Represents minimum 2 experiments per time point minimum n=4 per group.

Characteristics of cells responding to stimualtion with IL-12 and IL-18

At day 7 only 9.4+/-1.5% [mean+/-S.E.M] of CD8+ T cells respond to IL-12/IL-18 stimulation by secreting IFNγ compared to 21+/-1.1% [mean+/-S.E.M] CD8+ T cells expressing IL-18Rα. In order to better understand the type of cells responding to IL-12/IL-18 I carried out a set of experiments to examine their phenotype. C57BL/6 mice were infected with 1 x 10^6 pfu MCMV and splenocytes harvested 7, 50, 100 and 200 dpi and IL-18Rα expression and memory phenotype assessed.

The CD3-, CD4+ and CD8+ cells responding to IL-12/IL-18 by producing IFNγ all express high levels of IL-18Rα at all time points after MCMV infection with, on average, over 65% of IFNγ+cells co-expressing IL-18Rα+ (Figure 4.3 A).

The consistent expression of IL-18Rα by IFNγ producing cells compared with the varying frequencies of IL-18Rα+ cells producing IFNγ suggests with IL-18Rα+ cells only a certain subpopulation are capable of responding to IL-12/IL-18. I have demonstrated in chapter 3 that CD8+IL-18Rα+ T cells are found in both the central and effector memory compartments and similarly IL-12/IL-18 responsive CD8+IL-18Rα+ T cells are universally CD44+ but may or may not express CD62L. Overlaying flow cytometry plots showing the distribution of CD62L and CD44 on CD3+CD8+ T cells (grey) it is apparent in both naïve and MCMV infected (50 dpi) IL-12/IL-18 responsive cells are both effector and central memory (Figure 4.3 B).

I observe that CD8+/CD4+ T cells and CD3- cells responding to IL-12/IL-18 express high levels of IL-18Rα. In naïve and MCMV infected mice CD8+ T cells producing IFNγ after IL-12/IL-18 stimulation are all antigen experienced (CD44+).

IL12/IL-18 do not induce other common cytokines

In addition to IFNγ I examined whether the most effective concentration of IL-12 and IL-18 could stimulate production of other cytokines. Splenocytes from naïve, acute (7dpi) and

chronically (150 dpi) MCMV infected mice were stimulated with 10ng/mL IL-12 and IL-18 for 6 hours and production of TNFα, IL-2, IL-17A and IL-17F by CD8+ T cells was assessed. CD8+ T cells responding to IL-12/IL-18 produced low levels of other cytokines (less than 0.3% for any cytokine at any time point). TNFα producing cells did not alter in frequency with MCMV infection. CD8+ T cells producing IL-2 peaked at 7 dpi then by 150 dpi had returned to baseline levels found in naïve mice. Naïve splenocytes responding to IL-12/IL-18 displayed a higher frequency of IL-17A secreting cells whereas cells secreting IL-17F were more common in MCMV (Figure 4.3 C).

Broadly IL-12/IL-18 were unsuccessful at inducing significant production of any other cytokines.

IL-12/IL-18 responsive cells are found in peripheral tissues

Theorising that IL-12/IL-18 responsive cells may be protective against bacterial challenge means it is important to demonstrate that tissue resident cells (as opposed to cells in secondary lymphoid organs) respond to IL-12/IL-18, as it is early IFNγ production by these cells that will impact upon pathogens. Lymphocytes were extracted from the blood, liver, lungs and spleen of naïve C57BL/6 mice, stimulated with 10ng/mL IL-12 and IL-18 for 6 hours and IFNγ production assessed.

Between 2-5% of all CD8+ T cells and 3-4% of CD4+ T cells produce IFNγ in response to IL-12/IL-18. IFNγ producing cells are distributed throughout the tissues. (Figure 4.3 D).

The presence of IL-12/IL-18 responsive populations in the lungs and liver supports our hypothesis that MCMV induces a population of T cells that retain some innate like capacities, such as the ability to secrete cytokines without cognate antigen.

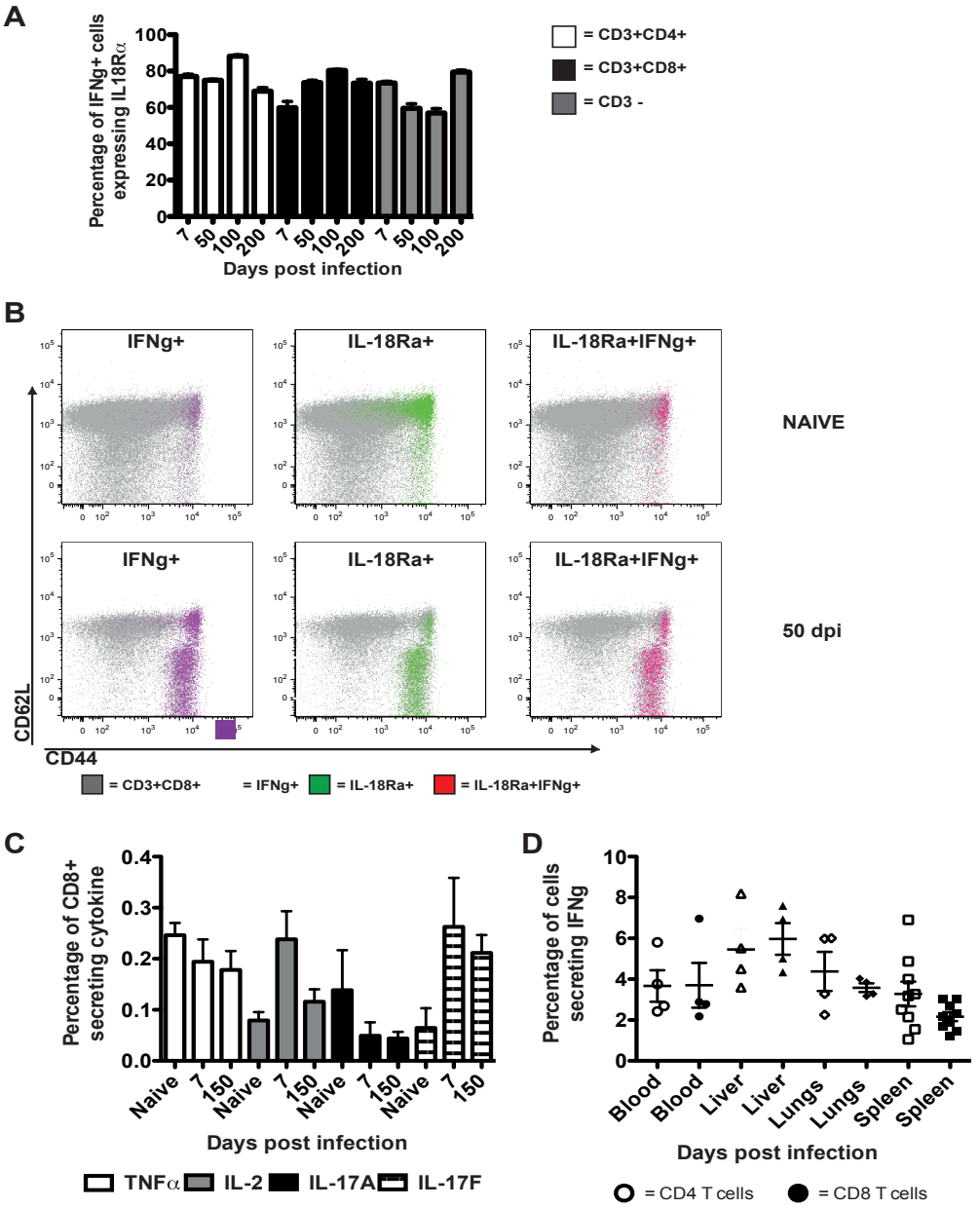

Figure 4.3 – Characteristics of cells responding to stimulation with IL-18 and IL-12 10ng/mL. C57BL/6 mice were infected intravenously with 1 x 10^6 pfu MCMV. Lymphocytes were isolated from naïve spleens or the spleens of mice 7, 50, 100 and 200 dpi and stimulated with 10ng/mL IL-12 and IL-18 for 6 hours and IFNγ production assessed. **A** Percentage of splenic CD3-, CD4+ and CD8+ IFNγ+ cells expressing IL-18Rα. **B** Flow cytometry plots showing CD44 and CD62L expression on IFNγ+, IL-18Rα+ and IL-18Ra+IFNγ+ CD8+ T cells. **C** Production of TNFα, IL-2, IL-17A and IL-17F by CD8+ T cells after IL-12 and IL-18 stimulation. **D** Lymphocytes were isolated from indicated tissues in naïve C57BL/6 mice, stimulated with 10ng/mL IL-12 and IL-18 and IFNγ production assessed. Black symbols = CD8+ Clear symbols = CD4+.

MCMV-specific cells respond to stimulation with IL-12 and IL-18

To examine whether the increase in IL-12 and IL-18 responsive cells observed in MCMV is antigen specific or a bystander effect I examined splenocytes from mice infected intravenously with 1 x 10^6 pfu MCMV 100 days previously. Splenocytes were incubated with 10ng/mL IL-12 and IL-18 for 6 hours then stained with MHC class I tetramers specific for the inflating M38 and non-inflating M45 specific CD8+ T cells and IFNγ production assessed.

Flow cytometry plots displayed in Figure 4.4 illustrate CD8+ T cells producing IFNγ (Figure 4.4 A) and distribution of tetramer positive cells amongst the IFNγ+ compartment (Figure 4.4 B). Approximately 1/3rd of IFNγ producing cells responding to IL-12 and IL-18 are either M38 or M45 specific (Figure 4.4 D) compared with 1/10th of all CD8+ T cells. This clearly delineates the increase in IL-12 and IL-18 responsive cells seen in MCMV infection is directly related to MCMV specific cell populations, although not necessarily restricted to these. In parallel a media control was used to ensure IL-18/IL-12 stimulation did not up or down regulate the TCR (data not shown).

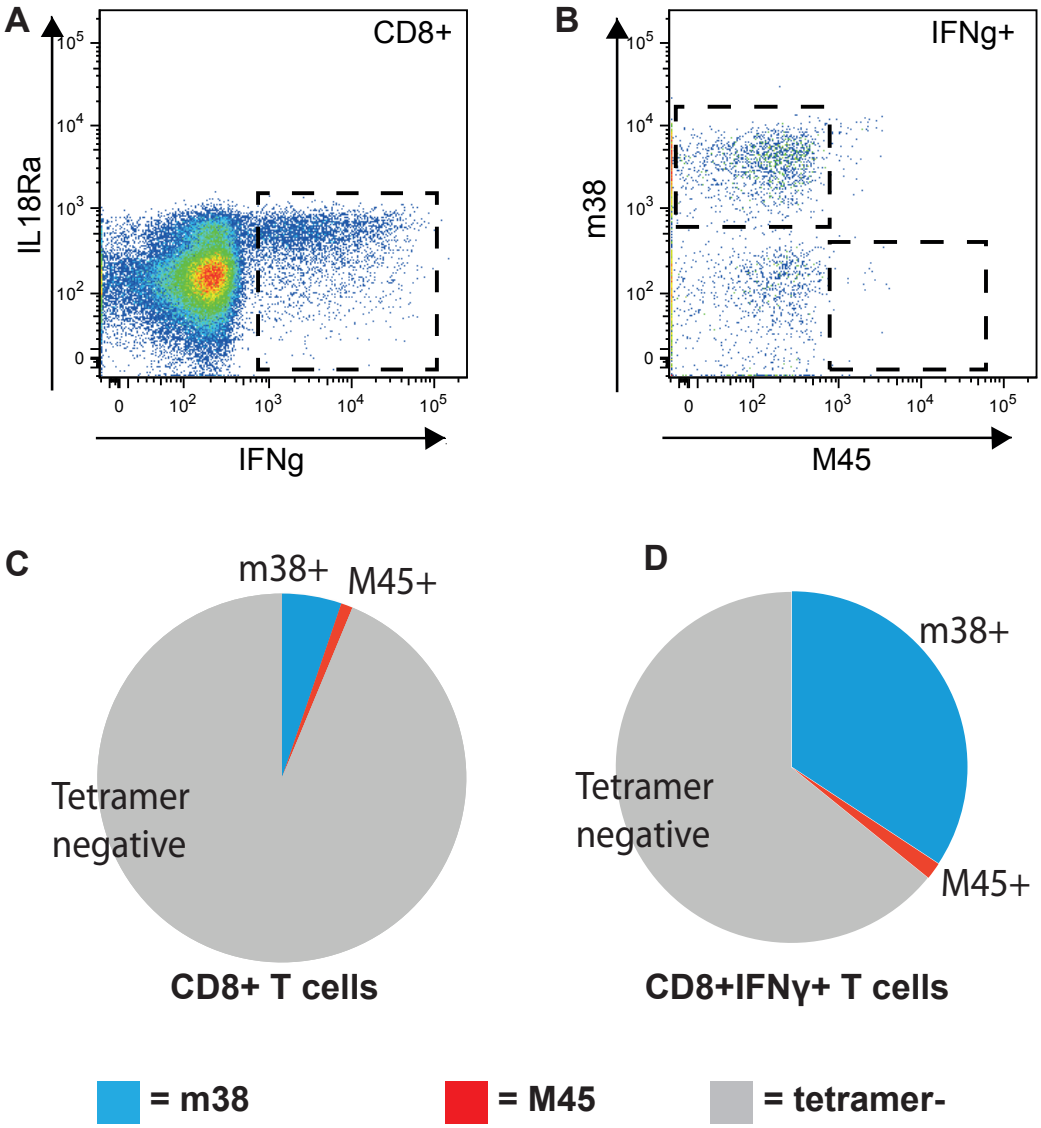

Figure 4.4 – MCMV specific cells respond to stimulation with IL-12 and IL-18. C57BL/6 mice were infected intravenously with 1 x 10^6 pfu MCMV. Lymphocytes were isolated from spleens 50 dpi and stimulated with 10ng/mL IL-12 and IL-18 for 6 hours (Brefeldin A was added after 2 hours) and IFNγ production assessed. Tetramers specific for M38 and M45 were added. Flow cytometry plots showing **A** IFNγ production by CD8+ T cells after stimulation and **B** M38/M45 tetramer binding by IFNγ+ cells. **C** Pie chart showing percentage of M38- and M45-specific CD8+ T cells. **D** Percentage of CD8+IFNγ+ T cells binding M38 and M45 tetramers.

Prolonged exposure to IL-12 and IL-18 results in up regulation of IL-18Rα upon CD8+ T cells

It has been previously reported in BALB/c mice that T cells incubated with IL-12 express higher levels of IL-18Rα mRNA whereas incubation with anti CD3 antibodies reduces IL-18Rα expression[189]. To investigate whether this occurs in C57BL/6 mice I infected mice intravenously with 1 x 10^6 pfu MCMV and 7 dpi harvested splenocytes. Cells were incubated with 10 ng/mL IL-12 and IL-18 for varying lengths of time and the frequency of IL-18Rα expression assessed.

In a "standard" 6 hour ICS IL-18Rα expression was reduced upon CD8+ T cells when splenocytes were incubated with PMA/ionomycin, although this was not statistically significant. Stimulation with IL-12/IL-18 did not affect IL-18Rα expression (Figure 4.5 A). I performed a time course experiment incubating splenocytes from mice 7 dpi with 10ng/mL IL-12 and IL-18 for 0, 30, 60, 120, 360 or 1080 minutes, after incubation cells were washed three times, resuspended in media containing Brefeldin A and rested for 4 hours before IL-18Rα expression and IFNϒ production examined. Frequency of IL-18Rα expression on CD8+ T cells appeared to increase as incubation time with IL-12/IL-18 increased (Figure 4.5 B). It is possible to show a direct correlation between the frequency of IL-18Rα expression and length of incubation with IL-12/IL-18 ($r=0.9$, $p<0.05$ Spearmans rank). Altered expression of IL-18Rα in response to IL-12/IL-18 stimulation is unclear when shorter incubation periods are observed however over night stimulation reveals IL-18Rα is indeed up regulated by prolonged exposure to cytokines although these experiments don't tell us which cytokine is definitively required.

CD8+ T cells respond to IL-12 & IL-18 rapidly

In the experiments detailed above splenocytes from mice 7 dpi 1 x 10^6 pfu MCMV were incubated with 10ng/mL IL-12 and IL-18 for increasing periods of time. In addition to IL-

18Rα expression we examined IFNγ production. If IL-12/IL-18 responsive cells were to be effective in defense against challenge with unrelated pathogens response to cytokines would need to be rapid. Within 30 minutes of exposure to IL-12/IL-18 CD8+ and CD4+ T cells were producing IFNγ (3.6+/-0.5% and 1.4+/-0.1% respectively [mean+/-S.E.M.]). After 360 minutes of IL-12/IL-18 stimulation IFNγ production peaked at 11.9+/-1.3% in CD8+ T cells and 8.44+/-1.7% in CD4+ T cells [mean+/-S.E.M.]. After an 18 hour overnight stimulation the frequency of IFNγ secreting cells had reduced to 5.2+/-0.57% CD8+ T cells and 1.94+/-0.12% of CD4+ T cells [mean+/-S.E.M.] (Figure 4.5 D).

Responses in acutely infected mice are of interest - however perhaps more physiologically relevant is the pattern of response in chronically infected mice (50 or more dpi) as individuals infected with a herpesvirus spend far longer in the chronic or latent phase. Splenocytes from mice infected 50 days previously were incubated with IL-12 and IL-18 for 0, 30, 60, 120, 360 or 1080 minutes and IFNγ production assessed. The kinetic of IFNγ secretion by CD8+ T cells from mice 50 dpi is nearly identical to that seen in mice 7 dpi. A notable exception is the 360 minute incubation where a significantly higher frequency of CD8+ T cells from chronically infected mice produce IFNγ compared to acutely infected mice ($p<0.0005$ Mann-Whitney U test).

Thus in MCMV infection cells responding to IL-12 and IL-18 by producing IFNγ do so rapidly. CD8+ T cells from the spleens of mice 50 days after MCMV infection produce significantly more IFNγ than CD8+ T cells from acutely infected mice after 6 hours of stimulation with IL-12/IL-18. The facts taken together suggest non-cognate mediated IFNγ secretion by T cells from mice latently infected with MCMV could affect responses to other pathogens.

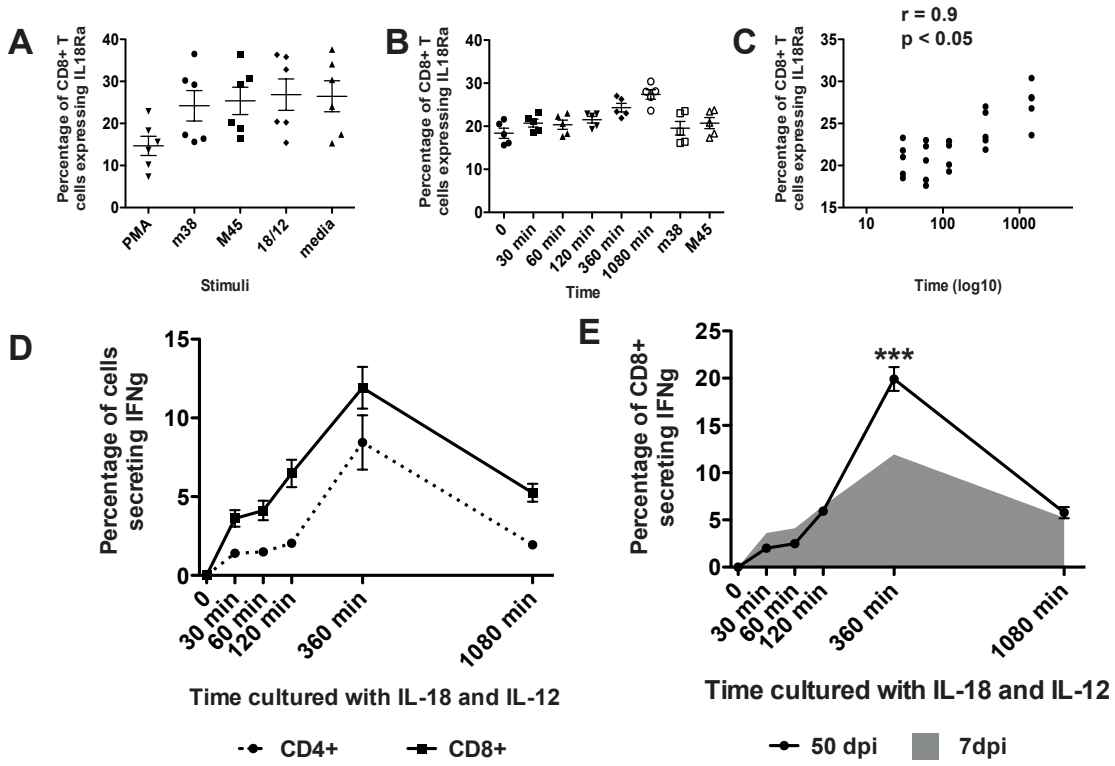

Figure 4.5 - IL-12 and IL-18 mediated IFNγ secretion involves up regulation of IL-18Rα and occurs rapidly. C57BL/6 mice were infected intravenously with 1 x 10^6 pfu MCMV. Lymphocytes were isolated the spleens of mice 7 dpi and stimulated with 10ng/mL IL-12 and IL-18 for different lengths of time **A** Standard 6 hour ICS (Brefeldin A added after 2 hours). Stimuli were PMA/ionomycin, M38 or M45 peptide, 10ng/mL IL-12/IL-18 and media control. Percentage of CD8+ T cells expressing IL-18Rα (individual values, mean and S.E.M displayed). **B** IL18Rα expression on CD8+ T cells after different length incubation. Splenocytes from the same mice were incubated with 10ng/mL IL-12/IL-18 for 30, 60, 120, 360 or 1080 minutes washed and then rested in media for 4 hours (Brefeldin A added after washing step)(individual values, mean and S.E.M displayed). **C** IL-18Rα expression on CD8 T cells versus length of exposure to IL-12/IL-18. R=0.9 p <0.05 Spearmans r. IFNγ secretion by splenocytes from mice **D** 7 dpi (CD4+ and CD8+ T cells) and **E** 50 dpi (CD8+ T cells with data from 7 dpi shown for comparison; p<0.0005 by Mann Whitney U test) (mean +/- S.E.M). Data show represents 2 experiments n = 4 per group.

Brefeldin A inhibits the production of IFNγ by CD8+ T cells responding to IL-12 and IL-18

In view of our findings that prolonged incubation with IL-12/IL-18 caused increased expression of IL-18Rα I sought to elicit whether IL-12/IL-18 stimulation caused changes in expression or secretion of other receptors including IL-12. Commercially available antibodies for the IL-12 receptor did not work in our hands so I elected to use Brefeldin A; a lactone antibiotic that inhibits transport of proteins from the endoplasmic reticulum (ER) to the Golgi apparatus.

C57BL/6 mice were infected intravenously with 1 x 10^6 pfu MCMV and 7 days post infection splenocytes harvested and incubated with 10ng/mL IL-12 and IL-18 either with Brefeldin A present from 0 minutes or added after 120 minutes. After 6 hours cells were assessed for IFNγ production.

Addition of Brefeldin A from the beginning of the stimulation significantly reduced the amount of IFNγ produced by CD8+ T cells from a frequency of 9.8+/-0.93% to 4.64+/-0.63% [mean +/-S.E.M]. Notably CD3-NK1.1+ cells were not affected providing an internal control. (Figure 4.6 A and B). It is not possible to determine reduction in IFNγ secretion is due to receptor up regulation being suppressed or whether a second cytokine is induced by IL-12 or IL-18 and required for optimal IFNγ secretion by CD8+ T cells.

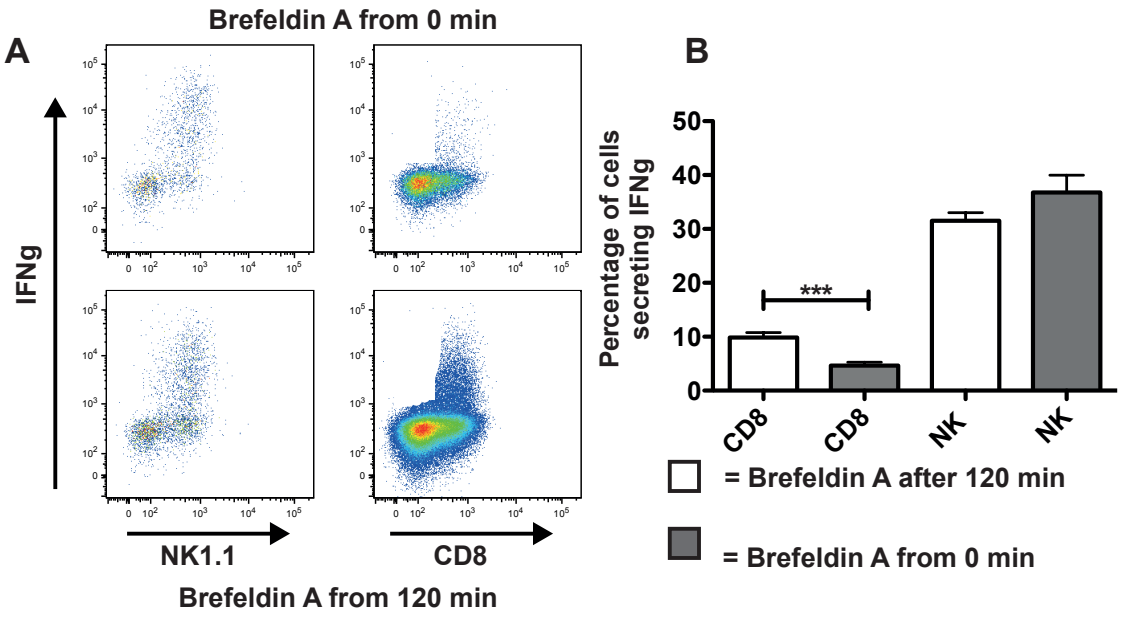

Figure 4.6 – Brefeldin A inhibits production of IFNγ by CD8+ T cells in response to IL-12/IL-18 C57BL/6 mice were infected intravenously with 1 x 10^6 pfu MCMV. Lymphocytes were isolated the spleens of mice 7 dpi and stimulated with 10ng/mL IL-12 and IL-18 for 6 hours with Brefeldin A added immediately or after 2 hours. **A** Flow cytometry plots showing IFNγ secretion by CD8+ T cells and CD3- NK1.1+ cells with Brefeldin A from 0 min or 120 min. **B** Percentage of CD8+ T cells and CD3- NK1.1+ cells producing IFNγ (*** p <0.005 by Mann Whitney U test, displayed are mean and S.E.M).

CD8+ T cells require exposure to IL-12 and IL-18 simultaneously for optimal IFNγ secretion

In BALB/c mice it has been shown that optimal secretion of IFNγ in response to IL-12/IL-18 requires cells to be exposed to IL-12 prior to IL-18 and this was in part due to the fact IL-12 induced higher levels of IL-18Rα expression[189].

In order to test the optimal conditions for IL-12/IL-18 induced IFNγ production a supernatant transfer experiment was performed.

C57BL/6 mice were infected with 1 x 10^6 pfu MCMV and 7 days later splenocytes harvested. Cells were stimulated with 10ng/mL IL-12 or IL-18 for 3 hours, washed three times then either the supernatant the transferred to the opposing cytokine (IL-12 supernatant into IL-18 stimulated well), fresh IL-18 or IL-18 added or R10 added (Figure 4.7A). By transferring supernatants I was seeking to determine whether IL-12 (or IL-18) caused secretion of a secondary cytokine which would affect IFNγ production or whether Brefeldin A blocked receptor up regulation.

It is apparent both IL-12 and IL-18 together stimulate optimal IFNγ secretion by CD8+ T cells however cells incubated with IL-12 which then had fresh IL-18 added produced significantly more IFNγ than any other stimulation condition ($p<0.0005$ by Kruskal Wallis with Dunns' correction; Figure 4.7 B). Supernatant transfer had little effect on IFNγ secretion – surprisingly IL-12 stimulated cells receiving the supernatant of IL-18 stimulated cells did not respond by secreting higher levels of IFNγ (Figure 4.7B). This may be due to IL-18 binding protein (IL-18BP), a novel secreted protein, which binds IL-18 and blocks its biological activity. Recombinant IL-18BP administered to mice causes a 90% attenuation of IFNγ production in mice after administration of LPS[190]. IL-18BP may be induced by IL-18 in a negative feedback loop although little is known about the mechanism of IL-18BP induction.

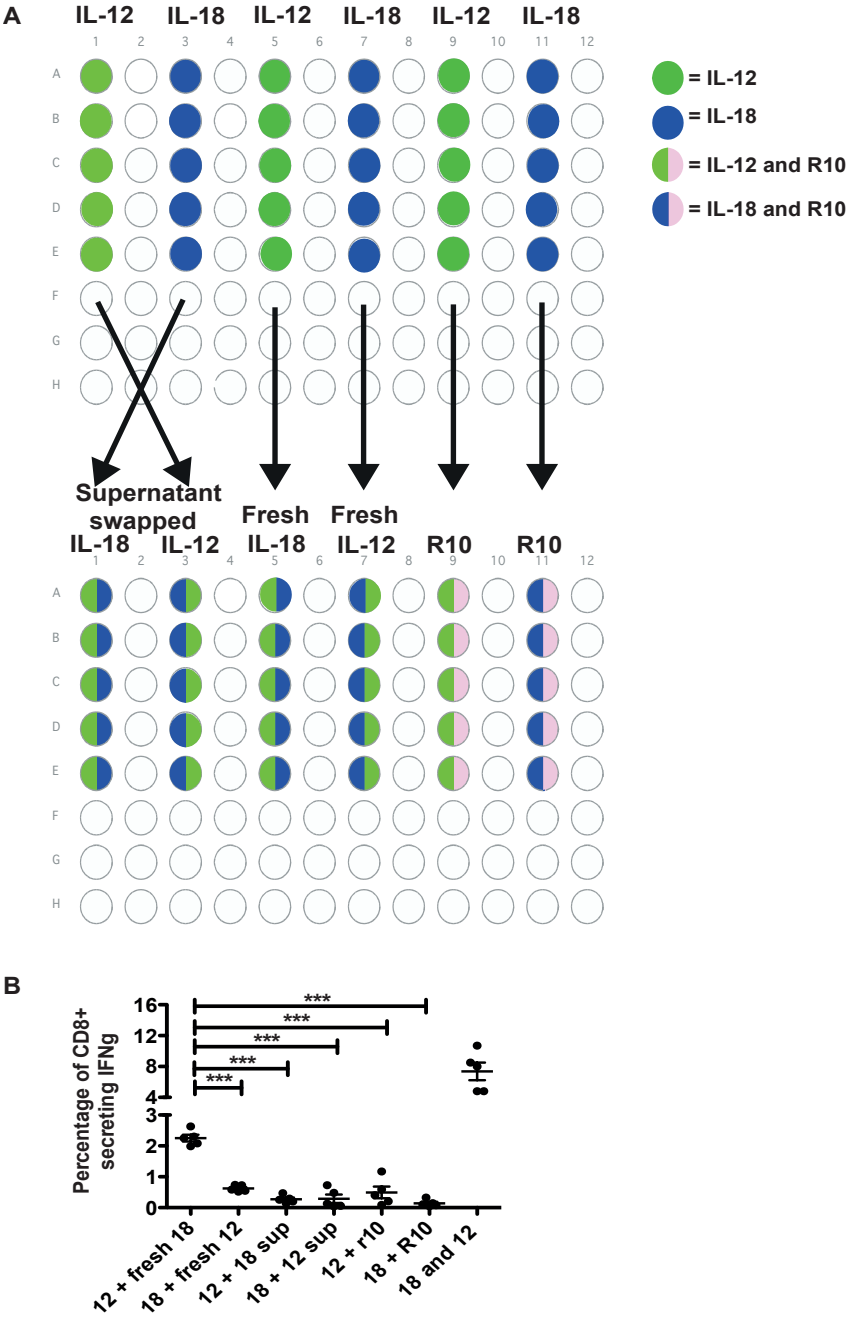

Figure 4.7 – CD8+ T cells require IL-12 and IL-18 exposure simultaneously for optimal IFNγ production. C57BL/6 mice were infected intravenously with 1 x 10^6 pfu MCMV. Lymphocytes were isolated the spleens of mice 7 dpi and stimulated with (i) 10ng/mL IL-12 (ii) 10ng/mL IL-18 for 3 hours, plates were centrifuged and supernatants aspirated, cells were washed x 3 with media and resuspended in (i) fresh IL-12 or IL-18 (10ng/mL) (ii) supernatants from IL-12 and IL-18 stimulated cells (iii) R10 for a further 3 hours. Brefeldin A was added for the final 2 hours (8 hour stimulation in total). **A** Schematic of experiment **B** Percentage of CD8+ T cells producing IFNγ (shown are individual values, and mean +/- S.E.M) *** p<0.0005 by Kruskal Wallis with Dunns correction.

MCMV infected mice have transiently lower bacterial counts after challenge

It has been shown that β and γ herpesviruses offer transient protection against bacterial challenge and in the case of γHV-68 this is postulated to be due to enhanced activation of macrophages secondary to raised serum levels of IFNγ. No clear mechanism has been illustrated for the protection observed in mice infected with MCMV[185].

I theorized that in MCMV infected mice protection against bacterial challenge is linked to the innate functions of MCMV-specific T cells producing IFNγ in response to IL-12 and IL-18. As displayed earlier (Figure 4.3A) 70 – 80% of CD8+ T cells producing IFNγ in response to IL-12/IL-18 stimulation express IL-18Rα thus I elected to test this hypothesis by infecting C57BL/6, IL-18$^{-/-}$ and IL18Rα$^{-/-}$ mice with 1 x 10^6 pfu MCMV and after 7, 50 or 100 days challenging these mice intravenously with 1 x 10^5 cfu *Listeria monocytogenes.* 72 hours after infection mice were culled and the bacterial load in liver and spleen counted. Injection stocks were cultured after each experiment to ensure comparability and ranged from 8 x 10^4 cfu to 2.6 x 10^5 cfu (data not shown). IL-18$^{-/-}$ and IL18Rα$^{-/-}$ mice are characterized by hyperphagia, obesity and insulin resistance thus all data reported is per gram of tissue rather than per organ[191].

Initially it was key to show that mice deficient in IL-18 and IL-18Rα did not have higher bacterial colony counts in the liver or spleen. I infected naïve C57BL/6, IL-18$^{-/-}$ and IL18Rα$^{-/-}$ mice intravenously with 1 x 10^5 cfu *Listeria monocytogenes.* After 72 hours colony counts in spleen and liver were indistinguishable (Figure 4.8 A and B). This support currently published data where no difference is seen either in the magnitude and function of antigen specific T cell responses or survival after *Listeria* challenge in IL-18 and IL-18Rα deficient mice[132].

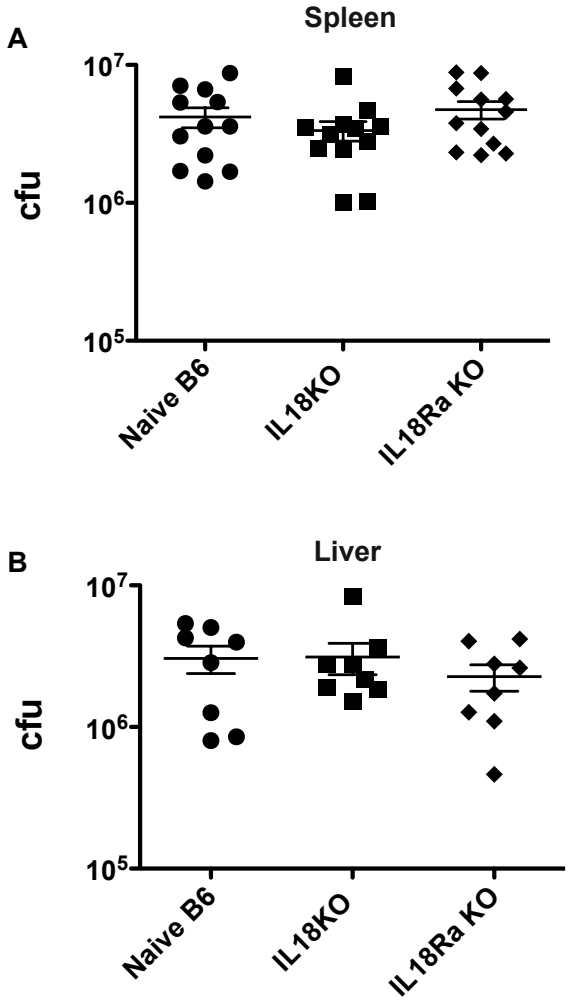

Figure 4.8 – Absence of IL-18 and IL-18Rα does not affect growth of Listeria monocytogenes following challenge in naïve mice. IL-$18^{-/-}$ and IL18R$\alpha^{-/-}$ and C57BL/6 mice were infected intravenously with 1 x 10^5 cfu *Listeria monocytogenes*. After 72 hours mice were euthanized and bacterial colonies enumerated in **A** spleen and **B** Liver. Data shown is per g of tissue (individual data points, mean and S.E.M)

After 7 days MCMV infected mice exhibited significantly lower bacterial colony counts in both spleen and liver when compared with age matched naïve C57BL/6 mice. MCMV infected IL-18$^{-/-}$ and IL18R$\alpha^{-/-}$ mice also show significantly lower bacterial counts in both liver and spleen although the average value are higher than MCMV infected C57BL/6 mice (Figure 4.9 A and B). γ-herpesvirus expression does not offer any protection during acute infection whereas I observe a reduction in bacterial counts. This may be due to generalised immune activation in diverse organs in acute MCMV.

After 50 days of MCMV infection, *Listeria* challenge led to bacterial counts in the spleen and livers of C57BL/6 mice, which are still significantly lower than naïve age matched controls (Figure 4.10 A and B). However this difference is completely abrogated in MCMV IL-18$^{-/-}$ and IL18R$\alpha^{-/-}$ mice, with bacterial counts being significantly higher than seen in MCMV infected C57BL/6 mice. This demonstrates that MCMV-induced resistance to Listeria is IL-18 dependent, and fits with our hypothesis that MCMV specific T cells responding to IL-12/IL-18 contribute to MCMV induced reduction in bacterial load.

120 days post MCMV infection MCMV no longer provides any reduction in bacterial colony numbers in the spleen and liver after challenge with *Listeria monocytogenes*.

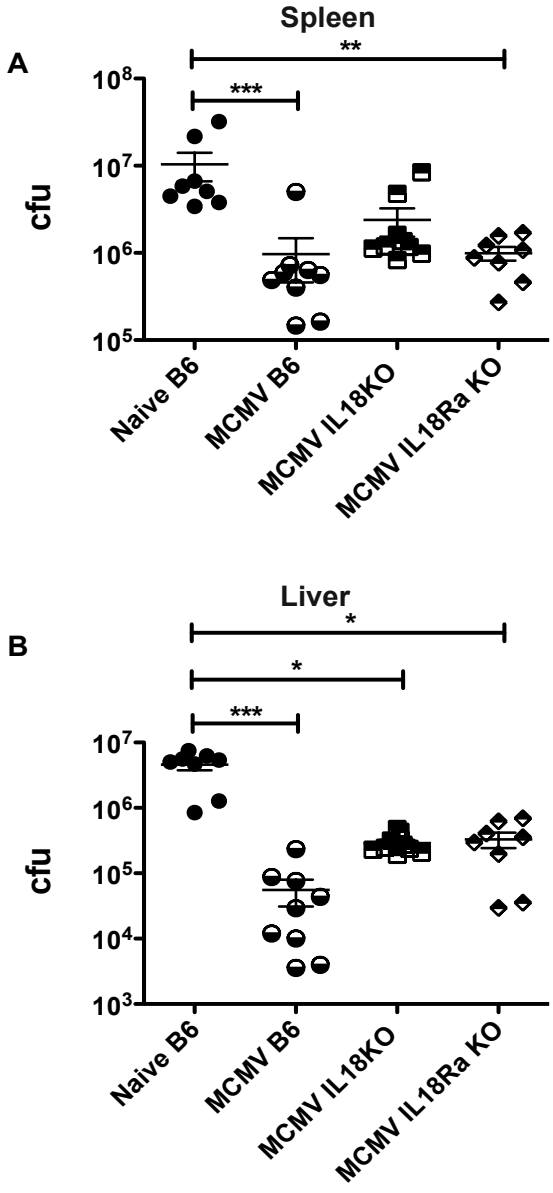

Figure 4.9 – Acutely MCMV-infected mice display lower growth of Listeria monocytogenes following challenge. IL-$18^{-/-}$ and IL18R$\alpha^{-/-}$ and C57BL/6 mice were infected intravenously with 1 x 10^6 pfu MCMV. 7 dpi these mice plus an age matched group of MCMV uninfected C57BL/6 mice were challenged intravenously with 1 x 10^5 cfu *Listeria monocytogenes*. After 72 hours mice were euthanized and bacterial colonies enumerated in **A** spleen and **B** liver. Data shown is per g of tissue (individual data points, mean and S.E.M) * $p<0.05$, ** $p<0.005$, ***$p<0.0005$ by Kruskal Wallis ANOVA with Dunns correction.

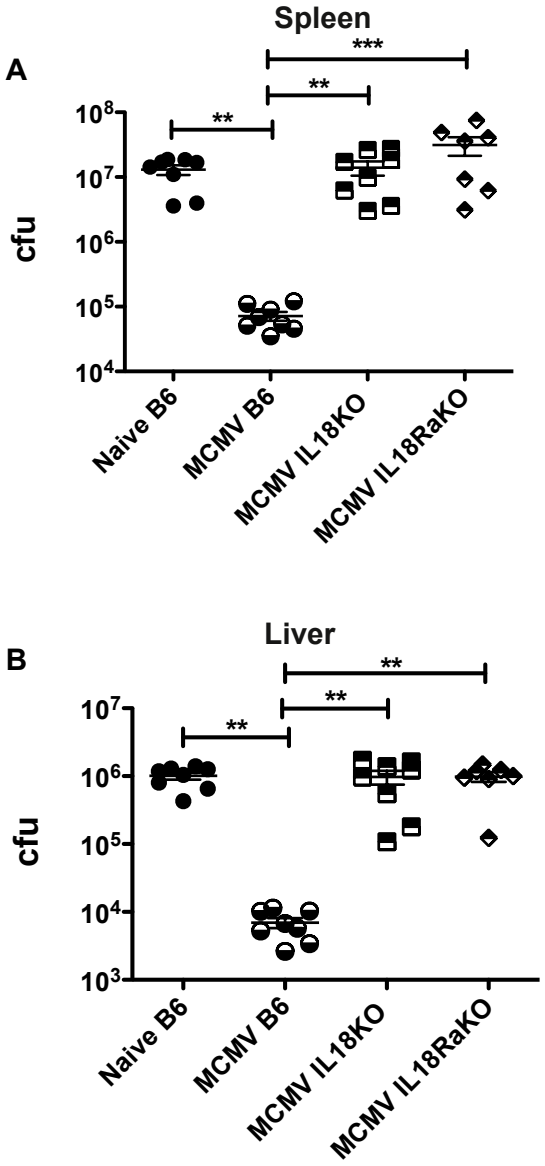

Figure 4.10 – 50 days after infection MCMV infected mice display lower growth of Listeria monocytogenes following challenge in an IL18-dependent manner. IL-$18^{-/-}$, IL-$18R\alpha^{-/-}$ and C57BL/6 mice were infected intravenously with 1 x 10^6 pfu MCMV. 50 dpi these mice, plus an age matched group of MCMV uninfected C57BL/6 mice were challenged intravenously with 1 x 10^5 cfu Listeria monocytogenes. After 72 hours mice were euthanized and bacterial colonies enumerated in **A** Spleen and **B** Liver. Data shown is per g of tissue (individual data points, mean and S.E.M) * $p<0.05$, ** $p<0.005$, ***$p<0.0005$ by Kruskal Wallis ANOVA with Dunns correction.

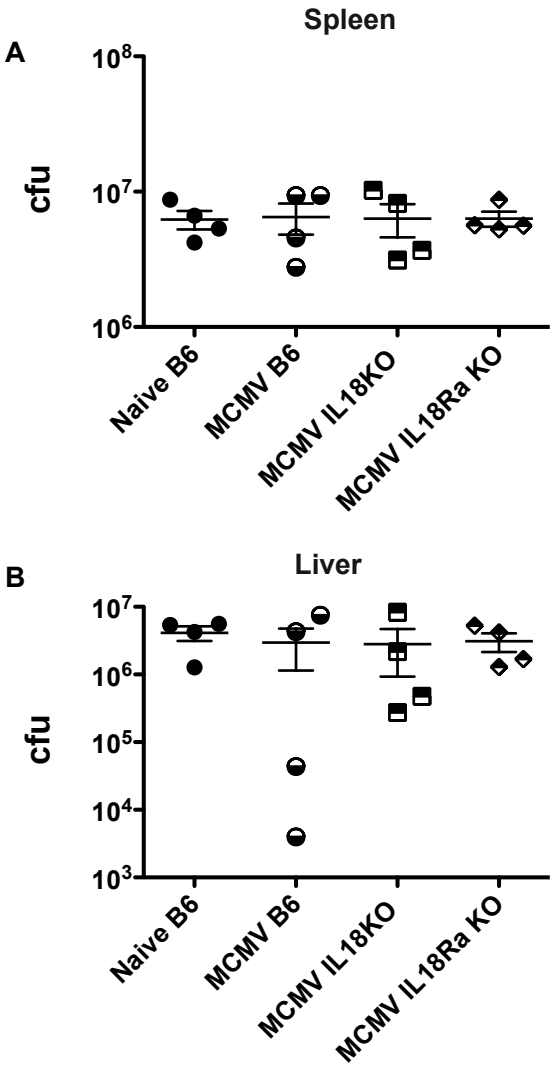

Figure 4.11 – 120 days after infection MCMV does not reduce Listeria monocytogenes growth following challenge. IL-$18^{-/-}$, IL-$18R\alpha^{-/-}$ and C57BL/6 mice were infected intravenously with 1 x 10^6 pfu MCMV. 120 dpi these mice, plus an age matched group of MCMV uninfected C57BL/6 mice were challenged intravenously with 1 x 10^5 cfu *Listeria monocytogenes*. After 72 hours mice were euthanized and bacterial colonies enumerated in **A** spleen and **B** Liver. Data shown is per g of tissue (individual data points, mean and S.E.M).

MCMV infection offers transient protection against challenge with Mycobacterium tuberculosis

To ensure MCMV induced protection is not due to cross-reactive antigenic epitopes between MCMV and *L.monocytogenes* a second pathogen was used for challenge. MCMV infected mice were challenged with an aerosol challenge delivering ~200 CFU per lung of *Mycobacterium tuberculosis* (Mtb*).* After 28-32 days Mtb infected mice were culled and bacterial colonies in the lungs and spleen enumerated.

In the initial series of experiments C57BL/6 mice infected with MCMV 30 days previously displayed a significant reduction in mycobacterial counts in the lung but not in the spleen (Figure 4.12 A). The reduction in Mtb was of a similar magnitude to that seen with BCG the current vaccine used against Mtb - an approximate reduction of 1 log and little difference observed in the spleen[192]. A repeat experiment where mice infected with MCMV 30 days previously showed a similar pattern with significant reduction in mycobacterial load in the lung but the magnitude of the reduction was reduced to just over 0.5 log. By 60 days after MCMV infection there is still a significant difference between MCMV infected and MCMV naïve mice but the magnitude is reduced to a difference of 0.25 log (Figure 4.12 B). MCMV infection offers transient protection against infection with *Mycobacterium tuberculosis* suggesting cross reactivity of antigens is not a factor in MCMV mediated protection.

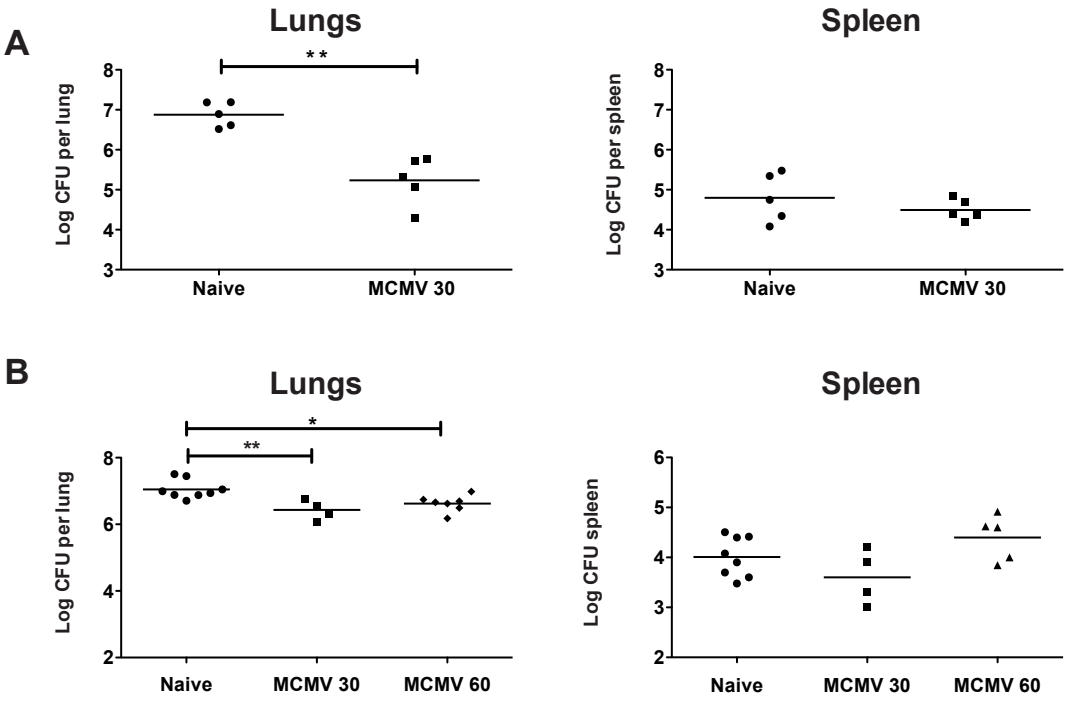

Figure 4.12 – MCMV infection offers transient reduction in Mycobacterium tuberculosis bacterial load in the lung. C57BL/6 mice were infected intravenously with 1 x 10^6 pfu MCMV. 30 dpi these mice, plus an age matched group of MCMV uninfected C57BL/6 mice were challenged via the aerosol route with approximately 200 cfu Mtb with. After 4 weeks mice were euthanized and bacterial colonies enumerated in spleen and lungs. A represents one challenge experiment 30 dpi and B a second experiment with mice 30 and 60 dpi with MCMV. * $p<0.05$, ** $p<0.005$, ***$p<0.0005$ by Kruskal Wallis ANOVA with Dunns correction.

Discussion

MCMV induces CD8+ T cells capable of responding to cytokines alone

Similarly to CD8+ T cells in the LCMV model, MCMV induces a population of CD8+ T cells that respond to stimulation with IL-12 and IL-18 by producing IFNγ though the frequency of IL-12/IL-18 responsive CD8+ T cells in MCMV is 7 times lower than observed in LCMV (9.4% CD8+ T cells secreting IFNγ in MCMV versus 74.4% 8 days post LCMV infection). Responses to stimulation with IL-12 and IL-18 alone are relatively weak, in comparison approximately 30 - 40% of CD8+ T cells respond to IL-12 or IL-18 alone 8 days after LCMV Armstrong infection[179]. Notably the authors did not find IL-12/IL-18 responsive CD8+ T cells in naïve mice whereas I found low level of cytokine responsive cells in naïve mice.

Only a proportion of CD8+ T cells secreting IFNγ in response to IL-12/IL-18 are M38- or M45-specific. There are two explanations for this. Firstly cytokine responsiveness may be a bystander phenomenon due to expansion of non-MCMV specific memory populations; clearly these already exist in naïve mice. Secondly, more likely, this also reflects our limited choice of tetramers - infection of C57BL/6 mice with MCMV produces a CD8+ T cell response, in viral latency, dominated by five singular epitopes that elicit inflationary responses, although there are numerous smaller responses to diverse epitopes also elicited[61].

Cytokine responsive T cells induced by MCMV are effector memory cells

In order to have maximal effect on any pathogen cytokine responsive memory T cells are best located in peripheral tissues where IFNγ can have immediate effect.

In the previous chapter I showed IL-18Rα is expressed by a high frequency of CD8+ T cells after infection which the frequency of expression declining over time. IL-18Ra+ CD8+ T cells are almost uniformly CD44+ and the majority CD62L- – supporting an effector memory phenotype. I have shown cytokine responsive cells express high levels of IL-18Rα and are

distributed throughout peripheral tissue such as lung and liver and begin to secret IFNγ within 30 minutes of exposure to IL-12/IL-18.

It is important to acknowledge that IL-12/IL-18 responsive cells express IL-18Rα although only a subset of CD8+IL-18Rα+ T cells produce IFNγ in response (consider chapter 3 whereby 7 dpi with MCMV 20% of CD8+ T cells are IL-18Rα+ but here only approx. 10% are cytokine responsive). Key to understanding this dichotomy would be accurate examination of IL-12 receptor expression and IL-18Rβ, which unfortunately was unsuccessful using flow cytometric techniques. Brefeldin A blocks production of IFNγ by cytokine responsive CD8+ T cells but not NK cells suggesting IL-12 receptor or IL-18Rβ chain up regulation may be required for the induction of IFNγ after cytokine stimulation. In vitro CD3 activated T cells are able to up-regulate IL-12Rβ2 expression in response to IL-18 in the absence of IL-12[193]. Exchange of supernatants from IL-12 and IL-18 stimulated cells and subsequent measurement of IFNγ production supports published data that IL-12 exposure is required to allow cells to respond to IL-18 but in our hands the converse (IL-18 exposure sensitizing cells to IL-12) did not occur, despite suggestion in the literature that IL-18 exposure increases IL-12Rβ2 expression on T cells[189,193]. Often work examining the role of IL-12 on IL-18Rα expression and IL-18 on IL-12Rβ1 or IL-12Rβ2 expression use anti CD3 to activate cells which may not be truly representative of physiological T cell activation[189,193]. It is clear in our model that IL-12 and IL-18, when considering IFNγ secretion by memory CD8+ T cells, are maximally effective together.

IL-18BP represents a unique soluble protein with the ability to bind mature IL-18, but not pro-IL-18, and prevent its interaction with cell surface receptors[194]. Interestingly IL-18BP production appears to be up-regulated by IFNγ in human epithelial cell lines, IFNγ induces the production and release of IL-18BP indicating, like other cytokines, IL-18 indirectly increases the production of its own inhibitor in a feedback loop[195]. IL-18BP is expressed

constitutively in the spleen in humans and mice with both endothelial cells and monocyte/macrophages also representing important sources of IL-18BP[119,120]. The kinetic of IL-18BP production is not known, however IFNγ stimulated IL-18BP production was detectable after 8 hours of culture, maybe explaining why IL-12/IL-18 mediated IFNγ production by CD8+ T cells in MCMV peaks after 6 hours exposure then begins to decline. Little is known of the role of IL-18BP in infections.

Clearly the interaction between IL-12 and IL-18 and their respective receptors is extremely complex. Any further work on this phenomenon should focus on elucidating the whether IL-12 receptor expression is up regulated on CD8+ T cells in MCMV and role of the IL-12 receptor and IL-18Rβ in IL-12/IL-18 mediated stimulation. In addition the role and mechanism of induction of IL-18BP must be addressed.

MCMV offers transient protection against bacterial challenge

Barton et al. postulate that protection offered by latent infection with either γHV-68 and MCMV is due to chronically elevated levels IFNγ secretion causing macrophage activation[187]. The results presented here show MCMV infection offers transient impairment in bacterial growth after challenge, which I surmise will have an effect on protection. This reduction in bacterial growth observed in latently MCMV infected animals is abrogated by the removal of IL-18 or the IL-18 receptor and this is supportive of a role for IFNγ production by IL-18Rα+ CD8+ T cells induced by MCMV.

T cells responding to initial encounters with cognate antigen proliferate and acquire effector functions including the secretion IFNγ, which activates macrophages and thus engenders a heightened state of innate immunity together pathogens encountered shortly afterwards. This is classical cross protection and unsurprisingly when effector cells differentiate into memory cells this phenomenon wanes[196]. It is suggested that γHV-68 mediated protection against pathogens is a prolonged state of classical cross protection.

In addition to classical protection it has been recently recognized that cross reactivity of T cells may also play a role. The T cell receptor (TCR) upon a CD8+ T cell can discriminate peptides of eight to 10 amino acids presented via major histocompatibility complex class I (MHC-I) molecules[197]. Structural studies have elucidated that only a few contact residues on the peptide presented by MHC molecules interact with the TCR and are required for activation and a TCR can tolerate amino acid substitutions in peptides and still be activated[198,199]. This suggests peptide ligands different from the initially encountered peptide may contain sufficient biding sites to activate a TCR. When cross-reactive epitopes between the antigens from primary and secondary pathogens result in antigen- specific memory lymphocyte activation after secondary infection this is termed heterologous immunity. Unsurprisingly heterologous immunity may occur in viral infections considering evolutionarily conserved sites within virus groups[200]. However it has also been proposed in non-related viruses such as LCMV and vaccinia virus (VV), influenza virus and hepatitis C virus (HCV)[201-204]. T cell cross-reactivity between viruses and bacteria has yet to be described in the literature. I observe MCMV mediate transient reductions in bacterial growth in *Listeria monocytogenes* and *Mycobacterium tuberculosis* strongly suggestive of non cross-reactivity.

The transient nature of protection seen in MCMV may be partially explained by the decline of IL-12/IL-18 responsive cells seen over time suggesting a threshold level is required to affect bacterial growth after challenge. Whilst CD8+ T cells are the dominant responders to IL-12/IL-18, CD4+ T cells are also responsive at a lower magnitude. When the total frequency of T cells responding to IL-12 and Il-18 are considered it is clear there is a near linear reduction in the frequency of IFNγ secreting cells over time supporting the theory of a "threshold" of IFNγ secretion. During acute infection however the frequency of IFNγ secreting cells is above the putative threshold yet the observed reduction in bacterial counts is of a lower magnitude. Barton et al. observe a similar phenomenon in γHV-68 infection where acute infection offers

no protection despite, presumably high level of IFNγ in the serum and activated macrophages. Perhaps macrophages already activated by γHV-68 and MCMV infection, both lytic in the acute stages, cannot be further activated nor increase bactericidal/viricidal capacity.

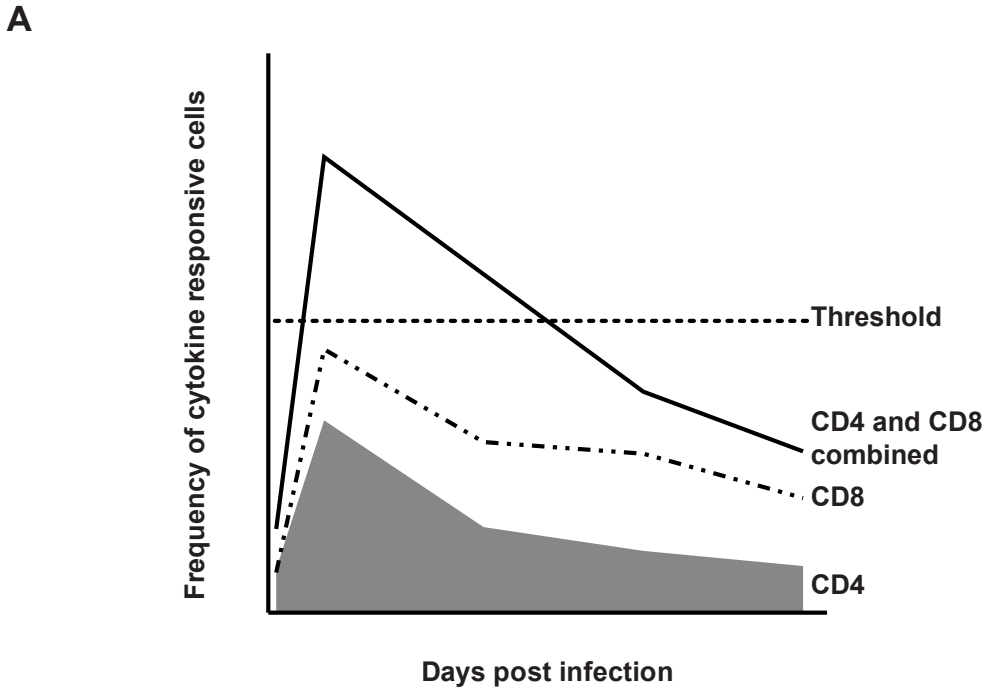

Figure 4.13 – Schematic illustrating the frequency of T cells secreting IFNγ in response to IL-12/IL-18 over time. Frequency of CD4 and CD8 T cells secreting IFNγ in response to 6 hours stimulation with 10 ng/mL IL-12 and IL-18. Shaded grey = CD4+ Dotted/dashed line = CD8+ Solid line = CD4+ and CD8+. Dotted horizontal line shows theoretical threshold for protection at challenge.

NK cells play a limited role in MCMV induced protection against bacterial challenge

Natural killer (NK) cells kill target cells displaying signs of infection, malignant transformation or cellular stress. Long classified as a major component of the innate immune system there is evidence, in mice, NK cells are capable of "memory"[205]. Recently it has been have demonstrated that antigen specific contact hypersensitivity (CHS) responses to chemical haptens are mediated by liver resident CXCR6+NK cells. CHS reactions only occur in in recombination-activating gene (RAG)-deficient mice (which lack B and T cells), but not in mice deficient for both RAG and γ_c (which also lack NK cells). Secondary responses in RAG mice were only elicited by the hapten to which mice had been originally exposed and not by a different hapten showing NK cells are capable of behaving in a similar fashion to T cells[206,207]. If capable of antigen specific memory it is thus theoretically possible NK cells may also be cross reactive however the fact protection is observed in both *Listeria monocytogenes* and *Mycobacterium tuberculosis* infections supports the hypothesis MCMV induced protection is not mediated by cross reactivity.

NK cells in MCMV perhaps warrant further special consideration as a clonal-like expansion has been described in the LY49H-bearing subset of NK cells during MCMV infection. LY49H NK cells in C57BL/6 mice undergo a 3- to 10-fold expansion during acute infection[208]. As MCMV is controlled however LY49H NK cell NK cells undergo apoptosis 1-2 weeks later contracting to form a "memory" pool maintained in blood, spleen, liver and lung, and various other tissues. MCMV "specific" NK cells thus behave in a similar fashion to classical memory T cells encountering antigen, proliferating then undergoing apoptosis and forming smaller pools memory cells[207]. It is entirely possible these cells may contribute to the MCMV mediated reduction in bacterial load observed here however during latent infection M38-specific CD8+ T cells have not contracted and are a significant constituent of tissue resident lymphocytes AND capable of responding to IL-12/IL-18 rapidly.

NK cells exposed to the IL-12 and IL-18 *in vitro* secrete IFNγ. When transferred in to naïve mice, and thus cytokine stimuli removed these cells cease to secrete IFNγ however when re-exposed they are capable of far more robust IFNγ production compared to NK cells not previously sensitized by IL-12/IL-18 and cytokine activated NK cells produce daughter cells never exposed to high doses of cytokine in vitro but capable of more rapid IFNγ secretion[209]. Interestingly these cytokine-activated memory NK cells do not express granzyme B protein and kill appropriate targets when compared to naïve NK cells.

Perhaps the strongest evidence suggesting NK cells do not play a role in this model system is that in MCMV naïve C57BL/6, IL-18$^{-/-}$ and IL-18Rα$^{-/-}$ mice no differences are observed in bacterial burden and notably in our hands in CD3- cells IFNγ production induced by exposure to IL-12 and IL-18 did not vary over the course of MCMV infection suggesting these cytokine driven memory NK cells do not play a role here. Furthermore in the Listeria monocytogenes OT-1 mouse model where ova-specific CD8+ T cells secreting IFNγ in response to IL-12 and IL-18 are protective during infection transfer of either NK or memory OT-1 T cells (specific for the OVA protein) into IFNγ-deficient mice showed OT-1 specific CD8+ memory T cells were more effective at providing innate immune protection as determined by bacterial load[210].

Conclusions

MCMV infection induces a population of T cells to produce IFNγ in response to the cytokines IL-12 and IL-18 without cognate antigen. The frequency of cytokine responsive cells decreases gradually over time but for a period offers protection against infection with bacterial challenges. IL-12/IL-18 responding cells are tissue resident and capable of rapidly secreting IFNγ in response to stimulation. The underlying mechanisms of IL-12 and IL-18 mediated IFNγ secretion are complex and up and down regulation of receptors not fully understood and worthy of further enquiry as is the role of IL-18BP in infection. Depletion of T cells and NK cells would allow elucidation of protective cell subsets. The knowledge IL-18R1 is highly expressed in CD8+ T cells after CMV infection in humans suggests that innate type functioning in human T cells should be investigated.

In this chapter I have shown

- Memory CD8+ T cells responsive to IL-12 and IL-18 are found in naïvemice and expanded by MCMV infection
- CD8+ T cells responding to IL-12 and IL-18 rapidly produce IFNγ but very low levels of IL-2, TNFα or IL-17
- IL-12 and IL-18 responsive CD8+ T cells are antigen experienced but may reside in either the effector or central memory compartment
- Prolonged exposure to IL-18/IL-12 causes up regulation of IL-18Rα
- IL-18Rα expressing tissue resident CD8+ T cells produced in MCMV infection are capable of short term protection against infections.

Chapter 5

IL-1Rα expressing T cell subsets

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Introduction

Defence against persistent viruses such as hepatitis C (HCV), hepatitis B (HBV), and cytomegalovirus (CMV) is critically dependent on CD8+ T cells but as yet the essential properties of these cells in man are not fully defined. A novel CD8+ T cells subset with distinct homing and functional properties in both healthy donors and those with HCV has recently been defined, named Tc17 due to their secretion of IL-17A[112]. These cells express high levels of the C-type lectin CD161 and a pattern of molecules consistent with Type 17 differentiation including the transcription factors RORγt, cytokine receptors (IL-23R, IL-18Rα) and chemokine receptor CCR6[106,113,211]. CD161+CD8+ T cells are markedly enriched in the livers of persons with chronic hepatitis C and can co-express IL-17A with high levels of IFNγ, and/or IL-22[113]. Representing up to one in six circulating CD8+ T cells in normal humans, and 50% of liver infiltrating CD8+ T cells in liver disease it is apparent these cells play a key role in the pathogenesis of chronic viral hepatitis and may potentially be involved in other major infectious and inflammatory diseases of man[112].

A phenotypically similar T cell subset – mucosally associated invariant T cells (MAIT) – has been shown in humans to express high levels of CD161, CCR6 and IL-18Rα together with a capacity to secret IL-17A in response to PMA/ionomycin[97,98,102]. MAIT cells have been described as CD4-CD8- (DN) or CD8αβ T cells although recently they have been defined as CD8αβ or CD8αα CD161+ T cells[99,106]. Detectable in both mice and humans these cells express an evolutionarily conserved invariant T cell antigen receptor (TCR) α-chain. In mice the invariant α-chain is composed of variable region iVα19 (associated with the α-chain joining region 33 (Jα33) segment) and in humans the 7.2 (iVα7.2) segment[100,101,103]. Selection and expansion of this population requires B lymphocytes expressing MR1, an MHC I related molecule. MAIT cells in humans have been detected in cord blood where they are in low numbers and display a naive phenotype. In contrast, post-natally they acquire a memory phenotype and expand dramatically forming between 1–4% of peripheral blood T

cells[102,103,106]. These cells to respond to bacteria- or yeast-infected APCs and MAIT cell frequencies were much lower in the peripheral blood of bacterially infected patients – perhaps due to migration of MAIT cells into infected tissue. A role in viral infections has not been delineated as yet[103,104,107].

In man, two sets of CD161+ T cells exist, those that express the highest levels of CD161++ (CD161hi) which is adults are dominated by MAIT cells and CD161+ (CD161dim), which do not secrete IL-17, but do display tissue homing characteristics. Virus specific T cells typically fall into the latter category and few are IL-17 producers[103,113].

CD4+ T cells capable of secreting IL-17 have been well described and characterised as Th17 cells: producing the cytokines IL-17A and IL-17F, expressing CD161, IL23R and CCR6 and requiring the transcription factor RORγt[212,213]. Demonstrable in murine models of disease they have been identified in patients with multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease and contact dermatitis[104,214].

IL-17 secreting CD8+ T cells have recently been described in the mouse, either in as pathogenic in LCMV model where both Eomes and T-bet knockout is required to produce Tc17 cells or as protective in a murine influenza challenge model where post infection Tc17 cells can be detected in the lung[114,215]. Using an Eomes/T-bet knockout mice Tc17 cells are generated and are unable to control LCMV replication. Subsequently when restimulated ex vivo with viral peptides LCMV-specific CD8+ T cells, from Eomes/T-bet knockout mice, exhibit substantial defects in IFNγ expression and target cell cytotoxicity in addition to impaired induction of granzyme B protein and perforin mRNA. In a murine influenza model however Tc17 cells, whilst barely detectable in naive mice, are induced in lung tissue by influenza challenge whereby although they comprise a relatively low frequency of CD8+ T cells their absolute numbers change 100-fold during an infection[114,215]. In vitro polarization of influenza specific Tc17 cells and transfer into naïve mice offer protection at influenza

challenge showing a clear role for Tc17 cells in protection although these cells do not maintain a pure IL-17A secreting phenotype.

Interestingly mice have very few MAIT cells and the lack of an antibody specific for the invariant Vα19 TCRα chain prevents analysis of MAIT cells in vivo or ex vivo and thus necessitated generation of a transgenic mouse expressing an invariant mVα19-Jα33 TCRα chain transgene; transgenic over-expression of the invariant TCRα chain induces a clear MR1-dependent bias in Vβ chain segment usage towards Vβ6 and Vβ8. This suggests the ontogeny of Tc17 cells in mice maybe significantly different than that in humans[99,106].

Chemokines control trafficking of cells into tissues, and chemokine receptor expression on the cell surface allows cells to be directed to relevant tissues by responding to a chemokine gradient. Most chemokine receptors bind to multiple chemokines, CCR6 is unique in that it can bind only a single chemokine ligand, CCL20. CCL20 is expressed at low levels by keratinocytes, pulmonary epithelial cells, and intestinal epithelial cells but in pro-inflammatory conditions expression is upregulated to facilitate lymphocyte trafficking to fight infection[216,217]

CXCR6 is another chemokine receptor which mediates the chemotaxis and adhesion of leukocytes to soluble and membrane-anchored forms of CXCL16; it is typically associated with liver and gut homing and thus of relevance when considering tissue homing T cells. CXCR6 can be found on the surface of classic αβ T cells, NK cells and NK T cells; indeed within murine livers NK T cells account for most CXCR6+ cells. CXCR6-deficient mice have reduced numbers of hepatic NK T cells and mice are resistant to Concanavalin A induced hepatitis (a murine NKT driven model of human autoimmune hepatitis)[214,218]. Like IL-18Rα, CXCR6 was specifically associated with CD161 expression on human CD8+ T cells.

As CD161 is also an important marker of the Type 17 lineage in human CD4+ and CD8+ T cells and thus defining equivalent C Type lectin homologues in the mouse could be of real

value in defining its role in vivo. Lectin-like transcript-1 (LLT1) / CLEC2D is a physiologic ligand for human CD161[219].

NK cell markers in mice

NK1.1 was defined using a monoclonal antibody, PK136 mAb, which demonstrated expression upon NK cells and certain T cell subsets[220,221]. Sequencing revealed that NK1.1 is expressed on the NKR-P1 family of type II transmembrane C-type lectin-like receptor. Located with the NKC gene complex on chromosome 6 the NKR-P1 cluster this contains five distinct murine NKR-P1 genes: NKR-P1A, NKR-P1B, NKR-P1C, NKR-P1D, and NKR-P1F[222,223]. Functional studies indicate that NKR-P1A, NKR-P1C, and NKR-P1F are stimulatory receptors and that NKR-P1B and NKR-P1D are inhibitory isoforms. Sequencing indicates the NKR-P1D gene represents a divergent allele of NKR-P1B; indeed C57BL/6 mice express NKR-P1D instead of the NKR-P1B found in other common mouse strains[224,225]. The NKR-P1B/D ligand is osteoclast inhibitory lectin (Ocil)/CLEC2D also known as Clr-b, a member of a previously cloned group of C-type lectin-related (Clr) proteins linked to the NKR-P1 receptors in the mouse NK gene complex (NKC)[226,227].

Aims

To investigate whether an equivalent subset of tissue homing CD8+ T cells exists in mice and whether it is altered in any way by MCMV infection. MCMV is used a model infection because it induces a pro-inflammatory cytokine response characterized by production of IFN-γ, TNF-α, IL-1α, IL-1β and IL-6 that occurs at the organ level as well as systemically and induces a transient mild hepatitis 33,34. In addition we have at our disposal a wide range of tetramers and peptides facilitating easy study of this model.

I aimed to examine

- What is the frequency, phenotype and function of the type 17 markers NKR-P1D+, CCR6+ and CXCR6+ cells in naïve mice?
- How does MCMV affect NKR-P1D+ T cells?
- What is the distribution and phenotype of Tc17 cells in mice?
- How does MCMV infection affect IL-17 secretion by T cells?

Results

Phylogenetic analysis and BLAST sequencing revels NKR-P1d as likely orthologue

BLAST sequence analysis examining amino acid conservation between human CD161 and members of the murine NKR-P1 family reveals similar levels of homology for all molecules (NKR-P1a 39%, NKR-P1b 44.7%, NKR-P1c 47.5%, NKR-P1d 45.2%, NKR-P1f 46.1%). Constructing a phylogenetic tree it is evident this molecule is conserved across species Figure 5.1. Considered together with the knowledge NKR-P1b and NKR-P1d possess an ITIM and bind the murine equivalent of LLT1 it makes NKR-P1d the most likely candidate molecule in C57BL/6 mice.

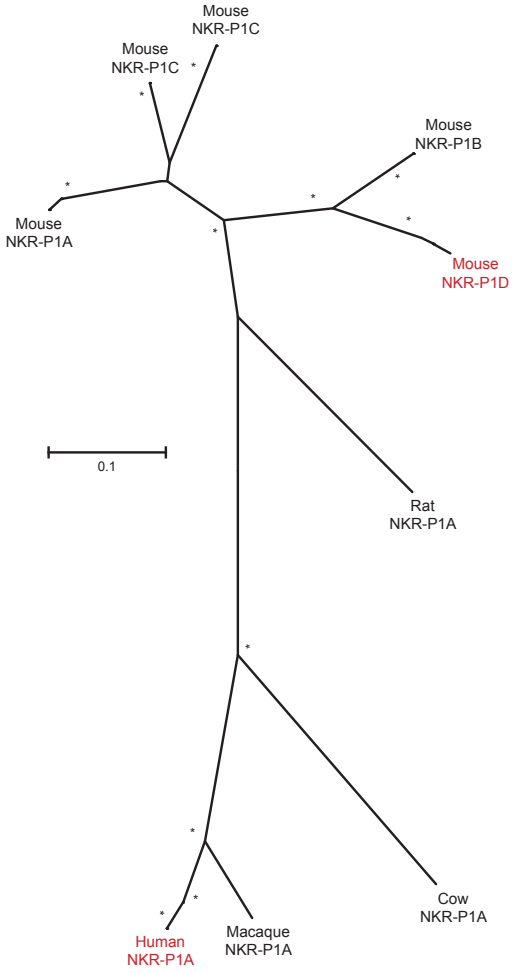

Figure 5.1 - Evolutionary relationships of taxa. Showing relationships between CD161 sequences from different species. Highlighted are murine NKR-P1D and human CD161. * indicates >80% of bootstrapped trees resolve to this design. The evolutionary history was inferred using the Neighbour-Joining method. The optimal tree with the sum of branch length = 2.05953270 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 30 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 113 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

MAIT cell phenotypic markers in MCMV

Lymphocytes were isolated from different tissues in uninfected mice or mice 7, 50, 100 and 200 days post infection with 1 x 10^6 pfu MCMV and subsequently stained for a panel MAIT cells / Tc17 typical marker surface markers including NKR-P1D, CCR6 and CXCR6 (IL-18Rα expression is discussed in chapter 3).

NKR-P1d

NKR-P1d is expressed on CD3-, CD4+ and CD8+ T cells from a wide range of tissues (Figure 5.2). The frequency of NKR-P1d+ cells is relatively low in CD3-, CD4+ and CD8+ cells in all tissue. Between 0.8 - 2.4 % CD3- cells in naive mice express NKR-P1d, 0.6 – 2.5% CD4 T cells and 0.2 – 1.1% of CD8+ T cells.

Infection with MCMV significantly increased NKR-P1d expression on CD3- cells in the liver (0.8+/-0.4% to 3.2+/-0.4% [mean+/-S.E.M]) but CD3- cells in other organs did not alter. Additionally no significant changes in NKR-P1d expression were observed on CD4+ or CD8+ T cells during MCMV infection.

M45-specific CD8+ T cells in the liver exhibit a significant increase in the frequency of NKR-P1d expression during acute MCMV infection (increasing from 1.1+/-0.3% M45 NKR-P1d+ to 3.4+/-1.4% [mean+/-S.E.M.]). Elsewhere NKR-P1d expression on both M38 and M45-specific cells does not vary significantly when compared with the frequency of NKR-P1d expression on naïve CD8+ T cells. Although in both CD3- and M45+ CD8+ T cells these percentages represent a small number of cells nonetheless this is suggestive that NKR-P1d + cells preferentially home to the liver during acute infection.

CCR6

CCR6 is expressed at low levels on CD3-, CD4+ and CD8+ T cells in naïve mice. The frequency of CCR6 expression varies between 0.8 – 2.1%, 1.1 -6.5%, 0.3 – 2.01% of CD3-, CD4+ and CD8+ T cells in the blood, liver lung and spleens of naïve mice. Infection with MCMV significantly

increases the frequency of CCR6+ cells in all subsets (CD3-, CD4+ and CD8+) most notably CD3- cells (from 0.8+/- 0.1% to 32.9+/-0.3% pulmonary CD3- cells expressing CCR6 [mean+/-S.E.M]). Both CD4+ and CD8+ T cells display higher levels of CCR6 expression during acute MCMV infection but like CD3- cells these differences are absent once acute infection has resolved. CCR6 expression on antigen specific cells does not vary significantly during MCMV infection.

CXCR6

CXCR6, initially described as a co-receptor for HIV, promotes T cell migration into tissues[228]. Expression of CXCR6 has also been detected on NK cells, NKT cells, dendritic cells, and activated T cells and is a marker for effector T cells in both humans and mice[229-231].

CXCR6 expression does not vary significantly between M38 and M45 specific cells during MCMV infection nor is it notably different from the levels expressed on CD8+ T cells throughout infection. In naive mice CXCR6 is expressed by all cellular subsets at low levels – CD3- (1.0-1.9%), CD4+ (1.4 – 1.9%) and CD8+ (0.4 – 2.2%) (arithmetic mean values from blood, liver, lung and spleen). Upon infection with MCMV CXCR6 expression is significantly enhanced in all cells most notably CD3- and CD4+ cells peaking at 6.0+/-1/4% CD3- cells in the blood and 7.0+/-0.96% hepatic CD4+ cells. In CD3- cells CXCR6 expression remains similar or declines over time however CD4+ and CD8+ T cells show much slower kinetics with heightened CXCR6 expression being maintained up to 100 days post infection although this is not statistically significant.

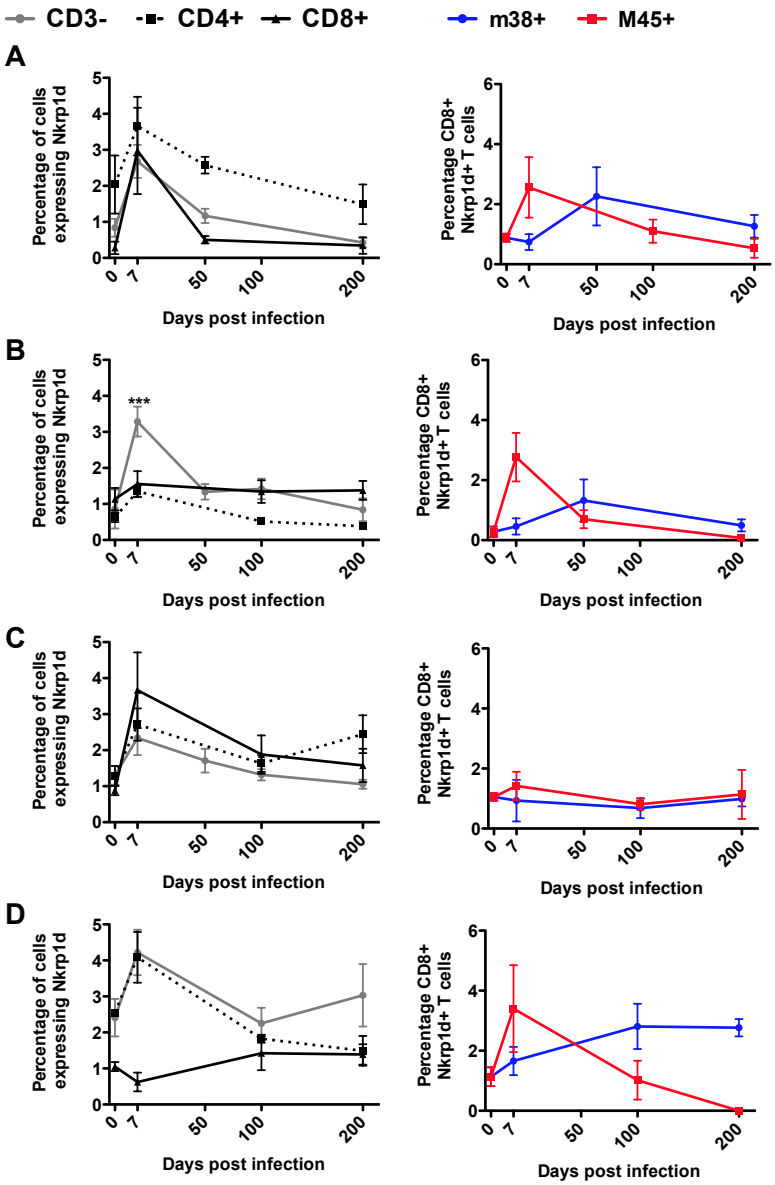

Figure 5.2 - NKR-P1d expression on T cells and CD3- cells after MCMV infection. C57BL/6 mice were infected with 1 x 10^6 pfu MCMV and organs harvested at indicated time points. NKR-P1d expression on CD3- (grey), CD4+ (dashed) CD8+ (solid) cells and on M38 (blue) and M45 (red) specific cells **A** Blood **B** Liver **C** Lung and **D** Spleen. (mean +/- S.E.M). * = $p<0.05$, ** = $p<0.005$, *** = $p<0.0005$ by Mann Whitney U test. Data represents 2 experiments per time point minimum n=4 per experiment.

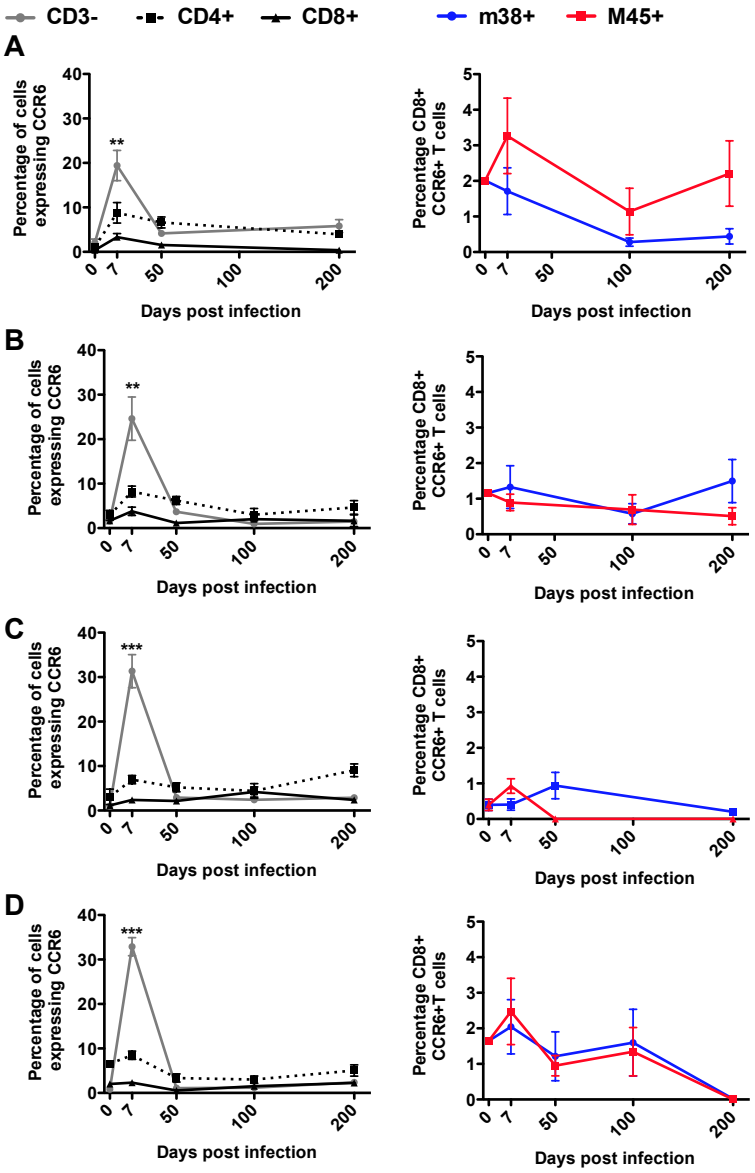

Figure 5.3 - CCR6 expression on T cells and CD3- cells after MCMV infection. C57BL/6 mice were infected with 1 x 10^6 pfu MCMV and organs harvested at indicated time points. CCR6 expression on CD3- (grey), CD4+ (dashed) CD8+ (solid) cells and on M38 (blue) and M45 (red) specific cells **A** Blood **B** Liver **C** Lung and **D** Spleen. (mean +/- S.E.M). * = p<0.05, ** = p<0.005, *** = p<0.0005 by Mann Whitney U test. Data represents 2 experiments per time point minimum n=4 per experiment.

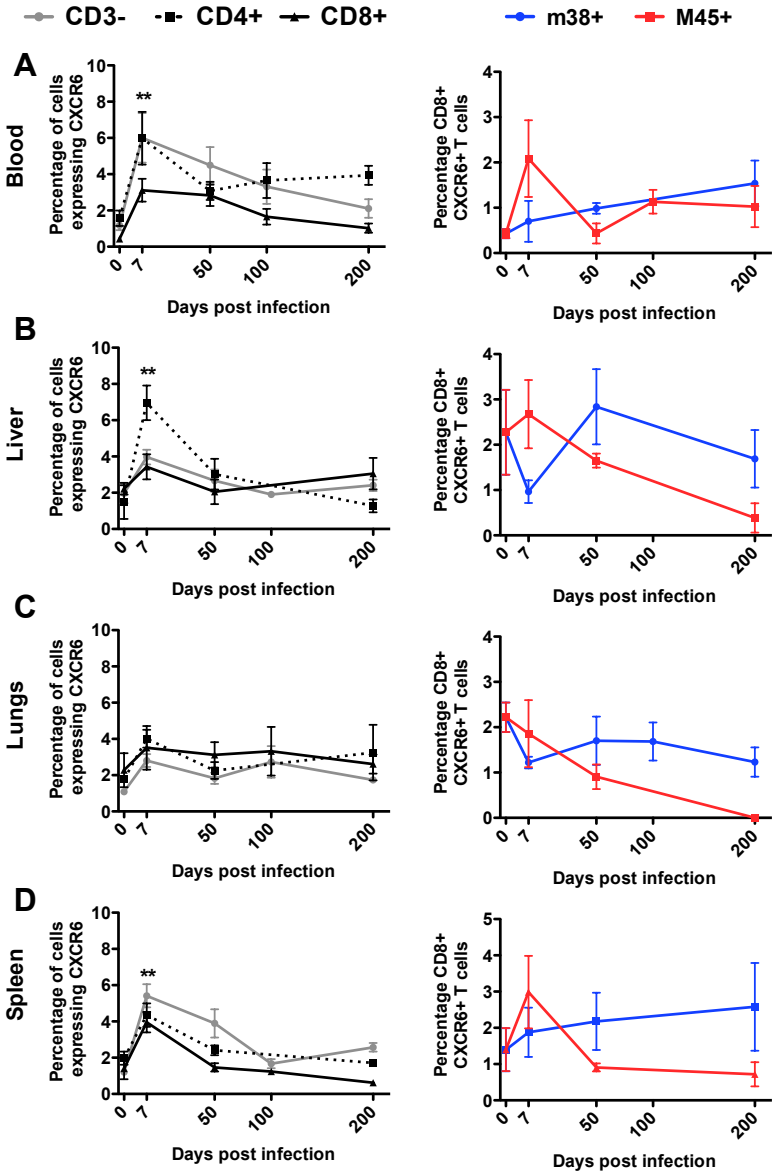

Figure 5.4 - CXCR6 expression on T cells and CD3- cells after MCMV infection. C57BL/6 mice were infected with 1 x 106 pfu MCMV and organs harvested at indicated time points. CXCR6 expression on CD3- (grey), CD4+ (dashed) CD8+ (solid) cells and on M38 (blue) and M45 (red) specific cells **A** Blood **B** Liver **C** Lung and **D** Spleen. (mean +/- S.E.M). * = p<0.05, ** = p<0.005, *** = p<0.0005 by Mann Whitney U test) Data represents 2 experiments per time point minimum n=4 per experiment.

CD8+ T cells secrete low levels of IL-17A except in liver and lung

CD3-, CD8+ and CD4+ T cells isolated from the spleens, livers, lungs, lamina propria lymphocytes (LPL) and mesenteric lymph nodes (MLN) of naïve mice can be induced to secrete IL-17A after stimulation with PMA/ionomycin (Figure 5.5).

CD3- and CD4+ cells from the lamina propria of the small intestine, liver and lung produce the highest amounts of IL-17A (0.2+/-0.06% of LPL CD3- and 1+/-0.22% LPL CD4+ [mean +/- S.E.M.]), with CD4+ cells secreting on average 5 times more IL-17A and than CD3- cells. Cells from the mesenteric lymph nodes of the gut were poor producers of IL-17A. CD8+ T cells from the liver and lung were the only CD8+ T cells capable of secreting IL-17A (0.38+/-0.049% and 0.21+/-0.029% respectively [mean+/-S.E.M.]).

Not shown here is data representing optimisation of these conditions where variation of stimulant (PHA, SEB, CON A and anti CD3 / anti CD28 antibodies), incubation time (6 hours versus 18 hours) and protein transport inhibitor (either monensin or brefeldin or monensin and brefeldin together) did not alter either the frequency of IL-17A secreting cells.

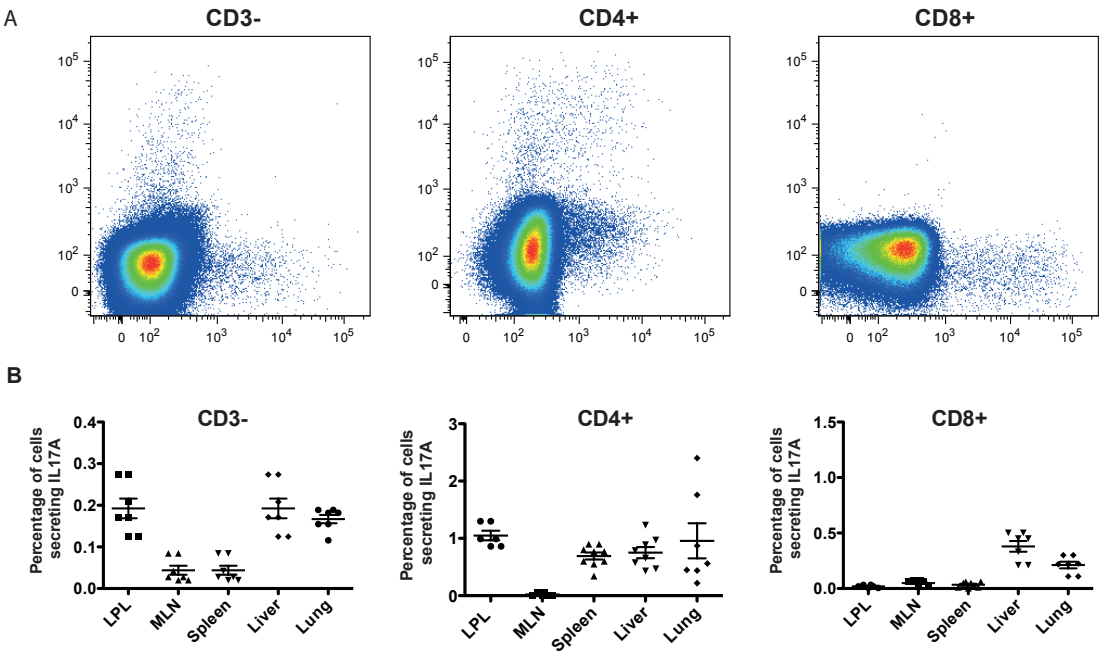

Figure 5.5 - IL-17A secretion by T cells and CD3- cells in naïve mice. Leucocytes were harvested from indication organs and stimulated with PMA / ionomycin for 6 hours. **A** FACS plots of hepatic leucocytes showing IL-17A secretion (y axis) versus TNFα (x axis) **B** Comparison of IL-17A secretion by different cell subsets in indicated tissues. LPL = lamina propria lymphocytes, MLN = mesenteric lymph nodes (mean +/- S.E.M). Data represents 2 experiments per time point minimum n=3 per experiment.

MCMV infection induces a small increase in IL-17A secretion

To examine if viral infection enhanced the amount of IL-17A cells that could secrete C57BL/6 mice were infected with 1 x 10^6 pfu MCMV and leucocytes examined for capacity to secrete IL-17A 7, 50, 100 and 200 days post infection (only 7 and 50 for LPL and MLN) in response to PMA/ionomycin, Additionally, at day 7 and day 50 peptide specific responses were examined to M38 and M45.

In MCMV infection CD4+ T cells displayed a significant increase in IL-17A secretion in the gut (both LPL and MLN), liver and lung when responding to PMA/ionomycin. In the spleen a similar increase was observed but was not significant. Within the liver CD3- cells significantly increased the amount of IL-17A secreted however, the magnitude was several fold less than observed with CD4+ T cells. (Figure 5.6).

CD8+ T cells showed a slight increase in IL-17A secretion in the LPL, MLN and spleen but at extremely low levels and did not reach significance. (Figure 5.6).

Peptide specific IL-17A secretion is shown in figure 5.7. In response to M38 and M45 a very low frequency of cells secrete IL-17A and results are highly variable. In acute infection IL-17A secretion in response to M38 can only be detected in the gut, by 50 dpi low levels can be detected in all tissues but is highly variable and does not rise above 0.2%. M45 specific responses are highest during acute infection in the LPL.

MCMV infection increases IL-17A secretion by CD4+ T cells in the gut, liver and lung although this is only transient. Peptide specific responses are low and variable. Coupled together these findings suggest tissue resident IL-17A CD4+ secreting cells are induced, albeit at low levels, in viral infections. CD8+ T cells responding to MCMV secrete little IL-17A in comparison to CD3- and CD4+ T cells. In the gut and spleen IL-17A secretion by CD8+ T cells increases from a baseline of 0 but interestingly IL-17A secretion in lung and liver is reduced after infection suggesting in mice IL-17A secreting CD8+ T cells are not tissue homing.

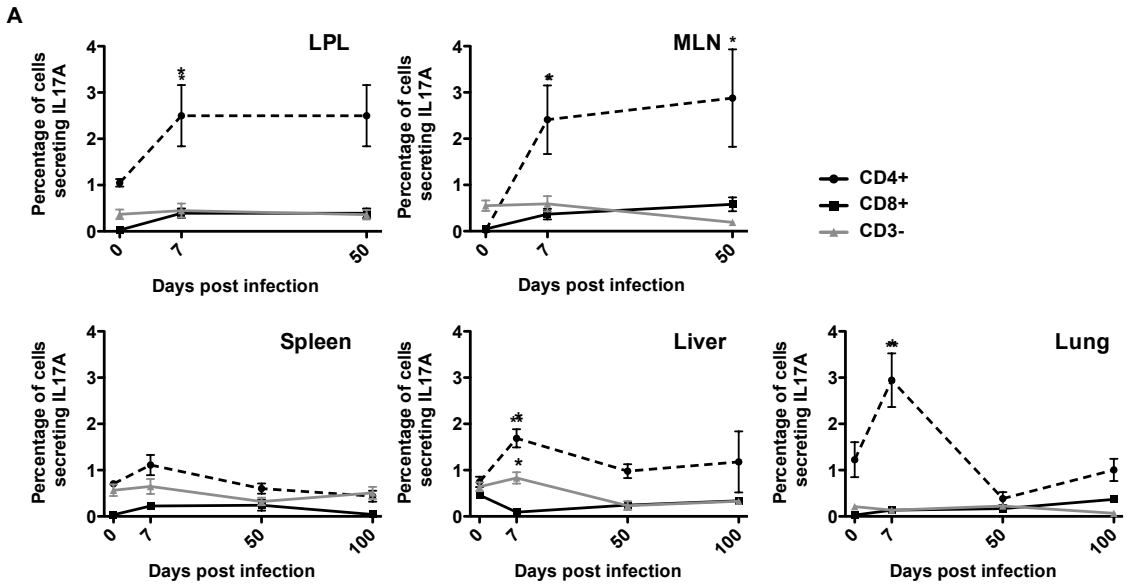

Figure 5.6 - Time course of IL-17A secretion by T cells and CD3- cells in MCMV infected mice. C57BL/6 mice were infected with 1 x 10^6 pfu MCMV and organs harvested at indicated time points. Leucocytes were harvested from indication organs and stimulated with PMA / ionomycin for 6 hours. **A** Comparison of IL-17A secretion by CD3- (grey), CD4+ (dashed) CD8+ (solid) in indicated tissues. LPL = lamina propria lymphocytes, MLN = mesenteric lymph nodes (mean +/- S.E.M). Data represents 2 experiments per time point minimum n=3 per experiment. . (mean +/- S.E.M). * = p<0.05, ** = p<0.005, *** = p<0.0005 by one way ANOVA with post test correction).

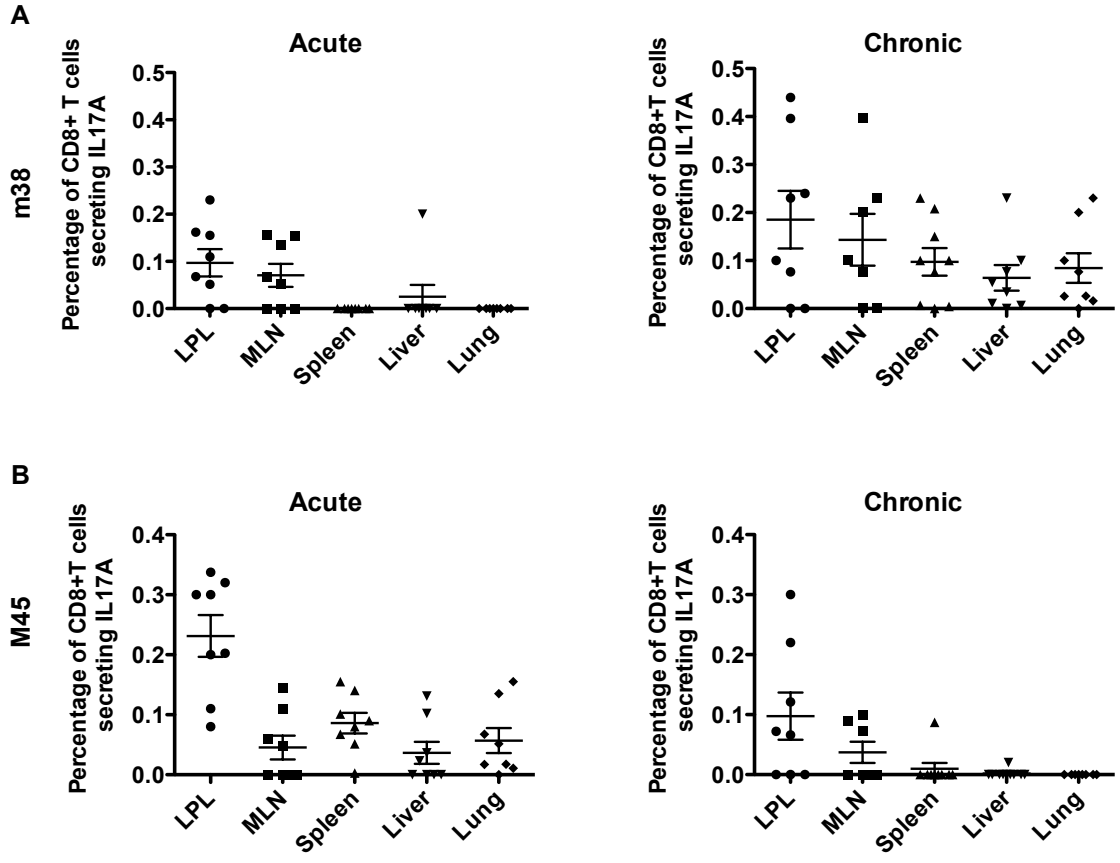

Figure 5.7 - IL-17A secretion in response to M38 or M45 peptide in MCMV infected mice. C57BL/6 mice were infected with 1 x 10^6 pfu MCMV and spleens harvested at 7 (Acute) or 50 (chronic) days post infection. IL-17A secretion in response to **A** M38 peptide and **B** M45 peptide. (mean +/- S.E.M). Data represents 2 experiments per time point minimum n=4 per experiment.

CD8+ T cells can be polarised to produce higher levels of IL-17A

Prolonged culture of either splenocytes or purified CD8+ T cells from mouse spleen in the presence of Tc17 polarising media, (20 ng/ml IL-6, 5 ng/ml TGF-β, 20 ng/ml IL-1β, 20 ng/ml IL-23, 10 μg/ml anti-IFN-γ, and 10 μg/ml anti-IL-4), leads to augmentation of both IL-17A secreting cells after stimulation with PMA/ionomycin in a standard ICS.

Splenocytes were harvested from naïve C57BL/6 mice and incubated in Tc17 polarising conditions for 5 days. In order to elicit whether other cells were inhibiting CD8+ T cell secretion of IL-17A a fraction of CD8+ T cells was purified (>88% CD8+) and exposed to the same conditions.

Higher frequencies of CD4+ T cells (3.9+/-0.4% [mean+/- S.E.M.]) could be induced to secrete IL-17A compared to CD8+ T cells (1.5+/-0.3% [mean+/- S.E.M.]) representing a significant increase in IL-17A secretion compared to a standard ICS. Enriching for CD8+ T cells did not significantly alter the frequency of IL-17A secreting cells (1.9+/-0.51 [mean+/-S.E.M.])(Figure 5.8 B).

To ensure the effect observed was a direct result was due to the polarizing conditions splenocytes were cultured in 10 fold dilutions on Tc17 media and IL-17A production measured after PMA/ionomycin stimulation. IL-17A secretion by CD8+ T cells decreased in parallel with the concentration of T c17 media 1.57+/-0.31% in full strength media, 0.63+/-0.2% at 1 in 10, 0.04+/-0.017% at 1 in 100 to undetectable levels at 1 in 1000 diluted Tc17 media [mean+/-S.E.M.](Figure 5.8 C).

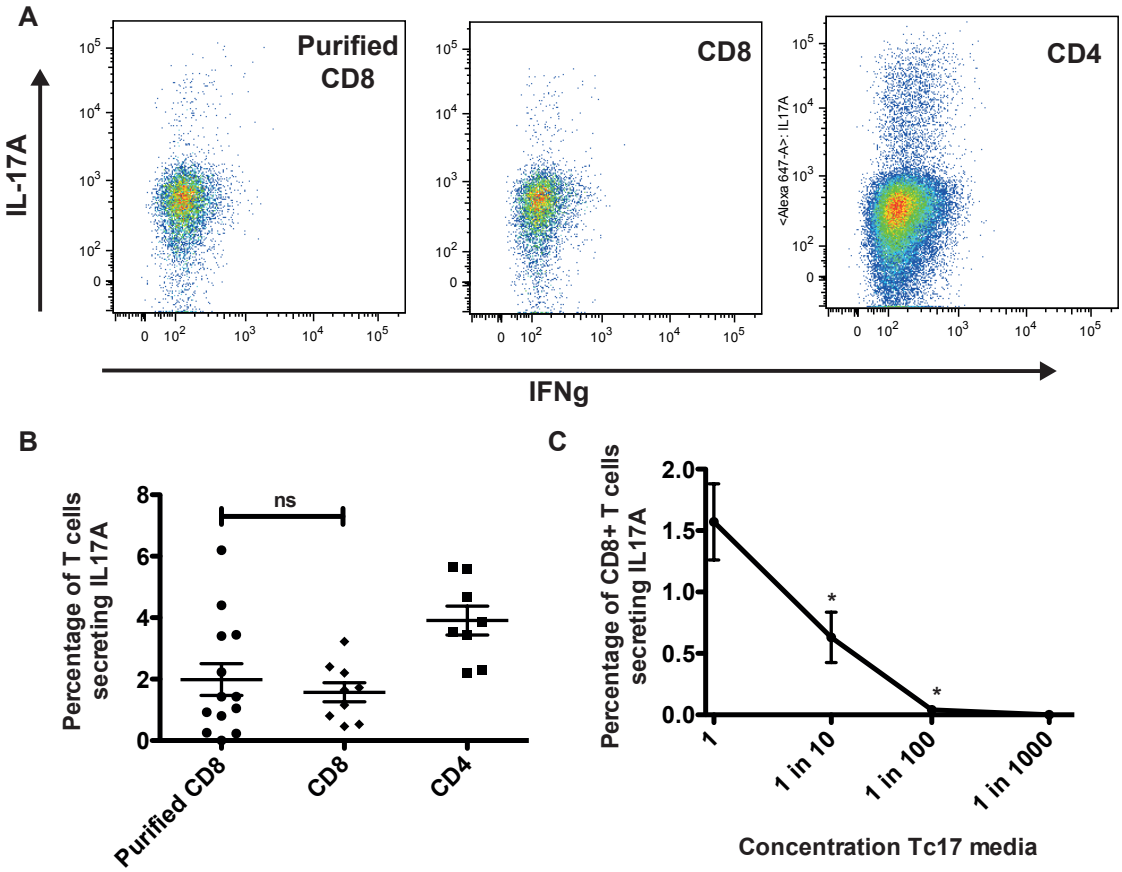

Figure 5.8 - T cells exposed to Tc17 polarising conditions secrete higher levels of IL-17A. Spleens were harvested from naïve C57BL/6 mice and cultured in Tc17 conditions. **A** FACS plots showing IFNγ (x axis) and IL-17A (y axis) secretion by purified CD8+ T cells and CD8+ or CD4+ T cells from cultured splenocytes. **B** Frequency of IL-17A secreting cells (mean+/- S.E.M) **C** Tc17 skewing media was serially diluted, splenocytes were cultured and IL-17A secretion measured in CD8+ T cells (mean+/- S.E.M.). Data represents **B** 2 -3 experiments per time point minimum n=4 per experiment **C** 1 experiment n=3. * ns = non significant by Mann Whitney U test.

Cells induced to secrete IL-17A express a similar pattern of markers seen in human Tc17 cells

Human Tc17 cells are characterized by the presence of several markers such as CCR6, IL-18Rα, CXCR6 and putatively NKR-P1d. In order to determine whether out polarized cells represented a similar population I examined the mean fluorescence intensities, (MFI) of these markers in IL-17A+ and IL-17A- CD8+ T cells.

IL-17A+ CD8+ T cells exhibit significantly higher MFI for CCR6 and IL-18Rα than IL-17A- CD8+ T cells. The MFI of NKR-P1d and CXCR6 did not vary between Il-17A+ and IL-17A- CD8+ T cells. (Figure 5.9).

This data suggests CD8+ T cells exhibiting some of the phenotypic markers associated with human Tc17 cells and MAIT cells can be generated in mice under strict conditions. Notably NKR-P1d expression was not increased on IL-17A secreting CD8+ T cells suggesting NKR-P1d may not be the correct homologue to human CD161.

Coexpression between Tc17 markers on CD8+ T cells in mice

A defining characteristic of MAIT cells in humans and mice and the larger Tc17 subgroup of cells in humans are the phenotypic characteristic described previously. Relatively small numbers of CD8+ T cells express NKR-P1d, CCR6 and CXCR6 and few secrete IL-17A or IL-17F. Certainly these phenotypic markers were not highly expressed on M38- or M45 specific CD8+ T cells. IL-18Rα, which is expressed at high levels after MCMV infection is discussed in Chapter 3 and 4. Analysis of co-expression of NKR-P1d, CCR6, CXCR6 and IL-17A (in a standard ICS) which would enable clear delineation of even a small subsets of Tc17 like cells in mice revealed extremely low levels of co-expression corresponding to less than 80 CD8+ T cells (data not shown).

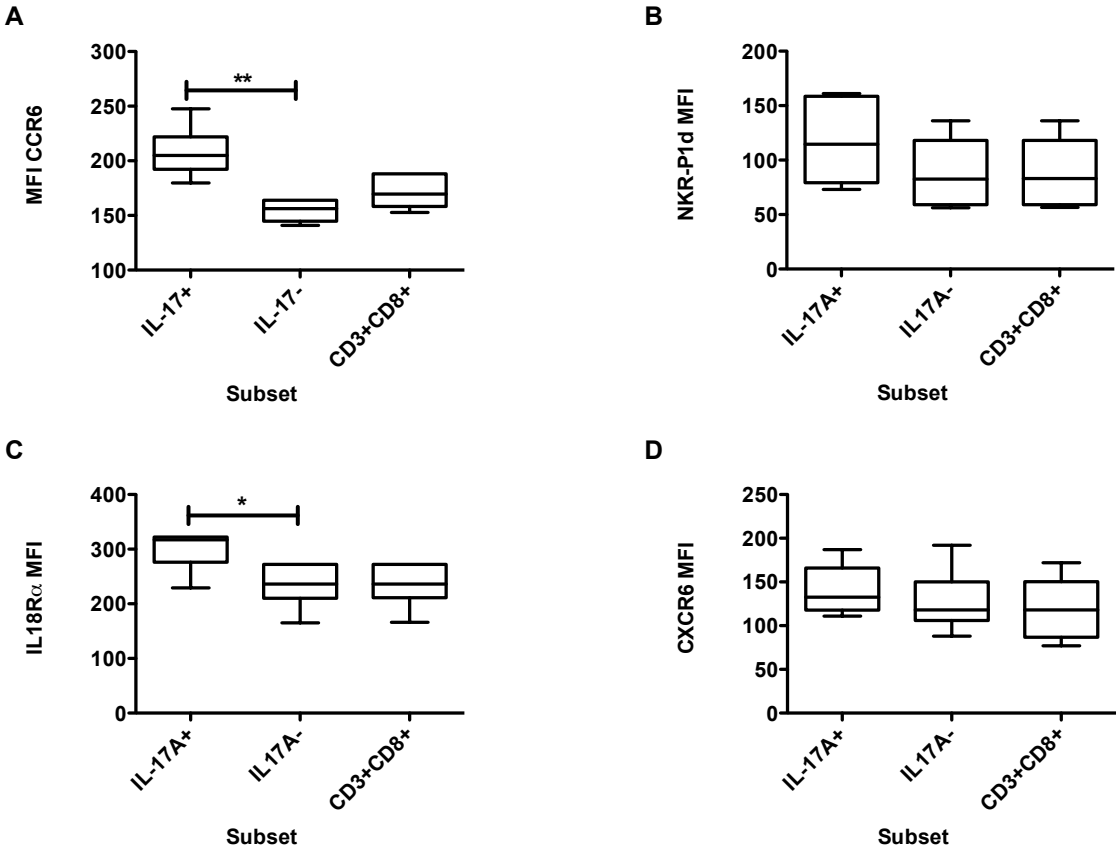

Figure 5.9 - IL-17A+CD8+ T cells exposed to Tc17 polarising conditions display some MAIT cell characteristics. Spleens were harvested from naïve C57BL/6 mice and cultured in Tc17 conditions. Mean fluorescence intensities of **A** CCR6, **B** Nkrp1d, **C** IL-18Rα and **D** CXCR6 was examined on IL-17A+ and IL-17A- cells (data from CD8+ T cells shown for comparison). Box and whisker plots showing minimum and maximum values. * = $p<0.05$, ** = $p<0.005$, *** = $p<0.0005$ by paired t test. Data represents 2 experiments per time point minimum n=4 per experiment.

CD8+ T cells from MCMV infected mice secrete higher levels of IL-17A in response to peptide

In view of the successful polarization of naïve splenocytes C57BL/6 mice were infected with 1 x 10^6 pfu MCMV and spleens harvested 7 or 50 days post infection and subjected to Tc17 polarising conditions in order to elicit if peptide specific IL-17A responses could be augmented.

Exposure to Tc17 polarising media significantly increased the amount of IL-17A secreted in response to M38 and M45 although once again the actual number of cytokine secreting cells was very low and insufficient to phenotype with typical MAIT/Tc17 markers (Figure 5.10).

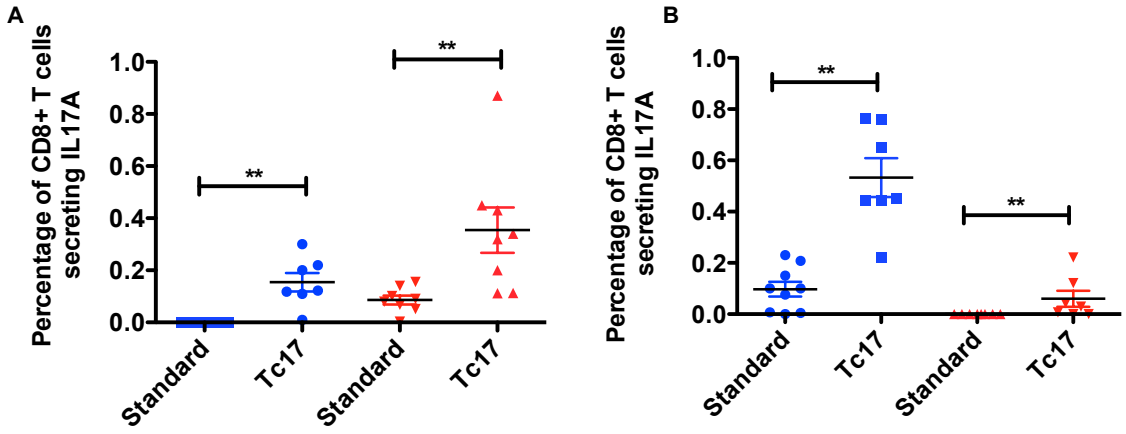

Figure 5.10 - Tc17 polarisation significantly increases peptide specific IL-17A secretion. C57BL/6 mice were infected with 1 x 10^6 pfu MCMV and spleens harvested at A 7 (Acute) or B 50 (chronic) days post infection. IL-17A secretion in response to M38 (blue) or M45 (red) peptide was assessed in a standard ex vivo ICS or after exposure to Tc17 polarising conditions. * = $p<0.05$, ** = $p<0.005$, *** = $p<0.0005$ by Mann Whitney U test. Data represents 2 experiments per time point minimum n=4 per experiment.

Adding IL-21 to Tc17 media significantly increases IL-17A and IL-17F secretion

IL-21 is the most recently identified member of the type I cytokine family. Produced by CD4+ T cells including Th17 cells, NKT cells and T-follicular helper cells found in the B-cell areas of secondary lymphoid tissue, IL-21 plays a role in innate as well as adaptive immune responses[232]. IL-21 can stimulate human CD8+ T cells to secrete IL-17 in the presence of TGFβ[232,233]. To elicit whether IL-17A or IL-17F secretion could be further augmented by other cytokines IL-21 was added to Tc17 media and splenocytes from naive mice cultured. 5 days later IL-17A and IL-17F secretion was examined.

Addition of IL-21 to Tc17 polarising media significantly increased the frequency of IL-17A and IL-17F secreting CD4+ and CD8+ T cells. (Figure 5.11).

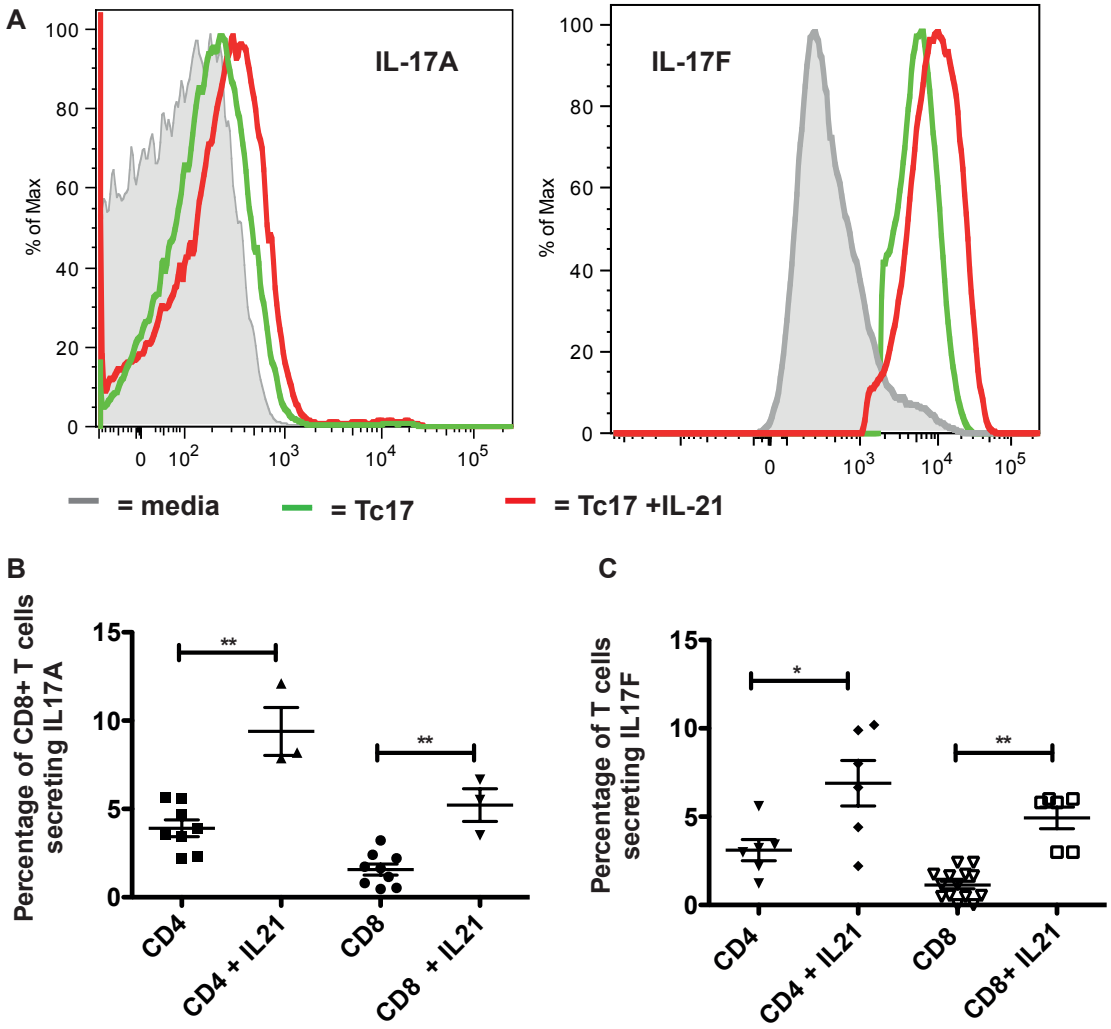

Figure 5.11 - Both IL-17A and IL-17F secretion is increased by addition of IL-21 to Tc17 polarising media. A Overlaid histograms illustrating IL-17A and F secretion by CD8+ T cells media control (shaded grey), standard T c17 (green) and Tc17 + IL-21 (red). Frequency of **B** IL-17A and **C** IL-17F secretion by CD4 and CD8 T cells exposed to different polarizing conditions.* = $p<0.05$, ** = $p<0.005$, *** = $p<0.0005$ by Mann Whitney U test. Data represents 2 experiments per time point minimum n=3 per experiment.

Discussion

NKR-P1d does not identify a CD161 bright or dim equivalent in mice

It is apparent that MCMV in immune replete mice does not provide an obvious orthologous subset of T cells to human CD161+CD8+ T cells or MAIT cells. Conservation of sequence between human and mouse is poor (approx. 50% homology at best in terms of amino acids). Whilst staining for NKR-P1d can be demonstrated and the frequency of NKR-P1d amplified by MCMV infection the pattern of expression on CD8+ T cells is of low magnitude and dissimilar to that found in humans with no bright and mid subsets of CD161+ T cells. However in hepatic tissue during acute infection there is an expansion of CD3- cells (likely representing NK cells) and M45-specific CD8+ T cells expressing NKR-P1d, which although of relatively low magnitude suggests a tissue homing subset of cells may exist in C57BL/6 mice. CCR6 expression was up regulated in MCMV infection in all tissues examined on both CD3- and CD4+ T cells but no significant change was observed in the CD8+ T cell subset. Similarly CXCR6 expression in MCMV infection did not identify a consistent subset of cells although increased expression was observed on hepatic CD3- and CD4+ T cells. IL-17A secretion was detectable in the liver and lung of naïve mice but this did not augment during MCMV infection where instead higher levels of IL-17A could be detected in the spleen and gut. To this extent there is a similarity between mouse and human data, where Tc17 cells are clearly enriched in liver tissue. As discussed previously, MAIT cells are found at low frequency in mice thus necessitating generation of a transgenic mouse; it is possible in an immune replete model these cells are occurring at such low frequency within the NKR-P1d subset they are not easily demonstrable using conventional techniques.

Cells expressing MAIT or Tc17 like phenotypic markers could be found in the CD3- and CD4+ subset suggesting in mice perhaps the ontogeny of Type 17 lymphocytes is different. With an absence of Tc17 cells. Most notably after MCMV infection a population of CD3- CCR6+ CXCR6+ cells were detected in the liver at higher frequencies.

The dearth of Tc17 cells in the model again could be a reflection of low frequency of cells in immune replete mice however review of current literature suggests Tc17 cell ontogeny in mice may be markedly different to humans. It is known that the intestinal microbiota can influence the presence and absence of Th17 cells whereby mice housed in germ free conditions do not generate Th17 cells but can be induced to by the introduction of certain types of bacteria. In addition, MAIT cells are not present in germ-free mice, indicating that commensal flora is required for their expansion in the gut lamina propria. Differentiation of Th17 cells correlated with the presence of cytophaga-flavobacter-bacteriodetes (CFB) bacteria in the small intestine or with the presence of segmented filamentous bacteria. When previously germ free mice were colonised by SFB, the gene showing the highest level of expression in the terminal ileum was that for serum amyloid A (SAA). This evidence suggests a close relationship between intestinal microbiota, and both Th17 and Tc17 cell lineages; commensals that may not be present in the mouse facilities we currently use. Additionally CD8+ T cells polarized in vitro to produce IL-17 when adoptively transferred in either vaccinia virus or influenza virus models either entirely lose capacity to secrete IL-17 or retain it but acquire capacity to secrete IFNγ, again suggesting this particular Tc17 phenotype is not fully stable in mice for reasons as yet unclear. In a collagen antibody-induced arthritis murine model MAIT cells are shown to exacerbate inflammation but these cells can only manipulated using mice deficient in MR-1 and transferring Vα19 transgenic T cells polarized in similar condition to above[234,235].

Conclusions

In this study I examined the phenotype and function of NKR-P1d+ T cells in mice to establish if they exhibited homologies to CD161+ subsets in man. I examined phenotype, function and distribution, especially in relation to the liver. In parallel I examined the frequency and distribution of Tc17 cells, which in man are a subset of CD161+ T cells and which home to the liver. Finally I examined and the impact of MCMV on NKR-P1d expression and on Tc17 responses. I addressed and answered the following questions.

1. Does NKRP1d identify a functionally equivalent set of cells to man? No
2. Is NKRP1d associated with liver homing populations? No.
3. Is NKRP1d associated with other phenotypic markers of a type 17 response? No.
4. Is NKRP1d expression influenced by MCMV? Yes. The most substantial changes were seen in CD3- (likely NK) subsets especially in the liver. Amongst T cells, an enrichment was seen in MCMV-specific populations but only in those homing to the liver in acute disease
5. Do Tc17 cells exist in naïve mice? Yes and they can be readily expanded in vitro.
6. Are Tc17 cells in mice enriched in the liver? Yes, compared to other organs analysed.
7. Do Tc17 cells associated with similar markers in mouse and man? Broadly speaking our data is inconclusive. IL18Rα and CCR6 up regulation is seen in Tc17 cells expanded in vitro mice, but not CXCR6 and NKRP1d.
8. Does MCMV impact on Tc17 subsets? No. The frequencies of antigen-specific T cells expressing IL-17 were detectable but very low. However, substantial and sustained impacts on CD4+ T cells expressing IL-17 were noted, especially in lung, liver and gut.

Chapter 6

Discussion

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Summary

In this thesis I have identified a transiently protective role for MCMV in secondary infections that is IL-18 dependent and appears to be mediated by memory T cells expressing IL-18Rα. Whilst non-cognate functions of memory CD8+ T cells have previously been shown this is the first time marked effects on two human pathogens have been observed and a mechanism defined.

I have observed that absence of IL-18 and IL-18Rα does not affect the magnitude, phenotype or function of memory inflating cells in MCMV infection. However primary responses to MCMV in IL18-/- and IL-18Rα consist of far higher numbers of leucocytes. My data shows IL-18Rα deficient mice do not control MCMV viremia unlike IL-18-/- mice supporting the popular hypothesis that IL-18Rα may be promiscuous and respond to other cytokines.

In healthy humans CD8+IL-18Rα+ T cells contain a subset of CD8+ T cells termed MAIT cells which express high levels of the C-type lectin CD161 and a pattern of molecules consistent with type 17 differentiation. I have shown in C57BL/6 mice NKR-P1d is not homologous to CD161 and indeed cells equivalent to the human CD161++ population are extremely difficult to detect in mice.

In this discussion I will examine the relevance of our findings in a broader context and identify the most promising research avenues generated by this thesis.

Does infection with human CMV induce memory cells capable of protecting against challenge?

I sought to elicit whether the MCMV induced protection observed by Barton et al. was dependent on IFNγ production by MCMV induced memory CD4+ and CD8+ T cells. Significantly any protection observed was abrogated in IL-18 and IL-18Rα deficient mice and I theorise this is due to the lack of IFNγ production by memory CD4+ and CD8+ cells responding to IL-12 and IL-18. The potential implications in terms of human health are large and will discuss these in this section.

Human cytomegalovirus (HCMV) is a ubiquitous β-herpesvirus and 60–80% of people in the developed world display evidence of infection by the age of 80 years[1]. However the incidence and prevalence of CMV infection varies between the developed and developing worlds. In Germany and the UK the seroprevalence of HCMV has declined in younger age groups and this correlates with the type of health insurance, gender, and unsurprisingly, HIV-status[236]. Comparing age matched adolescents in Malawi and the UK marked differences in CMV seroprevalence (100% vs 36% Malawi vs. UK) and notable differences in the proportions of naïve and memory T cells as one would typically expect after CMV infection[237]. Indeed in The Gambia CMV infection is endemic in infants, with 62% infected by 3 months and 85% by 12 months of age. In CMV infected infants antigen-specific cells identified using tetramers remained more activated and differentiated than the overall CD8 population (CD28-CD62L-Bcl-2loCD95+perforin+ and CD45RO+HLA-D+) and strikingly cells that emerged rapidly after infection remained stable after two years[238-240].

MCMV induced protection is only transient – it may be that memory T cell IFNγ secretion can offer a short-term boost to immunity in these infants and protect against common diseases such as diarrhoea or pneumonia (Figure 6.1). However profound differences induced in the CD8+ T cell compartments of such young infants may have long ranging effects though when considered in the context of vaccination using live or live attenuated vaccines.

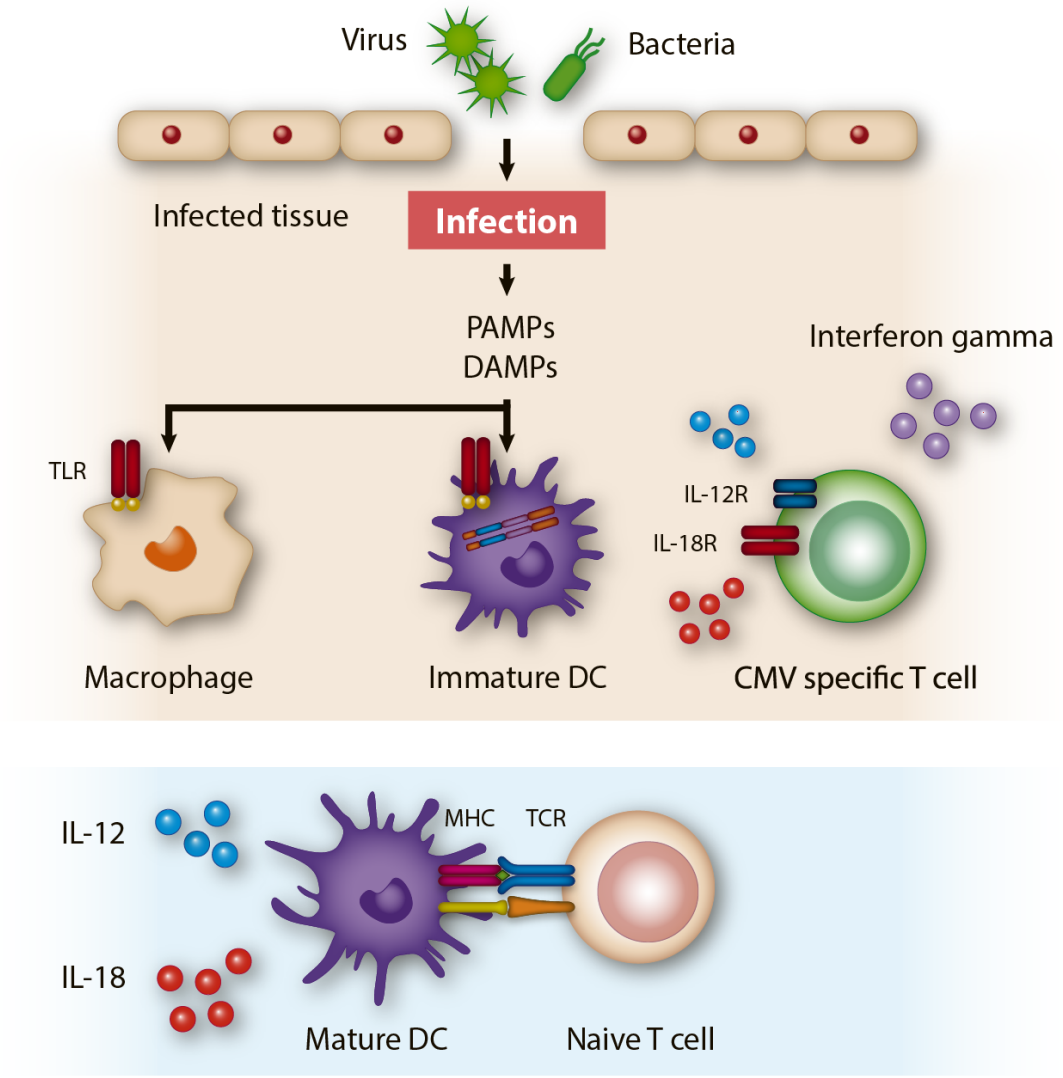

Figure 6.1 – Tissue resident CMV specific cells may mediate protection against infection. Breach of cutaneous or mucosal barriers by pathogens triggers TLRs and NLRs on macrophages and dendritic cells causing production of IL-12 and IL-18. Tissue resident CMV-specific T cells are stimulated by IL-12 and IL-18 and produce IFNγ, which aids the innate immune system to fight pathogens.

Widespread vaccination has been one of the most successful public health strategies to combat infectious disease worldwide. However, the efficacy of vaccines in Sub-Saharan Africa is lower than in high income settings e.g example efficacy of the live attenuated measles vaccine is typically over 90% in Europe and North America but below 70% in West Africa[241-243]. Vaccine efficacy may be affected by concurrent infections suggesting CMV may impair vaccine responsiveness[244]. Data from Gambian infants and children after measles vaccination is conflicting with one study showing the early T cell response to measles vaccine (1 week post vaccine) is lower in CMV infected children but this difference is absent 4 weeks post vaccination[245]. Furthermore EBV rather than CMV is shown to impair measles specific antibody responses after vaccination suggesting in this context non-cognate secretion of IFNγ does not play a role[245].

Given the transient nature of protection I observed it is possible effects on disease or live vaccines may only be truly noticeable when CMV infection is relatively recent – by 3 months post infection any MCMV protection had waned. Figure 6.2 illustrates the theory a threshold level for IFNγ produced by memory T cells may exist and this corresponds to a relatively short period of time in terms of the lifelong nature of CMV infection.

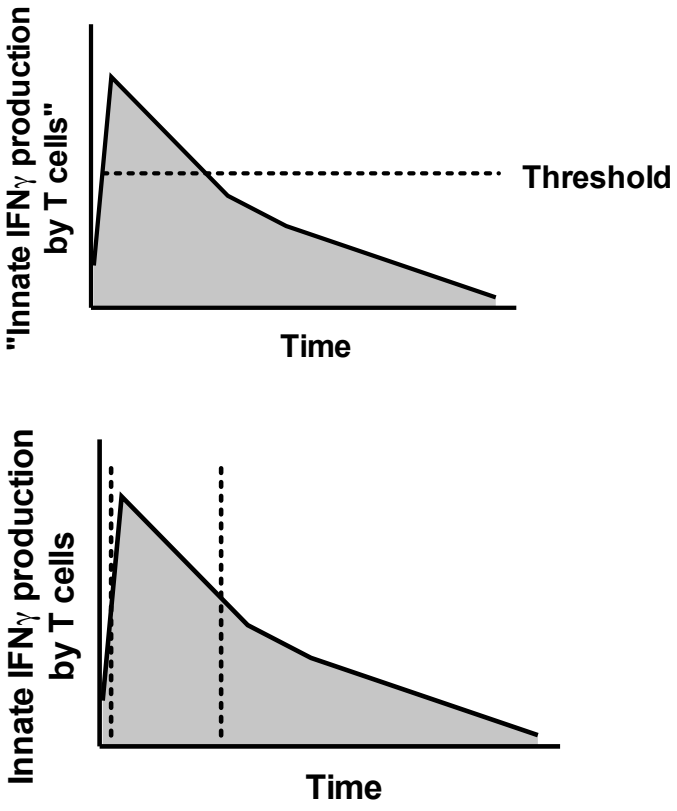

Figure 6.2 - Schematic showing A Threshold level of protection generated by memory T cells in CMV infection. **B** How this correlates to a relatively small window of time.

However CMV infection is not static, with frequent low-level reactivation occurring. As this occurs subclinically in healthy individuals it is difficult to detect but recipients of stem cell transplantation (HSCT) who are at risk of CMV disease are often closely monitored for both CMV viremia and the development of CMV specific immunity. In CMV+ individuals receiving CMV+ HSCT expansion of pp65 specific cells occurred rapidly suggesting in the presence of active disease CMV- specific T cells can respond quickly[246]. It has been shown that pp65 specific cells in acute and latent stages of CMV infection exhibit higher levels of IL-18R1 transcripts than naïve cells[69,247].

Superinfection can occur in MCMV despite adequate MCMV specific humoral and cellular immunity. Mice infected with the G4 naturally occurring variant of MCMV and then 28 days later with the K181 MCMV strain have mixed infections detectable in the salivary glands and lungs. MCMV-specific antibody in sera and G4 IE1-specific CD8+ T cells responses in the spleens of twice-infected mice failed to prevent reinfection[248]. Furthermore postmortem samples from humans shows that multiple human cytomegalovirus strains can be detected, supporting the idea superinfection in humans is possible[249]. Superinfection of rhesus CMV–infected rhesus macaques when using RhCMV as a vector show that CD8+ memory T cells responding to RhCMV IE-1 protein are boosted in frequency after superinfection as are CD8+ T cells responding to the vaccine insert[43,250].

Can memory T cells in humans behave in an innate fashion?

A subset of human CD8+ T cells can secrete IFNγ when stimulated with IL-12 and IL-18 although the magnitude of the IFNγ response by CD8+ T cells varied widely among individuals (2.3%+/-1.3% (mean+/- S.E.M.)) with 2/16 individuals not responding at all and 2/16 displaying 5-6% CD8+ T cells making IFNγ[251]. The authors had not assessed CMV seropositivity in this group. Other work reveals addition of IL-15 and/or IL-7 to cultures of human CD8+ T cells profoundly reduced the amount of IL-12/IL-18 required to generate IFNγ[252,253]. IFNγ production was exclusive to effector and central memory T cells. Importantly in both data sets discussed cells were stimulated in vitro for 15 – 48 hours prior to IFNγ quantification.

In total there is evidence suggesting CMV infection in humans produces a persistent T cell response, IL-18Rα is expressed by CMV–specific T cells and CMV–specific T cells are expanded during reactivation. When considered with data from Rhesus macaques showing CMV superinfection transiently expands the CMV specific T cell response and recent data showing human memory CD8+ T cells can respond to IL-12/IL-18 it is clear any further investigation of this phenomenon in humans would be worthwhile. Of note, recent data from the Oxford lab suggest that release of IFNγ in response to IL-12 and IL-18 is restricted to CD161++ T cells; however this is markedly enhanced in all T cell populations by addition of IL-15 (Willberg et al, manuscript submitted).

Could IFNγ from memory T cells be harmful?

$CD8_+$ T cells producing IFN γ in response to cytokines triggered by infection with *Listeria monocytogenes* and provide innate protection in a non–antigen- specific manner is obviously beneficial[177,178]. Conversely there also exists evidence memory CD8+ T cells secreting IFNγ may be pathogenic in certain states.

During the innate non-Ag-specific early phase of infections, IFN γ has generally been believed to be produced primarily by NK and NKT cells. However visualization of IFNγ secreting cells in the spleens and lymph nodes of LPS or polyI:C injected mice reveal CD8+CD44+ T cells represent 30 % of splenic and 70% of LN IFNγ secreting cells[253]. Gram-negative bacterial infections can elicit an endotoxin-mediated state of shock exacerbated by a cytokine storm that includes IFNγ-mediated immunopathology due to CD8+ T cells and NK cells. During LCMV-Armstrong infections, mice are far more sensitive to the lethal effects of bacterial endotoxin LPS, and significant mortality observed at up to 10-fold lower doses in LCMV infected than in uninfected mice. Profiling of serum cytokines indicated LCMV-infected mice had 3- to 50-fold higher peak serum concentrations of TNF, IL-12, and IFNγ levels after LPS administration. In this system NK and T cells were the primary sources of IFNγ and protection required depletion of both cell subsets[254].

Dengue viral infection is the cause of dengue fever (DF) and the more severe dengue hemorrhagic fever (DHF); secondary infection by dengue virus of different serotypes to the primary infection is significantly associated with severe DHF believed to be due to bystander T cell activation. In DHF T cell mediated production of IFNγ has been reported to influence the severity of disease and actually 50% of IFNγ production in these circumstances is due to IL-12/IL-18 and IL-15 dependent bystander T cell activation[255]. In HIV one current hypothesis for decline in CD4+ count is that activation-induced cell death occurring in CD4+

T cells is in part due to "bystander" activation[256]. The role of CMV in HIV disease is clearly complex, but it is plausible that the mechanisms described here could play a role.

IL-18 and IL-18Rα interaction

IL-18 and IL-18Rα have been clearly shown to be dispensable in the development of the adaptive immune response to MCMV. Most notably however, is the fact that despite a T cell response that is equal in magnitude and phenotype to that observed in IL-18Ra replete mice, IL-18Rα deficient mice fail to fully control MCMV viremia. Disparate behavior in murine models between IL-18 and IL-18Rα-deficient mice and cells has been reported previously. One study showed that IL-18Rα-deficient mice were resistant to experimental autoimmune encephalomyelitis (EAE), whilst IL-18-deficient mice were susceptible[159]. This suggests involvement of an IL-18Rα ligand other than IL-18 in the pathogenesis of EAE. In fact, engagement of IL-18Rα on antigen-presenting cells was required for the generation of pathogenic IL-17-producing Th17 cells, while IL-18 was dispensable. In a murine model of diabetes similarly, divergent responses of IL-18 or IL-18Rα-deficient pancreatic islets were observed[160]. In this context it has been postulated that IL-18Rα may convey an anti-inflammatory signal, independently of IL-18, in the context of inflammation-mediated islet injury. The IL-1 family of cytokines has not been fully characterized and six additional members of the IL-1 family of ligands have been identified in the last 10 years[257]. Transgenic mice expressing the newly discovered IL-1F6 in basal keratinocytes develop skin abnormalities dependent on IL-1Rrp2 and IL-1RAcP – which is closely related to IL-18Rβ - which can be ameliorated by IL-1F5[258]. It is entirely possible as yet undiscovered ligands for IL-18Rα, particularly in the mouse, exist.

The binding of IL-18 to IL-18Rα induces not only protective but also some pathogenic functions during infection, depending on the context in which it occurs. Several studies show that elevated serum levels of IL-18 are associated with poor clinical outcome in severe inflammatory and

septic shock type conditions[259-261]. IL-18 is highly expressed in the liver of C57BL/6 mice following lethal infection with highly virulent *Ixodes ovatus ehrlichia* (IOE), an obligate intracellular bacterium that causes acute fatal toxic shock-like syndrome. Infection of IL-18Rα$^{-/-}$ mice with IOE resulted in enhanced bacterial clearance, and prolonged survival coupled with significantly less host cell apoptosis, decreased hepatic leukocyte recruitment, compared with infected WT mice, suggesting a pathogenic role for IL-18/IL-18Rα in Ehrlichia-induced toxic shock[262]. Thus in this context IL-18 and IL-18Rα interaction is protective but in overwhelming infection or sepsis it appears it maybe pathogenic perhaps eliciting a cytokine storm suggesting manipulation of this axis may not be a feasible method of boosting immunity.

Chronic viral infections can produce a number of outcomes in man and mouse, and high levels of viremia and thus antigen can negatively impact T cell function. First noted in the LCMV model, one key outcome is loss of T cell functionality, such as autocrine cytokine production, leading ultimately to lack of proliferation and deletion of responses, a process termed exhaustion[263]. Related observations have been made in human infections such as HIV, and in both mouse and man T cell exhaustion is associated with high expression of inhibitory receptors notably PD-1. Although CMVs are persistent and the inflationary T cell responses show features of repetitive antigen exposure, they do not show features of exhaustion along the lines of the LCMV model[61,264]. Indeed in LCMV effector and memory, but not exhausted, CD8+ T cells produce IFNγ exposure to certain combinations of IL-12, IL-18, and IL-21. The unresponsiveness of exhausted CD8+ T cells is associated with downregulation of the IL-18Rα[265]. Loss of innate function by T cells may partially explain increased susceptibility to bacterial infection seen in HIV.

Of mice and men

MAIT cells are present in low numbers in mice and are naïve, in contrast to humans. The difference might come from environmental factors, such as specific microbial communities as discussed earlier. The genetic bottleneck of laboratory mice that results from the

establishment of experimental mouse lines from a few individual mice may have resulted in loss of the genes controlling MAIT cell development or survival. It is possible that the functions of MAIT cells in humans are dealt with by other cellular subsets in mice – such as NKT cells. NKT cells are narrowly defined as a T cell lineage expressing NK lineage receptors, including NK1.1 in the C57BL/6 background, in addition to semi-invariant CD1d-restricted αβ TCRs[137,266]. MAIT cells are numerous in human liver and NKT cells relatively scarce, whereas in mice high numbers of CXCR6 expressing NKT cells are found in mouse liver[266]. Indeed NKT cells in human tissues are found at 10 times lower magnitude than in mice[60,266]. The different usage of one or the other populations between species could be due to overlapping effector functions for MAIT and NKT cells or different evolutionary pressures.

Within this thesis it has been clearly demonstrated that phenotypic and functional characteristics associated with MAIT cells, tissue homing CD161+ cells or self renewing memory stem cells are not induced in CD8+ T cells by MCMV infection barring upregulation of IL-18Rα expression.

Conclusion

It would be entirely reasonable to question the importance of a transient effect on pathogens induced by MCMV. It is, however, worth remembering the transient immunosuppression caused by infection with measles virus is responsible for the vast majority of mortality associated with infection and is prevented by vaccination[267]. Thus a CMV induced transient protection from infection, particularly in the developing world could be highly significant and is worthy of further exploration.

In this thesis I have clearly shown

- IL-18 and IL-18Rα are dispensable in the generation of the adaptive immune response to MCMV. However, surprisingly, signaling through IL-18Rα – but not via IL-18 - appears to be required for full control of viremia. The mechanism responsible for the control of MCMV we have uncovered and the soluble mediator involved are worthy subjects of further study
- IL-18Rα memory T cells induced by MCMV can secrete IFNγ without cognate antigen at levels sufficient to be protective during bacterial challenge. I did not formally prove that T cell derived IFNγ is required for protection, but I did prove that IL18 is required for the MCMV-induced protection. Further experiments to define the pathway in more detail could be readily devised.
- In mice an extremely small proportion of CD8+ T cells display a Tc17 phenotype and this is not linked to expression of NKR-P1d. However, it is linked to liver homing CD8+ T cell populations. The specificity of such cell populations – although very small compared to man - requires further analysis.

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