The role of B cells during acute and latent infections with murine cytomegalovirus and Leishmania major

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Abbreviations

Aa	amino acid
Ab	antibody
APC	antigen presenting cell
approx.	approximately
BCR	B cell receptor
Bio	biotinylated
BM	bone marrow
b-ME	b-mercaptoethanol
bp	base pair
Breg	regulatory B cell
BSA	bovine serum albumin
BTLA	B and T lymphocyte attenuator
°C	temperature in degrees celsius
CD	cluster of differentiation
CFA	Complete Freund's Adjuvant
cDNA	complementary DNA
CFSE	carboxyfluorescein diacetate succinimidyl ester
Cre	site-specific recombinase (causes recombination)
CTLA-4	cytotoxic T lymphocyte antigen-4
Cyc	cychrome
d	day/s
DC	dendritic cell
DMEM	Dulbecco's modified Eagle medium
DNA	desoxyribonucleic acid
dNTP	desoxynucleotide triphosphate
dpi	days post infection
DTT	dithiothritole
DTx	Diphtheria toxin
e.g.	exempli gratia (for instance)
EDTA	ethylene-diaminetetraacetic acid
ELISA	enzyme-linked immuno-sorbent assay

ER	estrogen receptor
ES	embryonic stem
EtOH	ethanol
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
Fig.	Figure
FITC	fluorescein isothiocyanate
Flp	site-specific recombinase, product of yeast *FLP1*-gene
FoxP3	forkhead box protein 3
FRT	Flp recombination target
g	gramm
GITR	glucocorticoid-induced TNFR-related gene
Gp	glycoprotein
h	hour/s
hCMV	human cytomegalovirus
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid
i.e.	id est (that is)
IE	immidiate early
i.p.	intraperitoneally
i.v.	intravenously
IFN-γ	interferon-γ
Ig	immunoglobulin
IL	interleukin
kb	kilobase pair
l	liter
LN	lymph node/s
loxP	recognition sequence for Cre (locus of X-ing over of phage P1)
LPS	lipopolysaccharide
Ly6C	lymphocyte antigen 6 complex, locus C
M	molar
MACS	magnetic activated cell sorter
mCMV	murine Cytomegalovirus
MFI	mean fluorescence intensity
$MgCl_2$	magnesium chloride

MHC	major histocompatibility complex
min	minute
ml	milliliter
mM	millimolar
MOG	myelin oligodendrocyte glycoprotein
mRNA	messenger RNA
NaCl	sodium chloride
n	number
n	nano
NaOH	sodium hydroxide
neo	neomycin resistance gene
NK cell	natural killer cell
o/n	over night
ORF	open reading frame
PBS	phosphate buffered saline
PC	peritoneal cavity
PCR	polymerase chain reaction
PD-1	programmed cell death 1
pDC	plasmacytoid dendritic cell
PE	phycoerythrine
PFU	plaque forming unit
pg	picogramm
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
sec	seconds
SA	streptavidine
s.c.	subcutaneously
sc	spinal cord
SDS	sodium dodecyl sulfate
SN	supernatant
SPL	spleen
SSC	sodium chloride/sodium citrate buffer
TAE	Tris-acetic acid-EDTA buffer

Taq	polymerase from *Thermus aquaticus*
TCR	T cell receptor
TE	Tris-EDTA buffer
tg	transgenic
Tregs	regulatory T cells
Tris	2-amino-2-(hydroxymethyl-)1,3-propandiole
U	units
UV	ultraviolett
V	volts
vRAP	viral regulators of antigen presentation
vs	versus
v/v	volume per volume
w/v	weight per volume
WT	wild type
µl	microliter
µM	micromolar
3’	three prime end of DNA sequences
5’	five prime end of DNA sequences

1. Introduction

The immune system of vertebrates is a highly sophisticated system evolved to protect organisms against disease by identifying and killing pathogens and tumor cells. It needs to distinguish between the organism its own healthy tissue and cells infected by pathogens or malignants. Immune responses are mediated by cells of the innate immune system such as macrophages, dendritic cells (DCs), granulocytes, mast cells, and natural killer cells and by cells of the adaptive immune system, consisting of B and T lymphocytes. The detection of pathogens by the immune system is sometimes complicated because pathogens can adopt and evolve rapidly to avoid recognition. Therefore the interplay of the immune system in response to a pathogen needs to be investigated in detail.

1.1 B cells

B cells are an essential component of the adaptive immune system and play a key role in the humoral immune response. Like all cells of the immune system they develop from pluripotent hematopoetic cells in the bone marrow. They undergo positive and negative selection before they leave the bone marrow and circulate between blood and secondary lymphoid tissues.

Through somatic recombination B cells establish antigen receptors with an exclusive specificity (Nussenzweig et al., 1987). Antigen encounters activate B cells, which then either develops into antibody secreting cells or migrate into primary lymphoid follicle to form germinal centers to produce antibodies. Beside their antibody production, B cells are also able to activate antigen-specific T cells via their surface molecules, they are potent antigen presenting cells and able to secrete cytokines.

1.1.1. Antibodies

Antibodies play an important role in humoral immunity and are essential in establishing immunological memory. They are present in the serum of each individual, even without any exogenous antigenic stimulation. These circulating antibodies are predominantly IgM antibodies and referred to be natural antibodies. They are mainly produced by a subset of long-lived, self-replenishing B-1 cells. Their repertoire is shaped by molecular events that occur during rearrangement of the genomic Ig locus, followed by positive selection of the natural antigen-secreting cells (Baumgarth et al., 2005). The reactivity pattern of the repertoire stays relatively constant throughout life and is maintained by homeostasis mechanisms (Mouthon et al., 1995), also infections does not alter the repertoire. Natural antibodies, when bound to antigen can activate the classical complement system, which leads to the lysis of enveloped virus particles long before the adaptive immune response is activated (Baccala et al., 1989; Clarke et al., 1985). The activation of B cells, through recognition of the target antigen, initiates the adaptive B cell immune response, also generating antigen-specific antibodies of different isotypes. In the secondary lymphoid organs activated B cells undergo germinal center formation and class switch recombination to establish the production of antigen-specific antibodies (Wardemann and Nussenzweig, 2007). An increased affinity of antigen-specific antibodies is achieved by somatic hypermutation (SHM), which also increases the diversity of the antibody pool. Specific antibodies activate the complement system and via FCγR-signalling most of the effector cells of the immune system, like monocytes, macrophages, NK cells, mast cells, eosinophils, neutrophils and platelets, in this way they contribute to immunity against pathogens (Ravetch and Bolland, 2001). The antigen-specific antibodies generated through SHM are preserved by antibody secreting memory B cells or plasma cells, which prevents a secondary infection by the same pathogen, a phenomenon that highlights the central role of antibodies in immunological memory.

1.1.2. B Cells as antigen presenting cells

B cells express major histocompatibility complex (MHC) class I as well as class II molecules and therefore are potent antigen presenting cells (APCs). These two types of MHC molecules presenting peptides to T cells, MHC class I (MHCI) and MHC class II (MHCII) comprise different sources of protein antigens, MHCI is responsible for the presentation of cytosolic (intracellular) peptides, to CD8 T cells. MHCII presents extracellular antigens that have been taken by endocytosis to CD4 T cells. We were interested in the presentation of MHCI-loaded peptides by B cells, which can occur in two ways: direct presentation of epitopes synthesized by the presenting cell or cross-presentation, where the presented antigen is captured from the surrounding environment. In the past, peptide presentation by B cells on MHCII was extensively studied. In several autoimmune diseases (e.g. autoimmune type one diabetes and multiple sclerosis) B cells were shown to act as direct APCs, peptides are loaded on MHCII and presented to CD4 T cells (Finkelman et al., 1992; Harp et al., 2008; Serreze and Silveira, 2003). Lankar et al. discovered that antigen cross-presentation is mediated by clonally distributed B cell receptors (BCRs). These BCRs are composed of membrane immunoglobulin (Ig) associated with Ig-alpha/Ig-beta heterodimers and target antigens to MHC II-containing compartments (Lankar et al., 1998). Further, Crawford and coworkers observed in mice with a B cell-specific MHC class II deficiency a reduction of the clonal expansion of T cells, impairment of the differentiation to cytokine-secreting Th2 cells and a diminished memory T cell population (Crawford et al., 2006). This implies the direct antigen presentation by B cells, which stresses the strong impact of B cells on the CD4 T cell compartment. The influence of B cells on CD4 T cell function has often been shown, but cases of peptide presentation to CD8 T cells have rarely been described. Hoft and colleagues showed in a parasitic infection model with *trypanosoma cruzii* that B cells can directly induce an antigen-specific CD8 T cells response via cross-presentation (Hoft et al., 2007). Despite this, the role of B cells in antigen presentation to CD8 T cells in infections has not been thoroughly characterized and further studies are necessary.

1.1.3. B cells as IL-10 secreting cells

Besides antibody secretion and antigen presentation, activated B cells are capable of cytokine secretion. Similar to Th1 and Th2 T cell subsets, B cells can be divided into two different subsets depending on their cytokine secretion pattern. These subsets are called Be1 and Be2. Be1 B cells secrete interleukin 2 (IL-2), interferon-γ (IFN-γ), lymphotoxin, and IL-12, whereas Be2-polarized B cells secrete IL-2, IL-12 and IL-10 (Mosmann, 2000). Similar to T cells secreting IL-10, named regulatory T cells (Tregs), B cells secreting IL-10 have been termed regulatory B cells (Bregs). The main population of Bregs expresses high levels of CD21, CD23, CD24, IgM and CD1d (Mauri and Ehrenstein, 2008). IL-10 suppresses the synthesis of pro-inflammatory cytokines and antigen presentation, but stimulates B cell maturation and antibody production (Moore et al., 2001).

The first studies of the suppressive function of B cells suggested the production of inhibitory antibodies, which are responsible suppression (Morris et al., 1982). Later studies showed that the suppressive phenotype depends on the secretion of IL-10 (Mauri et al., 2003; Mizoguchi et al., 1997; Mizoguchi et al., 2002). For the generation of Bregs the engagement of CD40 on B cells was found to be a principal requirement (Fillatreau et al., 2002). This was confirmed by a study using arthritic mice in which the treatment with anti-CD40 increased the production of IL-10 and therefore ameliorated the course of disease (Mauri et al., 2000). The engagement of CD40 on B cells dictates their further differentiation into Bregs, memory B cells, or plasma cells. This depends on the stage of B cell maturation and the duration or strength of the mutual signals between T and B cells (Lee et al., 2002; Miyashita et al., 1997; Rousset et al., 1991; Saeland et al., 1993).

The group of Mangan showed IL-10 dependent protection in an experimental anaphylaxis model, using adoptively transferred Bregs (Mangan et al., 2004). In a viral infection less is known about the role of Bregs. It was shown that IL-10 secretion of $CD5^+$ B cells in *baculovirus* or *influenza* infection is triggered by TLR9 and can be enhanced by the crosstalk to plasmacytoid dendritic cells (pDCs) producing IFN-α (Zhang et al., 2007). In this thesis it is shown that B cell secreted IL-10 has a suppressive effect on the mCMV specific CD8 T cell

response (Madan et al., 2009; Majlessi et al., 2008; Mauri and Ehrenstein, 2008).

1.2. Different mouse models with deficiencies in the B cell compartment

The construction of knockout and knockin mice was an important achievement to investigate the function of single genes and to further investigate the interplay of the immune system. One of the most common techniques to generate knockout and knockin mice is conditional gene targeting using the bacteriophage-derived Cre-loxP recombination system (Hoess et al., 1982; Sternberg and Hamilton, 1981). This system is based on the Cre enzyme, which recognizes a sequence motif of 34bp called loxP site and cleaves this sequence (Hoess et al., 1982). If two loxP sites in the same orientation flank a DNA segment, Cre excises this segment from the DNA and just one loxP site remains. Appropriately positioning of the two loxP sites can generally be used to produce deletions (Gu et al., 1994; Gu et al., 1993; Ramirez-Solis et al., 1995), gene replacement/insertions (Hanks et al., 1995; Zou et al., 1994) and point mutations (Rajewsky et al., 1996; Torres et al., 1996). In this thesis the Cre/loxP recombination system was used to generate mice with a deficiency of IL-10 secretion specifically in B cells or DCs. Therefore a mouse strain with a loxP-flanked exon 1 of the IL-10 gene (Roers et al., 2004) was individually bred to two different Cre mouse strains. One carries the *cre*-coding gene under the control of the CD19 promoter (CD19-Cre), the lineage marker of B cells (Rickert et al., 1997), the other one under the control of the CD11c promoter (CD11c-Cre), a marker of DCs (Caton et al., 2007). This results in mice with either B cells or DCs incapable of IL-10 production.

Also the B cell-deficient J_HT strain was generated using the Cre/loxP system. Here the loxP sites were introduced into the J_H-E_μ locus (Fig.: 1), resulting in a J_HT strain, which is devoid of B cells (Gu et al., 1993).

Another method to investigate a mouse system lacking B cells is the Cre-inducible Diphtheria toxin receptor (iDTR) system. The generation of the iDTR

strain was achieved by inserting a loxP site-flanked stop-cassette followed by the gene sequence for the simian Diphtheria toxin receptor (DTR) under control of the ubiquitously expressed ROSA26 promoter. The resulting mouse strain was bred to a strain with a tissue-specific Cre. Therefore, the DTR is expressed in the respective tissue, and Diphtheria toxin (DTx) application depletes the DTR expressing cells (Buch et al., 2005). In this thesis the iDTR strain was bred to CD19-Cre and CD11c-Cre mice to achieve the specific depletion of B cells or DCs at a desired time during an ongoing experiment.

Finally two more mouse strains were used to investigate the role of B cells in mCMV and *Leishmania major* infection: The IgMi mice and the IgG1i mice. IgMi mice develop B cells expressing IgM as a B cell receptor, but these B cells are not able to undergo class switch recombination and thus cannot produce any other isotype than IgM. Also, the differentiation to plasma and to germinal center B cells is suppressed. The same is true for IgG1i mice. IgG1i mice develop B cells that exclusively express IgG1 as B cell receptor. The IgMi mice were generated through a deletion of the polyA responsible for the secreted form of antibodies in the genome of these mice, see also Figure 1 (Waisman et al., 2008; Waisman et al., 2007). Consequently, IgMi mice are not capable of secreting antibodies in contrast to IgG1i mice.

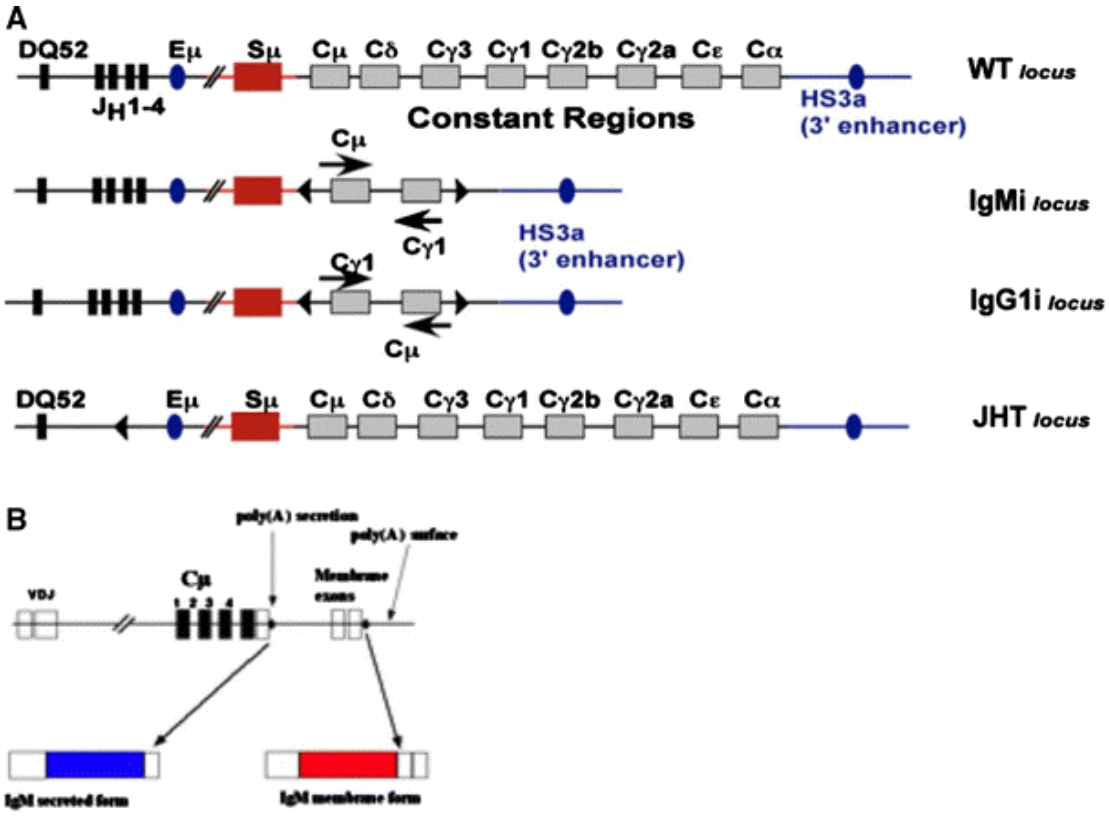

Figure 1: Loci of the B cell mutant mice. A Schematic representation of the IgH locus of wild type, IgMi, IgG1i and J_HT mice. **B** The first polyA signal, directly down stream of exon 4 was deleted in the IgMi mouse strain. The second polyA site is located down stream of the membranal exons and was not deleted. Therefore, this locus can only code for the membrane-bound form of the constant region of the IgM antibody, and not the secreted form like the wild type locus (Waisman et al., 2008).

1.3. Murine cytomegalovirus (mCMV)

The interest of this thesis is to elucidate the role of B cells in mCMV infection. mCMV belongs to the family of herpesviridae, which includes three subfamilies, the alpha-, beta- and gamma-herpesviridae with similar biological characteristics. The genome of mCMV codes for 170 open reading frames, but the function of most open reading frames (ORFs) is still unknown (Rawlinson et al., 1996; Tang et al., 2006). mCMV is a member of the beta-herpesviridae, a family with an uniform architecture of the virus particle (Plummer, 1967). It hosts a linear, double-stranded DNA-genome with different lengths regarding the individual virus strains. All viruses classified in this family reside in the host and establish latency (Roizman and Baines,

1991), and have typical characteristics like the establishment of latency, are strict species specificity and a long replication cycle in different host cells.

1.3.1 Infection of the host cell

To infect and enter a host cell the structure of the CMV-virion (Fig.: 1) is very important. The structure of the CMV-virion is a composition of a 100nm icosahedral capsid containing a linear 230kb-double-stranded DNA genome and a large tegument component surrounded by an envelope, which contains a cellular lipid bilayer with viral glycoproteins. A mature virion has a diameter of 150-200nm (Fig.: 2) (Streblow, 2006). The glycoproteins on the envelope (e.g. gB and gM/gN) are essential for the virus to attach to the host cell. The first attachment occurs between the glycoproteins gB and/or gM/gN and the host cell (Boyle and Compton, 1998). To stabilize the first attachment, further proteins get involved to launch the docking of the virus to the host cell. So far, the exact cellular receptors are unknown, but different receptors are in discussion to fulfill the docking processes. The interaction of these cellular receptors induces a clustering of the cellular component, inducing a fusion of the virus envelope and the cell membrane of the host cell (penetration). Next, the nucleocapsid and the tegument enter the cytoplasm of the host cell (Boehme, 2006). After uncoating, the tegument is removed and the remaining nucleocapsid translocates to the nucleus of the infected cell (Dohner and Sodeik, 2005). The viral genome enters via the nucleopore into the nuclearplasma, where the ends of the genome fuse and the viral genome remains in the nucleus of the host cell (Marks and Spector, 1988).

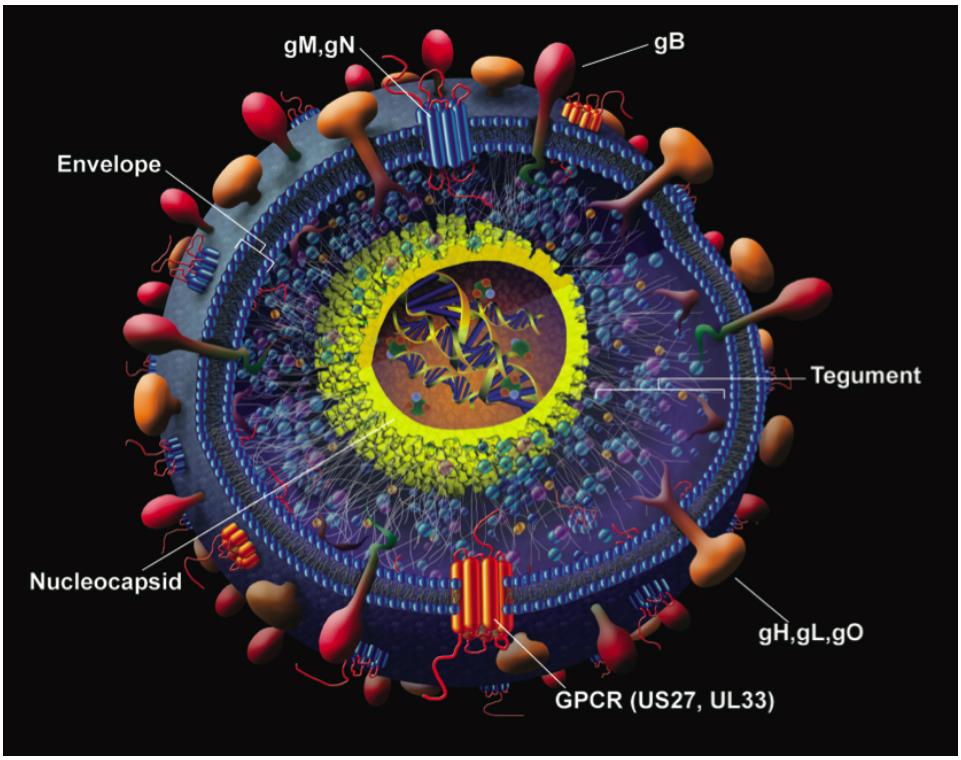

Figure 2: Structure of CMV. The first layer is the nucleocapsid containing the double-stranded viral DNA genome, which is surrounded by a proteinaceous tegument layer. The tegumented capsids are enveloped by a host-derived lipid bi-layer that is studded with viral glycoproteins (Streblow, 2006).

1.3.2. Gene expression and virus replication

The gene expression in mCMV follows the same scheme in all herpesviridae. The genes are separated into three subclusters with the same track of expression. The transcription starts with the immediate early (IE)- genes followed by early (E)- genes and at last the late (L)- genes (Emery and Griffiths, 1990; Honess and Roizman, 1974). The transcription of E-genes starts already 2h post mCMV infection. This is essential because the E-genes code for proteins, which are necessary for the virus replication. Therefore, the replication phase is initiated by the transcription of these genes (Keil et al., 1984; Messerle et al., 1997). The L-genes code for structural proteins of the virus particles. The replication in the host´s nucleus works on the principle of the rolling circle. Consequential the DNA-molecules consist of multiple copies of the viral genome (Marks and Spector, 1988). To start the virion morphogenesis these DNA-molecules are split into molecules with just one virus genome and processes of assembly and the packaging of the genomes

take place. The first developmental stage of the virion is formed through envelopment at the inner nuclear membrane and is set free through budding into the perinuclear cisterna (primary envelopment) (Gibson, 1996). This envelope is lost again by the translocation through the outer membrane (deenvelopment) and results in naked virions analogous to nucleocapsids in the cytoplasma of the infected cell (Sanchez et al., 2000). The secondary envelopment takes place in the cytoplasmic cisterna ensuing in viral particles with a double membrane. The outer membrane gets lost in a fusion with the plasma membrane to release viral particles with a single envelope membrane (Sanchez et al., 2000).

1.3.3 Latency and reactivation

Latency is defined by the presence of viral DNA without production of infectious virions (Bain, 2006). In human Cytomegalovirus (hCMV) infection, latency is established in many organs for example lung, liver, pancreas, kidney, and heart (Koffron et al., 1997). In mCMV infection the virus can be found in spleen, lung, salivary gland, kidney, adrenal gland, and also heart during latency (Balthesen et al., 1993; Collins et al., 1993; Klotman et al., 1990; Reddehase et al., 1994). The specific cell type for the virus to establish latency is still not defined. Latent hCMV genomes are found in granulocytes, monocytes, and DCs, but no viral DNA could be detected in B and T cells (Mocarski, 2006). Because of the high number of different organs in which latent mCMV can be found, it is most likely that additional cell types are the host of mCMV during latency. It has already been described that endothelial cells and tissue resident macrophages are involved (Koffron et al., 1998; Mercer et al., 1988). Also endothelial liver cells were described as a place of latent mCMV (Seckert et al., 2009).

Reactivation from latency causes a recurrent infection with the production of new infectious virions (Balthesen et al., 1994; Reddehase et al., 1994; Shanley et al., 1979). Reactivation is in most cases the consequence of immune suppression and results from a disturbed CD8 T cell response. The virus can be reactivated by cell differentiation and activation of myeloid cells

by proinflammatory cytokines (Hertel et al., 2003; Reeves et al., 2005). Especially TNF-α plays an important role in reactivation. The administration of TNF-α to latent infected mice leads to a recurrent virus infection (Cook et al., 2006; Simon et al., 2005).

1.3.4 Relevance of hCMV

In parts of the world, such as South-East Asia and Africa almost 100% of the people are infected with hCMV. In industrial states like countries in Europe only 40%- 60% are infected (Ho, 2008; Mocarski, 2006). The virus spreads via transmission, which can occur horizontal and vertical. Horizontal transmission occurs by the exchange of body liquids especially saliva (Adler and Marshall, 2007; Grundy et al., 1990; Pass, 1985), whereas vertical transmission occurs from mother to newborn during birth or later by breast feeding (Fitzgerald et al., 1990; Mocarski, 2006).

The first infection with hCMV after birth is usually symptomless. The reactivation in case of immunosuppression can result in an invasive CMV infection causing pneumonitis, esophagitis, encephalitis, hepatits, pancreatitis, adrenalitis, gastritis, enteritis, colitis, and retinitis (Hsieh et al., 2001; Lilleri et al., 2003). Virus reactivation is common in patients with organ transplantations, where hCMV is believed to cause graft injury and shorten the graft survival (Crawford et al., 1993; Rubin, 1990). Taken together, there are several reasons why hCMV needs to be further investigated to understand the mechanisms of reactivation and memory responses.

1.3.5 mCMV as model system for CMV infection

hCMV infection cannot be investigated directly in an animal model, because of the strict species specificity of all CMVs. Therefore mCMV serves as model system for hCMV infection. This is based on common features of all CMVs such as species specificity, latency and especially the analog development of adaptations to the host of mCMV and hCMV. In addition the viruses are structurally, biologically, and genetically similar (Holtappels et al., 2006;

Rawlinson et al., 1996; Reddehase, 2002). Both viruses ascertain a life long latency after the primary infection. In addition the virus can establish latency in organs like lung and salivary glands (Balthesen et al., 1993; Krmpotic et al., 2003; Mocarski, 2006; Reddehase, 2002; Reddehase et al., 1994). Furthermore the kinetics of the immune control is comparable, and mediated by CD8 T cells in both systems (Holtappels, 2006; Reddehase, 2002). Consequently investigation of mCMV allows to draw conclusions about hCMV infection.

1.3.6 Immunologic control of CMV infection

mCMV infection is controlled by both innate and adaptive immune system. Especially natural killer cells (NK cells) as major component of the innate immune system are important in the first three days after mCMV infection (Arase et al., 2002; Bukowski et al., 1985; Jonjic, 2006). NK cells show two general types of antiviral activity, direct mechanisms via the exocytosis of cytolytic granules and indirect noncytolytic mechanisms via the secretion of cytokines such as IFN-γ and TNF-α (Raulet, 2003) The adaptive immune system is necessary for the clearance of the infection. Here, particularly the antigen-specific immune response of CD4 and CD8 T cells is needed to terminate the first infection. B cell-secreted Igs play an important role in limiting the spread of the virus during recurrent infections. These Igs were educated during the first infection, which then protects against secondary infections (Jonjic et al., 1994; Klenovsek et al., 2007; Rapp, 1993; Reddehase et al., 1994; Wirtz et al., 2008).

1.3.7 Importance of CD8 T cells

In mCMV infection CD8 T cells are activated by MHC-class I presentation of pathogenic antigen and the additional interaction of costimulatory molecules (Rudolph et al., 2006). The activation induces effector functions and proliferation of the CD8 T cells. These effector functions include cytolytic activity and secretion of different cytokines such as TNF-α, IFN-γ, as well as

chemokines (Doherty, 1993; Harty et al., 2000). This mediates the control of infection to CD8 T cells and leads to the clearance of the virus. After the clearance, a population of memory CD8 T cells remains to re-establish the effector function after presentation of the same pathogen (Masopust et al., 2001).

The protective role of CD8 T cells in mCMV infection was shown by an adoptive transfer experiment of virus-specific T lymphocytes to susceptible immune suppressed mCMV infected mice, which protected these mice (Bohm et al., 2008a; Holtappels et al., 2001; Holtappels et al., 2002; Pahl-Seibert et al., 2005; Reddehase et al., 1985).

In 2006 Munks and coworkers identified 24 peptide epitopes from 18 antigens, the combination of these epitopes accounts for the mCMV-specific CD8 T cell response in C57BL/6 mice (Munks et al., 2006b).

The presentation of pathogenic antigen via MHC class I can occur in two different ways. First by direct antigen presentation and second by cross-presentation (see 1.1.2). The mechanism and the loading of these extra cellular proteins to a MHC class I molecule is still not completely analysed yet (den Haan and Bevan, 2001; Heath and Carbone, 1999; Shen and Rock, 2006). DCs, macrophages, B cells, neutrophile granulocytes, and endothelial cells are able to present antigen to CD8 T cells via MHCI presentation (Basta and Alatery, 2007; Heath et al., 2004).

1.3.8 Immune evasion

Immune evasion is the ability of a pathogen to protect itself by modulating immunological defense mechanisms of the host. For herpesviridae several different immune evasion mechanisms are known and several components of innate and adaptive immunity are involved. To evade the host immune responses CMV encodes a diverse arsenal of proteins focused on altering and/or mimicking leukocyte migration, classical and non-classical major histocompatibility complex (MHC) protein functions, activation, and cytokine responses and also host cell susceptibility to apoptosis (Mocarski, 2002; Reddehase, 2002). So far, four proteins (m145, m152, m155 and m138) are

characterized to diminish the expression of NKG2D, an activation marker of NK cells, with consequence of a diminished NK cell response, directly after infection the NK cell response is the most important one (Hasan et al., 2005; Krmpotic et al., 2005; Lodoen et al., 2003; Lodoen et al., 2004). The CD8 T cell response is also one of the main targets for this modulation. Here CMV codes for proteins, which can diminish the viral antigen presentation via MHC class I. These proteins are called vRAPs (viral regulators of antigen presentation) (Holtappels et al., 2006) and are coded by m152, m06 and m04 (Doom and Hill, 2008; Reddehase, 2002; Reddehase et al., 2004; Yewdell and Hill, 2002). A virus mutant without these genes mounts a higher virus-specific CD8 T cell response (Bohm et al., 2008a).

1.3.9 Memory inflation

Memory inflation is a new field in mCMV investigation and was first mentioned in 2000 (Holtappels et al., 2000). It describes the accumulation of virus-specifc CD8 T cells in latent mCMV infection. The same phenomenon is found in hCMV latency and is a consequence of the importance of the CD8 T cell response in CMV infection. Four different memory patterns of virus-specific CD8 T cell responses are identified in the mCMV response in C57BL/6 mice (Munks et al., 2006a), but maintenance, development, and necessity of this memory inflation are unclear.

Three hypotheses about the population of the inflationary CD8 T cell populations are existing: First the CD8 T cells accumulate over time (Karrer et al., 2003), second expansion as the reason for the inflation (Karrer et al., 2004), and third a continuous production of short-lived functional T cells as cause for the inflation (Snyder et al., 2008).

1.4. Leishmaniasis

Leishmaniasis is a disease mostly spread in South-America, around the Mediterranean Sea, and in Africa. 350 million people in 88 different countries are affected. Three different manifestations of Leishmaniasis are described.

The most common form is the cutaneous form, to which about 90% of all cases belong. It is transmitted by several *Leishmania* species, which cause mild or severe dermal ulcers with the course of disease depending on the patient ´s immune response. In general it is non-lethal and spontaneously self-healing, but scarring, ulcerative plaques and/or nodles can remain at the infection site. Even if *Leishmania´s* cutaneous form is normally restricted to the dermis, it may also promote more severe forms of the disease (Sacks and Noben-Trauth, 2002). The second form is the mucocutaneous form, which is caused by *L. braziliensis*. It starts as cutaneous form with a spontaneous healing, but the residual parasites in the body develop chronic ulcers after months or even years post infection. This form is more aggressive than the cutaneous one; it also spreads to the mucosa, e.g. mouth and nose, and is able to destroy the cartilage and underlying tissue. The third form is the visceral form, the most aggressive form, which is lethal in 100% of cases when untreated. In this form the parasites spread and invade inner organs, like liver, spleen, bone marrow, and lymph nodes. The visceral form of leishmaniasis is treated for example with amphotericin B, an antifungal drug used for systemic fungal infection but the medication gives rise to severe side effects with very low trearment success (Cascio et al., 2004).

1.4.1 The immunological Th1/Th2 concept

The course of *Leishmania major* infection differs in BALB/c and C57BL/6 mice. BALB/c mice succumb to the infection and die, C57BL/6 mice are able to clear the infection, heal the lesions, and establish a lifelong resistance. The difference is explained by a genetic predisposition, resulting in a dominant IL-4 driven Th2 response in BALB/c with the consequence of disease progression and death. The resistance in C57BL/6 mice is mediated by an IL-12 driven IFN-γ dominated Th1 response, which promotes healing and parasite clearance. Because of this difference in the immune response, C57BL/6 mice are used as a model for cutaneous leishmaniasis and the susceptible BALB/c mice serve as a model for the visceral form of leishmaniasis both induced by a *Leishmania major* infection. The visceral

leishmaniasis is induced normally by *L. donovani* and other *Leishmania spp*.

1.4.2 Life cycle of *Leishmania major*

The life cycle of *Leishmania major* consists of two different forms: The extracellular metacyclic promastigote and the intracellular amastigote form. The extracellular metacyclic promastigote is transmitted by the bite of a female sand fly. These promastigotes are opsonized by serum components and phagocytosed by macrophages. In the phagolysosomes, the promastigotes are able to transform into replicating amastigotes that are able to replicate by simple division, where they can lyse the infected macrophages to infect more macrophages, they can also be taken up again by sand flies during the blood-meal of infected people. In the sand fly the amastigotes are released to the midgut, and transform into the procyclic promastigotes, where they turn into metacyclic promastigotes by simple division (Fig.: 3) (Gossage et al., 2003; Sacks and Noben-Trauth, 2002). They migrate to the pharyngeal valve and are transmitted by blood-meal. Between 10 and 1000 promastigotes can be transmitted by one bite. The phagocytosis of the macrophages is normally a powerful mechanism to prevent the infection with incoming pathogens, but *Leishmania* is an intracellular parasite and adapted to take advantage of this uptake. The parasite is also resistant to the lytic complex of complement (Lohoff et al., 1998); otherwise the transmission of metacyclic promastigotes would not lead to leishmaniasis.

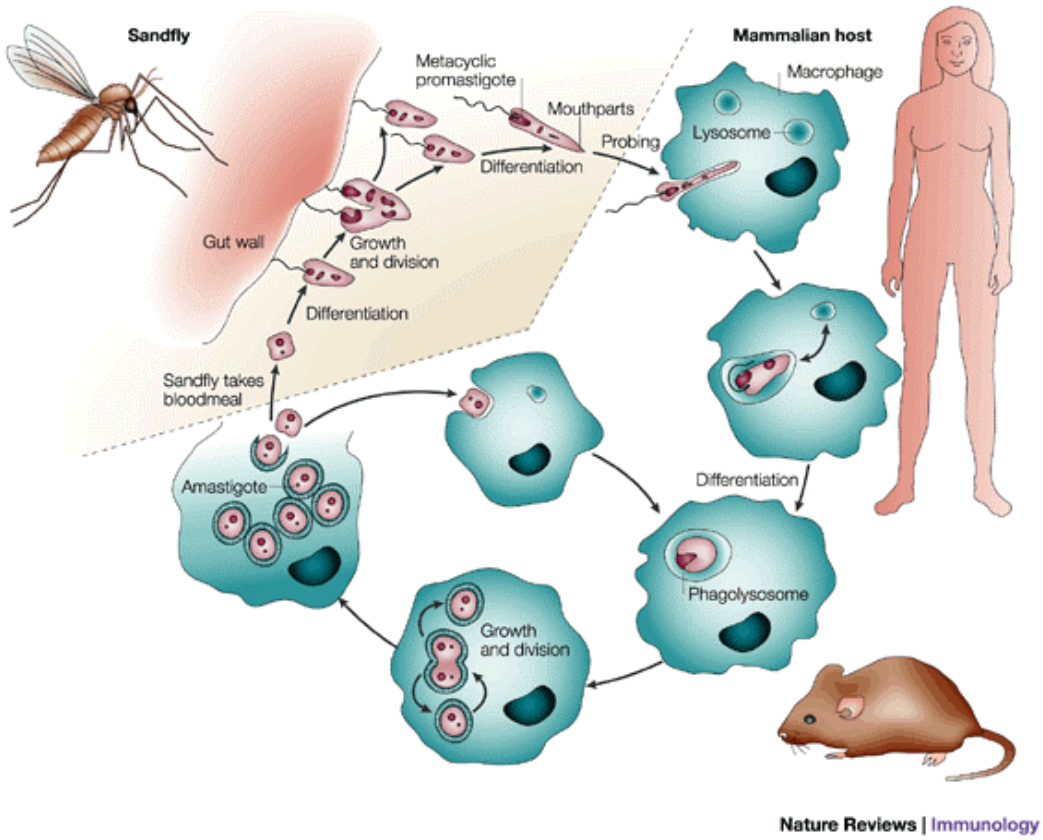

Figure 3: Life cycle of *Leishmania major* infection (Sacks and Noben-Trauth, 2002).

1.4.3 Leishmaniasis in resistant C57BL/6 mice

The infection of resistant C57BL/6 mice with a subcutaneous low dose infection of *Leishmania major* can be divided into three to four distinct phases. The first phase is the silent phase, which is characterized by the absence of lesions starting after the inoculation of *Leishmania*, when the parasite is taken up by macrophages and fibroblasts through a CR3-mediated mechanism (Bogdan et al., 2000). In the macrophages the parasites start to replicate and to transform into amastigotes. At this time point, the infection is not recognized by the immune system. The macrophages are inactivated and host the parasite until the cell is ruptured. Inactivated macrophages are not able to initiate a T cell-mediated immune response. The presentation of antigen is not possible, and no co-stimulatory molecules including IL-12 are expressed (Liew et al., 1997; Prina et al., 1993; Reiner et al., 1987; Reiner et

al., 1994). This stops with the lysis of the macrophages and the release of amastigotes four to five weeks post infection. The second phase is accompanied by the development of lesions starting with redness and swelling of the infected area caused by the innate immune system including neutrophils, eosinophils, and macrophages fighting the free amastigotes (Belkaid et al., 2000). This is initiated by complement and cutaneous mast cells. The third phase is characterized by the migration of DCs to the site of infection. The mast cell derived mediators are responsible for this migration (Maurer et al., 2006), but also cytokines, chemokines, and IgG-mediated mechanisms initiate the migration of DCs to the site of infection (Sato et al., 2000; Woelbing et al., 2006). The presence of DCs at the site of infection goes along with the first leishmania-specific T cells. Parasite elimination is initiated, and ulceration of the infected tissue is characteristic for this phase. The fourth phase describes the memory and persistent phase that starts with the healing of the lesions and continues also without any noticeable symptoms of leishmaniasis.
In BALB/c mice different phases are not distinguishable due to the susceptibility of these mice, which suffer from severe lesions and succumb the infection.

1.4.4 B cells in *Leishmania major* infection

The role of B cells in a *Leishmania major* infection is still elusive. On the background of C57BL/6 μMT mice (deficient for B cells) are able to mount a Th1 response to the same extent as wild type (wt) mice and control the infection. On the susceptible background of BALB/c, μMT mice and wt mice mount a Th2 response, again despite the presence or absence of B cells (Brown and Reiner, 1999). B cells do not affect the T cell polarization, but by using these mice a role for B cells in the IFN-γ production was shown, which influences the pathology of a secondary infection (DeKrey et al., 2003). Furthermore, antibodies were shown to be necessary for the development of an optimal protective immunity against *Leishmania major*, because DCs, when covered with parasite-reactive IgG take up the parasite more efficiently.

The interaction of IgG with FcγRI and FCγRII on DCs provides an optimal recognition and ingestion of the parasite, a prerequisite to an immune response (Woelbing et al., 2006).

1.5 Objectives

The role of B cells and antibodies in the acute response to viral or parasitic infection has been insufficiently described. In most cases it is known that the antibody response by memory B cells and plasma cells mediates protection against secondary infections with the same pathogen. In this thesis the role of B cells in leishmaniasis and mCMV infection was investigated. Therefore, different mouse models were used, the J_HT mice – a strain devoid of B cells, and the IgMi mice - that have B cells but lack serum antibodies and are incapable of class switch. A comparison of the same infection in these mice allowed us to draw conclusions about the function of antibodies and B cells in virus clearance. Further mouse strains such as the CD19-Cre/IL-$10^{fl/fl}$ mice were used to investigate the role of B cell-secreted IL-10 in mCMV infection. Additionally, the CD19-Cre/iDTR mice, which allow for B cell depletion upon DTx administration, provided new insights. This mouse line represents a direct system for investigations in the absence of B cells, but in the presence of remaining immunoglobulins.

2. Materials and Methods

2.1 Chemicals and biological material

Chemicals were purchased from Sigma (Steinheim), Fluka Chemie (Switzerland), Merck (Darmstadt) or AppliChem (Darmstadt) unless stated otherwise. Solutions were prepared with double distilled water (ddH_2O). Bacterial media were autoclaved prior to use. Sterility of solutions and chemicals used in cell culture was maintained by working under a sterile hood (Heraeus, Germany).

Table 1: Chemicals

Name of Chemical	Supplier
Agar	Gibco Life Technologies GmbH, Karlsruhe
Agarose, electrophoresis grade	AppliChem, Darmstadt
Ampicillin	Sigma-Aldrich, Steinheim
Bovine serum albumine (BSA)	Sigma-Aldrich, Steinheim
Calcium chloride	Sigma-Aldrich, Steinheim
Chloroform	Merck, Darmstadt
Citric acid	Fluka Chemie GmbH, Switzerland
2'-Deoxyguanosine Monohydrate	AppliChem, Darmstadt
Dextrane sulfate	AppliChem, Darmstadt
Dextrose	Merck, Darmstadt
Diethylpyrocarbonate (DEPC)	AppliChem, Darmstadt
Dimethylsulfoxide (DMSO)	Merck, Darmstadt
Dithiothreitol (DTT)	Boehringer Mannheim GmbH, Mannheim
dNTPs	Pharmacia Biotech, USA
Ethanol, abs.	AppliChem, Darmstadt
Ethidium bromide	Sigma-Aldrich, Steinheim
Ethylendiamine tetraacetate (EDTA)	Fluka Chemie GmbH, Switzerland
Fetal calf serum (FCS)	Boehringer Mannheim GmbH, Mannheim

Ficoll 400	Amersham Pharmacia, Freiburg
Glacial acetic acid	Fluka Chemie GmbH, Switzerland
Hydrochloric acid (37%)	Merck, Darmstadt
Isopropanol	AppliChem, Darmstadt
Magnesium chloride	Sigma-Aldrich, Steinheim
Magnesium chloride (for PCR)	Gibco Life Technologies GmbH, Karlsruhe
b-Mercaptoethanol (b-ME)	Fluka Chemie GmbH, Switzerland
Mineral oil	Sigma-Aldrich, Steinheim
Orange G	Chroma Gesellschaft Schmidt Co,Stuttgart
Phenol	Sigma-Aldrich, Steinheim
Potassium chloride	Merck, Darmstadt
Potassium hydrogenphosphate	Merck, Darmstadt
Proteinase K	Roche, Switzerland
Salmon sperm DNA	Biomol, Hamburg
Sodium azide	Fluka Chemie GmbH, Switzerland
Sodium carbonate	Fluka Chemie GmbH, Switzerland
Sodium chloride	AppliChem, Darmstadt
Sodium citrate	Fluka Chemie GmbH, Switzerland
Sodium dodecyl sulfate	AppliChem, Darmstadt
Sodium hydrogencarbonate	Fluka Chemie GmbH, Switzerland
Sodium hydrogenphosphate	Fluka Chemie GmbH, Switzerland
Sodium hydroxide	Fluka Chemie GmbH, Switzerland
Tris base	Fluka Chemie GmbH, Switzerland
Tris/ HCl	AppliChem, Darmstadt
Tween 20	Sigma-Aldrich, Steinheim

2.2 Molecular biology

2.2.1 Preparation of genomic DNA

Cells or tail biopsy (0,5 cm) were lysed over night (o/n) at 56°C in lysis buffer (10 mM Tris- HCl, pH 8; 10 mM EDTA; 150 mM NaCl; 0.2% (w/v) SDS; 400 mg/ml proteinase K). Subsequently, DNA was pelleted by centrifugation, washed in 70% (v/v) EtOH, dried at RT, and resuspended in TE buffer (10 mM Tris-HCl, pH 8; 1 mM EDTA).

2.2.2 Polymerase chain reaction (PCR)

PCR (Mullis and Faloona, 1987), (Saiki et al., 1988) was used to screen mice for the presence of targeted alleles or transgenes from tail tip DNA (primers shown in Table 1). Reactions were performed in PCR machines either APOLLO Instrumentation, ATC201 or peqlab, primus 96. Genotyping of mice tail tip DNA was generally performed in a total volume of 30 µl in the following reaction mix: 10 pmol of each primer, 0,25 U of *Thermus aquaticus* (*Taq*) DNA polymerase (1U/µl, Segenetics, Bonn, Germany), 25 mM dNTPs, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM MgCl2, 100 ng template DNA. Amplification started with denaturation for 4 min at 94 °C followed by 30-35 cycles of 94 °C for 30 sec, 52-60 °C for 30 sec, according to the melting temperature of the used Primer pair, 72 °C for 30 sec – 1 min and a final extension step at 72 °C for 10 min.

Table 2: List of primers routinely used for genotyping. Sequences of oligonucleotides are shown from 5´ to 3´.

Name of primer	Sequence (3´-5´)	$T_{Ann.}$ (°C)
Cre3	TCC AAT TTA CTG ACC GTA CAC	58
Cre7	TCA GCT ACA CCA GAG ACG G	58

Cre allg. fwd	GGA CAT GTT CAG GGA TCG CCA GGC G	58
Cre allg. rev	GCA TAA CCA GTG AAA CAG CAT TGC TG	58
CD11c-Cre_Boris_s	ACT TGG CAG CTG TCT CCA AG	63
CD11c-Cre_Boris_as	GCG AAC ATC TTC AGG TTC TG	63
CD19c	AAC CAG TCA ACA CCC TTC C	60
CD19d2	CCA GAC TAG ATA CAG ACC AGG A	60
RosaFA	AAA GTC GCT CTG AGT TGT TAT	58
RosaRA	GGA GCG GGA GAA ATG GAT ATG	58
SpliAcB	CAT CAA GGA AAC CCT GGA CTA CTG	58
DTR-F	CGA ATT CGC CAC CAT GAA GCT	
DTR-R	CGG GTG GGA ATT AGT CAT CGG	
WSS	GGC TAC TGC TGA CTC TCA ACA TT	
Luz6	CCT TCC TCC TAC CCT ACA AGC	
Luz8	GAG ACG AGG GGG AAG ACA TTT	
g-389	CAG ATG GGG GTG TCG TTT TGG CTA C	
IL-10flox (MCO2)	CCA GCA TAG AGA GCT TGC ATT ACA	
IL-10flox (IL-10EX2)	GAG TCG GTT AGC AGT ATG TTG TCC AG	

2.2.3 RNA isolation and quantitative Real-Time PCR

RNA from mouse T cells was prepared using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA was removed by 'on column' DNaseI digestion (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA was subsequently used for Quantitative Real-Time PCR in an iCycler (Light Cycler 1.2, Roche) using the QuantiTect SYBR Green RT-PCR Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Primers for Quantitative Real-Time PCR were obtained from Qiagen as described on their homepage https://www1.qiagen.com/GeneGlobe/Default.aspx. Expression was normalized to that of the 'house-keeping' gene GAPDH.

2.2.4 Agarose gel electrophoresis and DNA gel extraction

Separation of DNA fragments by size was achieved by electrophoresis in agarose gels (0.8% - 2% (w/v); 1x TAE (Sambrook, 1989); 0.5 mg/ml ethidium bromide). DNA fragments were recovered from agarose gel slices using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.3 Cell biology

2.3.1 Preparation of single cell suspensions from lymphoid organs

Organs of interest were taken and placed into ice-cold PBA (PBS-BSA-azide (NaN3); PBS, 0.1% (w/v) BSA, 0.01% NaN3). Spleen and lymph nodes (LN), were passed through a nylon cell strainer (40 µm, BD Falcon, Heidelberg, Germany) to obtain single cell suspensions. Bones were flushed with medium (DMEM, 10% (v/v) FCS, 2 mM L-glutamine) to extract bone marrow cells. Erythrocytes were lysed from spleen and thymus preparations in 140 mM NH4Cl, 17 mM Tris-HCl pH 7.65 for 2 min at RT. To stop lysis, cells were washed with PBA, centrifuged (6 min, 1200 rpm, 4°C), resuspended in the appropriate amount of PBA and kept on ice. For isolation of dendritic cells (DCs), LN or spleens were subjected to mild collagenase type II digestion (1 mg/ml; (Gibco) solved in PBS without Mg and Ca) for 30 min at 37°C. Then, normal preparation of single cell suspensions by passing digested tissues through nylon cell strainers followed. Blood from the tail vein was collected in a tube with heparin (Liquemin, Roche, Mannheim, Germany) and then layered on top of 7% (w/v) Ficoll 400 (Pharmacia, Freiburg, Germany). After 1400 g centrifugation at RT for 15 min, lymphocytes were recovered from the interphase of the gradient, resuspended in PBA and kept on ice.

2.3.2 Cell counting

Viable cells were assessed using the trypan blue dye exclusion test and counted using a Neubauer chamber (Assistent, Sondheim, Germany). To this end, an aliquot of the cell suspension was diluted with physiological trypan blue solution (Gibco, Long Island, NY, USA). Dead cells are stained blue whereas live cells cannot take up the dye due to their intact membrane. After counting 16 single quadrants, the counted cell number (N) was multiplied by the dilution factor (V) and the 'chamberfactor' (10^4) resulting in the number of live cells per ml (N x V x 10^4 =cell number/ml).

2.3.3 Flow cytometry

Single cell suspensions were prepared from all tested organs and the cell number was determined. Red blood cell lysis was performed - if necessary - as described above. The appropriate amount of cells (1-10 x 106 per sample) was surface-stained in 25-100 µl PBA with combinations of fluoresceine isothiocyanate (FITC), phycoerythrine (PE), CychromeTM (Cyc), allophycocyanin (APC) and biotin-conjugated monoclonal antibodies (mAbs) for 20 min at 4°C. Stainings with biotinylated mAbs were followed by a secondary staining step with Streptavidin (SA)-CychromeTM or SA-PE-Cy7. After staining, the samples were washed and resuspended in PBA. Stained cells were analysed on a FACSCalibur or a FACScan (Becton Dickinson, Heidelberg, Germany) and events in a live lymphocyte gate were analysed with CellQuest Software (Becton Dickinson, Heidelberg, Germany). Dead cells, if analysed by a FACSCalibur, were labeled with Topro-3 (10 nM; Molecular Probes, Göttingen, Germany) and excluded from the analysis. Monoclonal Abs, listed in Table 6, were either lab-made (C. Uthoff-Hachenberg, B. Hampel, Institute for Genetics, Cologne, Germany) or purchased from Pharmingen (San Diego, USA) or Natutec (Frankfurt, Germany).

Table 3: List of antibodies used for flow cytometry

Specificity	Clone	Reference and Supplier
CD4	GK1.5/4	(Dialynas et al., 1983), BD biosciences
CD8	53-6.7	(Ledbetter and Herzenberg, 1979), BD biosciences
CD11b	M1/70	(Ault and Springer, 1981), ebiosciences
CD11c	HL3	(Fagarasan et al., 2002), BD biosciences
CD19	1D3	(Springer et al., 1979), BD biosciences
CD21	7G6	(Heyman et al., 1990), BD biosciences
CD23	B3B4	(Heyman et al., 1990), BD biosciences
CD24	M1/69	(Alterman et al., 1990), BD biosciences
CD25 (IL2Ra)	7DA	(Malek et al., 1985), BD biosciences
CD28	37.51	(Gross et al., 1992), BD biosciences
CD38	90	(Howard et al., 1993), eBiosciene
CD40	3/23	(Noelle et al., 1992), BD biosciences
CD43	S7	(Gulley et al., 1988), BD biosciences
CD44	KM114	(Miyake et al., 1990), BD biosciences
CD62L (L-Selectin)	MEL-14	(Gallatin et al., 2006), BD biosciences
CD69	H1.2F3	(Yokoyama et al., 1988), BD biosciences
CD80	16-10A1	(Hathcock et al., 1994), BD biosciences
CD86	GL1	(Inaba et al., 1994), BD biosciences
CD90.1/Thy1.1	OX-7	(Mason and Williams, 1980), BD biosciences
CD90.2/Thy1.2	53-2.1	(Ledbetter and Herzenberg, 1979), BD biosciences
CD122	TM-b1	(Tanaka et al., 1991), BD biosciences
CD127	B12-1	(Noguchi et al., 1993), BD biosciences
CD138	281-2	(Jalkanen et al., 1985), BD biosciences
AA4.1	AA4.1	BD biosciences
B220	RA3-6B2	(Leo et al., 1987), BD bioscience
F480	BM8	eBioscience
IFN-γ	XMG1.2	(Cherwinski et al., 1987), BD biosciences
IgD	G12-	(Seemann et al., 1981), lab-made

	47/30	
IgM	R33-24.12	(Gruetzmann, 1981), lab-made
IgG1	A85-1	BD bioscience
MHCII	M5/114	(Bhattacharay et al., 1981), BD bioscience
PD-1	RPM1-30	(Agata et al., 1996), eBioscience

2.3.4 Magnetic cell sorting

Specific cell populations were either sorted or cells were depleted from a heterogenous cell suspension by magnetic cell sorting (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany). Cell populations were labeled with Ab-coupled microbeads (10 µl beads, 90 µl PBA per 107 cells) and separated on LS, MS or LD MACS columns in a magnetic field (Miltenyi et al., 1990). For cell sorting, T cells were purified by MACS and then stained with antibodies against various cell surface markers. T cells of individual T cell subsets were then sorted using a dual laser FACStar (Becton Dickinson, Heidelberg, Germany) or a FACSAria (Becton Dickinson, Heidelberg, Germany). The purity of isolated populations was subsequently tested by FACS analysis: MACS-isolated T cells were normally > 90% pure and sorted T cell subpopulations were > 95% pure.

2.3.5 Culture of *ex vivo* lymphocytes

The spleens were aseptically removed from mice and then pressed through a sterile sieve. Erythrocytes were lysed for 2 min by NH4Cl (140 mM NH4Cl, 17 mM Tris-HCl pH 7.65). Splenocytes were kept in RPMI 1640 (supplemented with 10% (v/v) FCS (decomplemented), 1 mM sodium pyruvate, 2 mM L-glutamine, 1x non-essential amino acids, 0.1 mM 2-β-mercaptoethanol, and 10 mM HEPES (Gibco), supplemented with various infection specific activation compounds (SBA; SLA; and mCMV Peptides (M45; m139; M57; m141; M38; M78; M33 and IE3) for not longer than two days.

2.3.6 Generation of peptide pulsed bone marrow-derived DCs

To extract bone-marrow (BM), bones (femur and tibia) were flushed with PBA, erythrocytes lysed as described above, and cells cultured in DMEM (Gibco), supplemented with 20 ng/ml of GM- CSF (PeproTech Inc., Rocky Hill, New Jersey, USA). On day 7, DCs were checked for purity and activation status (staining with CD11c, CD86 and MHCII). To mature the DCs LPS (100 ng/ml; Sigma-Aldrich, Steinheim, Germany) was added overnight incubation. The next day the DCs are harvested, counted, and cultured. At harvest, the purity of DCs was evaluated by flow cytometry for murine DC surface markers, CD11c and MHCII, and was 80-90%. To pulse the BMDCs, the 10µM peptide of interest was added to 1*10^6 cells/ml and incubated for 1 hour at 37°C.

2.3.7 ELISA immunoglobulin

Ig serum concentrations to NP were determined with ELISA as described previously (Roes and Rajewsky, 1993). Briefly, microtiter plates (Greiner, Frickenhausen, Germany) were coated with NP14-BSA at 4°C o/n, and subsequently blocked at RT for 30 min in 0.5% (w/v) BSA. Next, serially diluted sera samples were applied to the wells and incubated at 37°C for 1 h together with the standard. Secondary biotinylated antibody was then added for 1 h at 37°C. Detection of the biotinylated anti-sera was achieved with SA-conjugated alkaline phosphatase (AP, Boehringer: 30 min at RT) and p-nitrophenylphosphate as substrate (Boehringer). Following each incubation step, unbound antibodies or SA-conjugated AP were removed by five washes with tap water. The OD405 was measured with an ELISA-photometer (Anthos 2001, Anthos Labtech Instruments, Salzburg, Austria) and the relative antibody concentration was determined by calculating the association constant as described by Cumano and Rajewsky (Cumano and Rajewsky, 1986), following a method developed by Herzenberg et al. (Herzenberg and Black, 1980).

Common Ig serum concentrations were determined as described previously, but with a Ig specific coating, the other steps remain the same.

2.3.8 MCMV

The infection of the used mice took place with following viruses:

mCMV-WT (Strain Smith; ATCC VR-1399): This Labstrain is completely sequenced and the Genome is described in (Rawlinson et al., 1996). It was used for all experiments with just one virus.

MW97.01 (mCMV-WT.BAC): This is a through BAC technology recombined chimeric virus (Messerle et al., 1997; Wagner et al., 1999). It is comparable with the Labstrain mCMV-WT Smith (Wagner et al., 1999), just a HindIII´-E-fragment in this virus has its origin in a different clone K181 of this strain. It was used as control for all experiments using other with virus mutants.

mCMV-Dm04+m06+m152 (mCMV-DvRAP): It was cloned based on the mCMV-WT.BAC. The three ORFs m04, m06 and m152 were completely deleted. This mutant was generated by Markus Wagner (Wagner et al., 2002).

mCMV-SIINFEKL and mCMV-SIINFEKA: The K^b-resticted ovalbumin (Ova)-derived peptide SIINFEKL and its non-antigenic analog SIINFEKA were introduced as a replacement of the D^d-resticted antigenic m164 peptide 167-AGPPRYSRI-175 (Lemmermann et al., 2010).

All used viruses were provided by the group Reddehase and were supplied as virus stocks purified through a Saccharose gradient.

2.3.9 Isolation of *Leishmania* amastigotes and cultivation

The amastigote *Leishmania* are gained from intra fodpad infected mice. The mice are killed and the infected foot is taken off and kept in PBS containing glucose and EDTA. To get a clean culture of amastigotes it is necessary to exclude necrotic parts (containing LPS and bacteria). The remaining foot without skin is mashed and grinded. The fluid part of the grind is passed through a cell strainer (70 µm, BD Falcon, Heidelberg, Germany) and the

mashing and passing through a cell strainer is repeated. To separate the cells from the amastigotes, 10ml of PBS is added and the solution is centrifuged at 200g for 8 min. The amastigotes resides in the supernatant, which is centrifuged a second time. To get the amastigotes out of the solution they are centrifugated at 3000g for 8 min. The resulting pellet of amastigotes is resuspended in 1-3ml of DMEM according to the size.

2.3.10 Preparation of metacyclic *Leishmania major*

The preparation of metacyclic *Leishmania major* should start with a *Leishmania* culture in the age of 6-9 days. The suspension of the promatigotes is diluted 1:1 with DMEM before a 8 min/200g centrifuging step is following. The supernatant contains the *Leishmania* and is taken to a new tube. Two extract the *Leishmania* promastigotes from this solution, it is centrifuged for 8 min/3000g. The promastigote-containing pellet is now resuspended in 2 ml of DMEM to separate the parasites over a Ficoll-gradient. Therefore 2 ml of a 10% Ficoll solution is layered above 2 ml of a 20% Ficoll solution, the *Leishmania* solution is added as the last layer. After a centrifugation without brake at room temperature for 15min and 2000g the *Leishmania* are recovered from the interphase of the gradient and are kept in 5-10 ml of DMEM. To concentrate them a second centrifugation 8 min/3000g step at 4°C follows. The Pellet is resuspended in 1 ml DMEM. To prevent the adherence of the promastigotes to the counting chamber 1:100 paraformaldehyd needs to be added, this kills the promastigotes, just the long slim promastigotes are counted.

2.3.11 Parasite load spleen

The spleen is mashed with forceps and passed through a 70µm cell strainer. The used things are flushed with 5 ml PBS to prevent loosing of parasites. The cell solution is then centrifuged for 10 min at 200 g. The pellet is resuspended in 2,5 ml of PBS the fifth part of this 500 µl are taken to be mashed with a special mixer for 1 min. To extract the parasites from this a

centrifugation step for 8 min at 3000g follows. The resulting pellet is resuspended in 100 µl Schneiders medium (Schneiders drosophila medium, 2% human urine (v/v), 10% FCS (v/v), 4mM L-glutamine, 1% penicilline/streptamycine, 100 mM Hepes) and given in a dilution row, with a basis dilution of 1:5.

2.3.12 Parasite load ear

Same as described in parasite load spleen, but with a preceding collagenase digestion. The skin needs to be separated on the lesion side of the ear and the ear is digested dermis down in the digestion medium (DMEM, penicilline/strepamycine collagenase) for 2 h at 37°C and 5% CO_2. Adjacent the procedure is the same as described in parasite load spleen.

2.3.13 Immunohistochemistry

Shock frozen tissues were sectioned in 5-8 µm slices, fixed in ice cold acetone for 10 min and air dried for 10 min. Using a Pap pen, the tissue was outlined on the glass slide, placed in a wet chamber, and TBS was added for 5 min at RT. Slides were then incubated with quenching buffer containing 0.3% (v/v) H2O2 for 30 min and washed once with TBS. The sections were incubated with avidin solution and biotin solution for 15 min each and subsequently washed three times with TBS. The sections were then incubated with primary antibodies for 60 min. Sections were washed three times with TBS.

2.3.14 Preparation and immuno-staining of cytospins

In order to evaluate the expression levels of different proteins in B cells, cytospins were prepared. Therefore, 2x105 B cells were centrifuged on glass cover slips (5 min at maximum speed) resulting in cell spots and stored at –80°C.

Cytospins of purified B cells were fixed in acetone for 10 min at –20°C and

stained according to standard methods, using rabbit IgG Bcl-3 antibody (Santa Cruz, Heidelberg, Germany). All incubation steps during the staining procedure were carried out at RT. The cytospins were counterstained with Hoechst 33258.

2.4 Cytokine determinations

2.4.1 ELISA

Detection of cytokines (IFN-γ, IL-4, IL-10 and IL-17A) was performed with ELISA (BDBiosciences, Heidelberg, Germany) according to the manufacturer's instructions.

2.4.2 FlowCytomix

Multiple cytokine and chemokine levels were detected using FloxCytomixTM technology (BenderMedsystems, Vienna) according to the manufacturers instructions.

2.5 Mouse experiments

2.5.1 Mice

C57BL/6 mice were obtained from Charles River or Jackson Laboratories, C57BL/6 Thy1.1 mice were taken from breedings in our animal facility. CD19-Cre mice (Rickert et al., 1997); CD11c-Cre mice (Probst et al., 2003), iDTR mice (Buch et al., 2005), J_HT mice (Gu et al., 1993), intercrossed with mice in conventional facilities.

2.5.2 Depletion of cells with diphtheria toxin (DT)

As it is described in Buch et al. it is possible to deplete specific cell population while inbreeding of a Cre mouse strain with an iDTR mouse strain. A dose of 25mg/g bodyweight was used in every DT injection. To get the best result DT was injected once a day, four days in a row. The strains CD11c-Cre/iDTR and CD19-Cre/iDTR were used.

2.5.3 Adoptive transfer

After enrichment via magnetic cell sorting (2.3.4) of the desired cell population, cell suspensions were washed with PBS, counted, and diluted to the desired concentration. Cells were injected in PBS intravenously (i.v.) into the tail vein of recipient mice (500 µl/mouse). To better track transferred T cells by FACS, Thy1.1^{+} cells were transferred to Thy1.2^{+} recipients.

2.5.4 Bone marrow chimeras

The C57BL/6 recipient mice were irradiated with a lethal dose of 9,5 Grey, followed by i.v. adoptive transfer of bone marrow cells from donor mice consistent of 80% J_HT bone marrow and 20% of either TRIF/MyD88$^{-/-}$; CD40$^{-/-}$; MHCI$^{-/-}$; J_HT or C57BL/6. The bone marrow mixture of just J_HT and J_HT + C57Bl/6 was necessary as positive and negative control. The Reconstitution was analyzed in blood 6-8 weeks after the transfer. The mice were infected with 2x10^5 pfu of mCMV-WT intra food pad eight weeks after reconstitution and analyzed 7 days after infection.

2.5.5 Reconstitution with B cells

J_HT mice were reconstituted with magnetic sorted B cells from C57BL/6, modified from (Pasare and Medzhitov, 2005). 50*10^6 MACS purified B cells with at least 95% of CD19^{+} Cells (flow cytometry checked) were adoptively transferred via i.v. injection into the tail vein of the recipient mice. Seven days

after the reconstitution the mice were infected.

2.5.6 Reconstitution with serum

In both used infection models *Leishmania major* and mCMV were reconstitutions with serum performed, but with different protocols, because of the different courses of infection. In both infections the mice were reconstituted with normal mouse serum (NMS) and with immunized mouse serum (IMS). The IMS was harvested from mice three weeks after the regarding infection. The first reconsitution with serum was done in the week before the infection. The mice got 100 µl Sera diluted with 100 µl PBS in an intra peritoneal injection. The reconstitution was continuously carried out throughout the infection time until the analysis. After the infection the mice got one a week a serum reconstitution.

2.5.7 Infection with mCMV

8- to 10-week- old mice were infected subcutaneaous and intraplantar at the left hind footpad (Kabelitz), with 10^5 PFU of the according virus (mCMV-wt-Smith; mCMV-BAC; mCMV-SIINFEKL; mCMV-SIINFEKA; mCMV-ΔvRAP; mCMV-ΔvRAP-SIINFEKL; mCMV-Δm157Luc).

2.5.8 *In vivo* bioluminescence imaging

Mice were shaved and injected intravenously with 0.5 mg D-luciferin in 200 µl PBS and anaesthetized using isoflurane. Two minutes after luciferin injection, bioluminescence was recorded over a 300-second integration period by a cooled CCD camera system (Hamamatsu C4742– 98; Hamamatsu Photonics, Okayama City, Japan). Anesthesia was maintained during imaging by nose cone delivery of the anesthetic. SimplPCI Software (Compix, Cranberry Township, PA) was used for acquisition and images were processed in ImageJ (Wayne Rasband; National Institutes of Health, Bethesda, MD). Relative intensities of transmitted light from the in vivo bioluminescence were

represented as pseudocolor imaging. Corresponding gray scale photographs and color luciferase images were superimposed using Adobe Photoshop software (Adobe Systems, San Jose, CA).

2.5.9 Infection with *Leishmania major*

All experiments were carried out with a low dose *Leishmania major* infection. That means approximately 1000 metacyclic *Leishmania* (10µl of $0{,}1*10^6$/ml) were injected subcutaneously into the ear.

2.5.10 NP-CG immunisation and boost

Primary T cell dependent responses were induced with alum precipitated NP-CG (4- hydroxy-3-nitrophenylacetyl chicken-g-globulin) (Weiss and Rajewsky, 1990). The antigen was prepared by mixing 1 volume of NP-CG (1 mg/ml in PBS) with 1 volume of 10% (w/v) KA1(SO4)2. The solution was adjusted to pH 6.5 and kept 30 min on ice. Then, the precipitate was washed three times in PBS and resuspended in PBS. Mice were immunized by i.p. injection of 50 µg NP17-CG in a volume of 200 µl.

2.6 Statistics

Values are typically represented as mean ± SEM (standard error of mean). Statistical significance was assessed using 2-tailed Student's *t*-test. p-values < 0.05 were regarded significant, displayed by '*' in the figures (** = p-value < 0.05; *** = p-value < 0.005).

3. Results

3.1 The role of B cells in murine cytomegalovirus (mCMV)

Our intention was to elucidate the role of B cells in acute and memory mCMV infection, with focus on the acute phase. So far the role of B cells in the early mCMV response is elusive. To explore this role, we took advantage of different mouse models with different deficiencies in the B cell compartment. We analyzed the virus-specific CD8 T cell response to mCMV infection in J_HT, IgMi, IgG1i, CD19-Cre/iDTR, CD11c-Cre/iDTR, CD19-Cre/IL-$10^{fl/fl}$ and CD11c-Cre/IL-$10^{fl/fl}$ mice.

3.1.1. B Cells play a direct and an indirect role in acute and memory mCMV

B cells primed during mCMV infection have several possibilities to influence the immune response. To investigate the role of B cells and antibodies in the immune response to mCMV, C57BL/6, IgG1i, IgMi and J_HT mice (Gu et al., 1993; Waisman et al., 2008) were infected with mCMV-WT.Smith. General screening of the CD8 T cell activation markers CD25, CD69, CD62L, PD-1, and CD127 seven days post infection showed no differences between the mouse strains (data not shown). Therefore, seven days post infection we analyzed the IFN-γ secretion of CD8 T cells upon stimulation with virus-specific peptides, which have been described to be immuno-dominant in mice of the C57BL/6 background (Fig. 4 A). In J_HT mice we observed a significant decrease in the percentage of responding CD8 T cells seven days post infection, compared to wild type (wt) controls, whereas the percentage of IFN-γ CD8 T cells in IgMi and IgG1i mice was unaltered (Fig. 4 A). The virus-specific response of CD8 T cells towards e.g. M38 was in C57BL/6 about 4,1%, in IgG1i 3,9%, and in IgMi 3,1%, but less than 1% of CD8 T cells of J_HT mice were able to respond to this virus peptide. Since IgMi mice have B cells but lack antibody secretion this result indicates, that secreted antibodies do not play a role in the IFN-γ response of CD8 T cells. The decrease of this

virus-specific cell compartment in J_HT mice implies a need for B cells, not antibodies, for the establishment of the IFN-γ secreting virus-specific CD8 T cell response during acute mCMV infection.

Further we investigated the virus-specific CD8 T cell response in the memory phase, 135 days post mCMV infection. No differences in the CD8 T cell response of J_HT and IgG1i mice compared to C57BL/6 mice were observed (Fig. 4 B), in contrast to the IgMi strain, which showed an increased response (Fig. 4 B). In comparison to about 0,4% responding CD8 T cells to peptide M38 in C57BL/6 mice, around 0,6% in IgG1i, and 0,3% in J_HT mice responded, but in IgMi mice about 3% of CD8 T cells responded to M38. In particular the response to five virus-specific peptides (M38; m141; m139; M78 and IE3) was elevated (Fig. 4 lower panel). While in the acute response the presence of B cells is essential for the virus-specific CD8 T cell compartment, the memory response is affected by the absence of immunoglobulin. J_HT mice lack immunoglobulin as well as IgMi mice, but the CD8 T cell memory response in J_HT mice is disputable, because of the already altered acute response. However, this data showed immunoglobulin having a high impact on the CD8 T cell response in the memory response in IgMi mice.

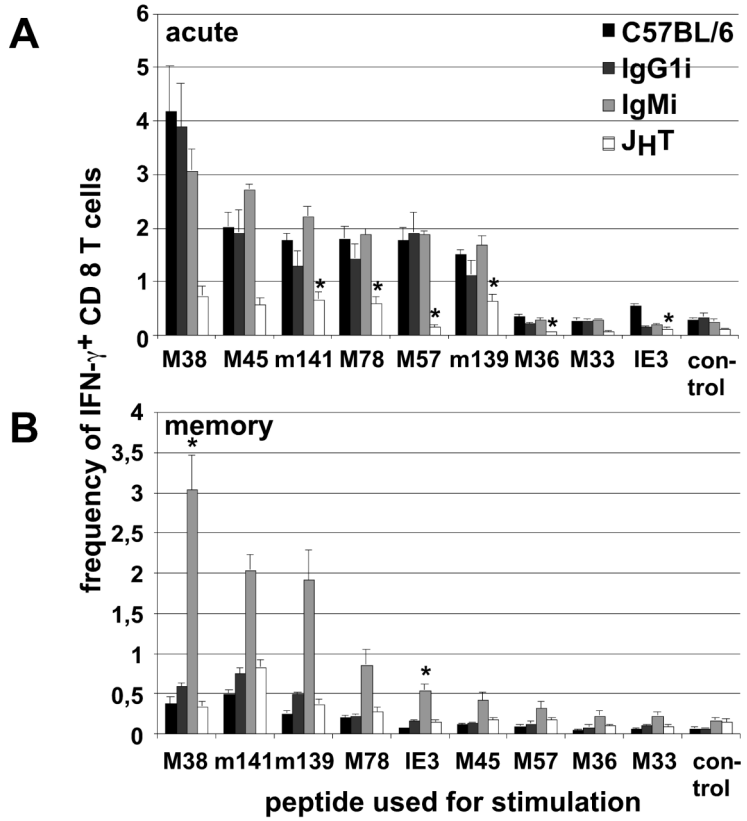

Figure 4. Frequency of responding mCMV specific CD8 T cells during acute and memory infection. A. C57BL/6, IgG1i, IgMi and J_HT mice (n=5) were infected with $2x10^5$ pfu mCMV-WT.Smith for seven days. The response to nine epitopes was determined by intracellular staining (ICS) for IFN-γ after stimulation of splenocytes with peptide in vitro for 6h in presence of Brefeldin A. Results are graphed as average ±SEM, and are typical for at least 3 experiments. **B.** Same as (A), mice (n=5) were infected for 135 days. Results are typical for at least two experiments. Asterisks denote responses significant in comparison with responses of C57BL/6 mice.

A direct comparison of the virus-specific CD8 T cell response in the acute and the memory phase regarding IFN-γ secretion showed four epitopes (M38, m141, m139 and IE3) having the same or even larger responding memory CD8 T cell population in IgMi mice, when compared to wt mice (Fig.5). The IFN-γ^+ memory CD8 T cell response in wt mice 135 days post infection was in all cases decreased compared to the response during the acute infection. For example the CD8 T cell population specific to the m141 epitope was during acute response in IgMi mice about 2,3% and in memory response about 2%. The size of this specific population in the acute response of wt mice was about the same, 2%, but 135 days post infection a population of just 0,5 was left. This phenomenon is called memory contraction and is founded and

induced by the clearance of the acute infection, IgMi mice apparently lack the memory contraction in the memory response to specific virus peptides. IgG1i mice underwent a memory contraction as seen in wt mice and hardly any virus specific CD8 T cells could be found. As already mentioned a clear interpretation of the response of J_HT mice during the memory response is not possible, since the initial response is low, it cannot be defined if J_HT mice undergo a memory contraction. Probably the virus-specific immunoglobulin in wt mice terminate recurrent virus directly, without the involvement of CD8 T cells. In IgMi mice these immunoglobulin are lacking and the recurrent virus stimulates directly the memory response of CD8 T cells, showing comparable populations sizes to acute response.

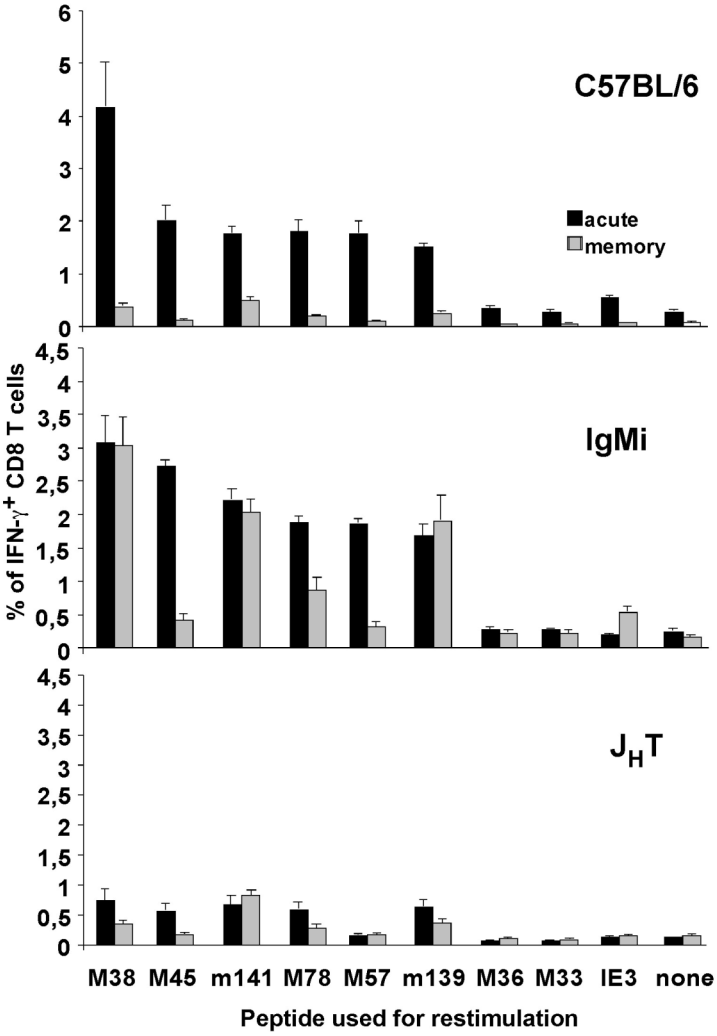

Figure 5. Comparison between the acute and the memory CD8 T cell response. Same experiment as in Fig. 4. C57BL/6, IgG1i, IgMi and J_HT mice (n=5) were infected with $2x10^5$ pfu mCMV-WT.Smith for seven days. The response to nine epitopes was determined by intracellular staining (ICS) for IFN-γ after stimulation of splenocytes with peptide in vitro for 6h in presence of Brefeldin A. Results are graphed as average ±SEM.

3.1.2. Virus clearance is not affected by the absence of B cells

J_HT mice showed a significantly decreased virus-specific CD8 T cell population in the acute response to mCMV infection (see 3.1.1). IFN-γ producing virus-specific CD8 T cells are normally directly responsible for the killing of virus-infected cells. Therefore the diminished CD8 T cell response in J_HT mice could affect the virus clearance. To verify this possibility, mice were infected with mCMV-Δm157-Luc. In this virus the locus of m157 is replaced by another open reading frame (ORF) coding for luciferase (luc). Luc is an enzyme the substrate luciferin and the progression of luciferin by luc results in bioluminescence, which can be recorded *in vivo* with a camera system (Klenovsek et al., 2007). The bioluminescence was observed in C57BL/6, IgG1i, J_HT, IgMi and $RAG^{-/-}$ mice over ten days during the acute infection. The clearance of the virus in all experimental mouse groups was as fast as in C57BL/6 control mice. The $RAG^{-/-}$ strain represented the positive control succumbing to the virus infection lacking virus clearance (Fig. 6).

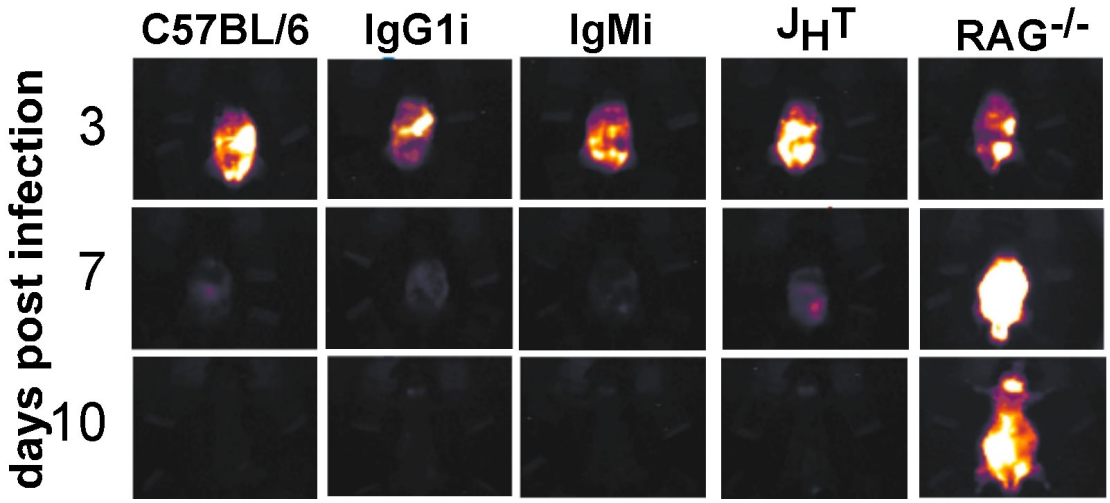

Figure 6. *In vivo* imaging of mCMV spread during the course of infection. C57BL/6, IgG1i, IgMi, J_HT, and $RAG^{-/-}$ mice were infected with $2x10^5$ pfu mCMV-Δm157-Luc intra peritoneal. Luciferin injection visualized the virus *in vivo*. Pictures were taken with a 300 second integration period. The bioluminescence of the virus was observed on day (d) 3, d7, and d10 post infection. C57BL/6 served as negative controls, $RAG^{-/-}$ mice served as positive controls.

3.1.2.1 Reconstitution of J_HT mice with normal mouse serum (NMS) and immunized mouse serum (IMS) did not affect the acute CD8 T cell response

The comparison of the CD8 T cell response of J_HT and IgMi mice does not indicate an involvement of immunoglobulin in the establishment of the cytotoxic lymphocyte (CTL) response in acute mCMV infection as can be seen in Fig. 4 A. In order to directly prove the role of immunoglobulin in the acute mCMV-specific CD8 T cell compartment, we reconstituted J_HT mice with sera of naïve (NMS) and mCMV-infected mice (IMS) from C57BL/6 mice. The reconstitution started three days before infection, followed by one serum reconstitution during the seven days of infection. Analysis of the virus-specific response seven days post infection showed no evidence of immunoglobulin being responsible for the decreased CD8 T cell response in J_HT mice (Fig. 7). All J_HT groups regardless of the treatment showed the same low response as observed in Fig. 4 A. The frequency of responding virus-specific CD8 T cells was below 1% in all J_HT groups in contrast to 3% and more in the C57BL/6 controls according to the peptide used for stimulation. This raised the question, whether B cells play a role as antigen presenting cells (APCs).

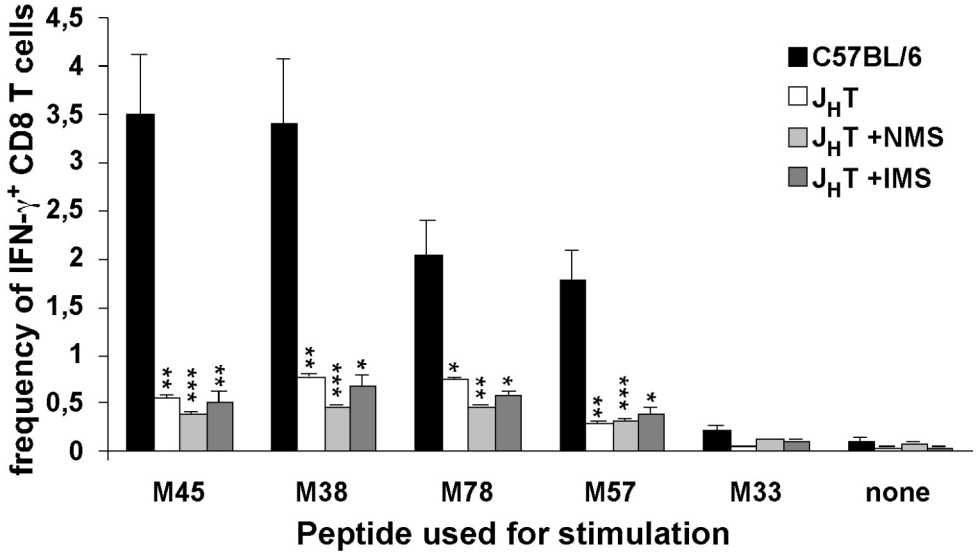

Figure 7. Examination of the influence of immunoglobulin on the virus-specific CD8 T cell population in acute infection. C57BL/6, J_HT and J_HT mice (n=5) either reconstituted with IMS or NMS at d-3, d0, and d4 were infected with with $2x10^5$ pfu mCMV-WT.Smith for seven days. The response to five epitopes was determined by intracellular staining (ICS) for IFN-γ after stimulation of splenocytes with peptide in vitro for 6h in presence of Brefeldin A. Results are graphed as average ±SEM, and are typical for at least three experiments. Asterisks denote responses significant in comparison with responses of C57BL/6 mice.

3.1.3 B cells as antigen presenting cells (APCs) in mCMV infection

B cells were described as APCs *in vivo*, especially in autoimmune diseases, (Finkelman et al., 1992; Harp et al., 2008; Serreze and Silveira, 2003). They were also shown as mediators of cross-presentation and can directly influence the cytotoxic T cell response (Hoft et al., 2007). One requirement for a successful antigen presentation in all infections is the presence of the virus in lymphocytic organs such as draining lymph nodes. Thus we had to investigate whether the virus is able to enter the draining lymph node in the absence of B cells.

3.1.3.1 mCMV enters the lymph system and replicates normally in J_HT

A blockage of the virus entry to the lymphatic system could be an explanation for the low virus-specific CD8 T cell response in J_HT mice. If B cells were the shuttle cells needed for the virus entry the lymphatic system, the low CD8 T

cell response in J_HT mice would be the consequence of a lacking infection of lymphocytic cells and little inflammation would be detectable. Without inflammation the adaptive immune response would not be initiated and the diminished CD8 T cell response could be explained. The lymph node of interest in an intra footpad infection is the draining popliteal lymph node. To demonstrate a role of B cells in the virus entry into the draining popliteal lymph node, the day with highest viral load post infection had to be assigned via RT-PCR of immediate early 1 (IE1) transcripts in wt mice. Figure 8A shows day two post infection as the relevant day. A comparison of the viral load in J_HT and wt mice two days post infection showed no significant difference in the viral load of the draining lymph node (Fig. 8B). Also in the absence of B cells the virus is able to enter the lymphatic system. Consequently, B cells are not involved in the trafficking of the virus to the draining lymphnode, and the inflammation starts B cells independently.

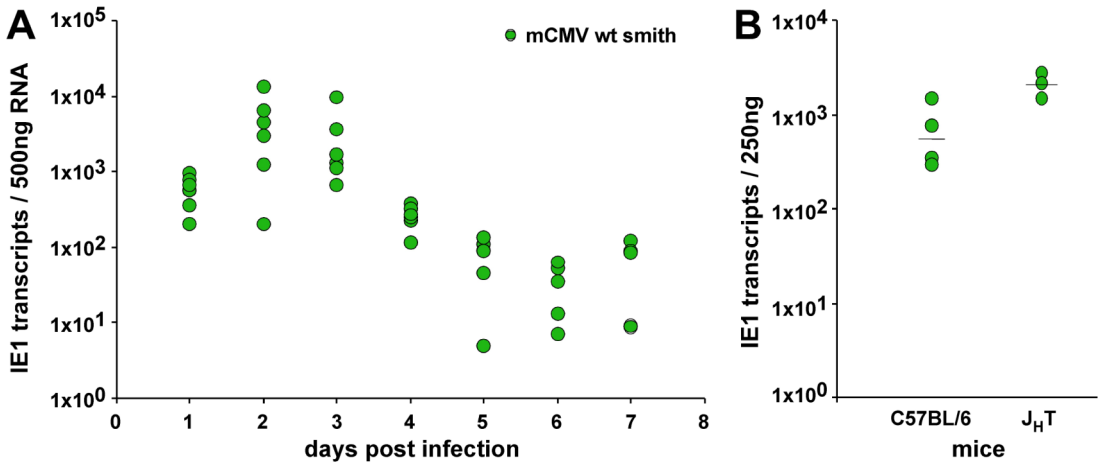

Figure 8. Viral load of draining popliteal lymph nodes two days post infection. **A.** Verification of the time point with the highest viral load in the popliteal lymph node. C57BL/6 mice (n=35) were infected with $2x10^5$ pfu mCMV-WT.Smith, each day the popliteal lymph node of five mice was taken RNA isolation and subjected to RT PCR for IE1 transcripts of mCMV-WT.Smith to measure the viral load. Each point represents the viral load of one individual popliteal lymphnode. **B.** Number of IE1 transcripts in the draining popliteal lymph node in J_HT and C57BL/6 mice (n=5) two days post infection.

3.1.3.2 B cell depletion in the CD19-Cre/iDTR system

Several studies show an abnormal T cell development and behaviour in mice deficient for B cells throughout their lives (Sun et al., 2008). By using the

CD19-Cre/iDTR system, we wanted to investigate, whether an abnormal T cell development might be the reason for the diminished CD8 T cell response in J_HT mice. The CD19-Cre/iDTR system offers the possibility to express a simian diphtheria toxin receptor (DTR) under the control of the CD19 promoter, a B cell lineage marker (Nadler et al., 1983; Palacios et al., 1987). After diphtheria toxin (DTx) application nearly all B cells in this system are depleted. Therefore the development of the T cells occurs in the presence of B cells in contrast to J_HT mice with a lifelong absence of B cells, but after depletion the mice are very similar to the B cell deficient J_HT mice. When the CD8 T cell response in this system after depletion of B cells is diminished as it is in J_HT mice, a developmental defect of the CD8 T cells caused by the absence of B cells can be excluded.

3.1.3.2.1 The functionality of the CD19-Cre/iDTR system

The expression of the DTR depends on the efficient excision of the stop cassette by the Cre-recombinase, which is expressed under the control of the CD19 promoter. Therefore, the efficiency of the DTR expression is directly combined with the strength of the CD19 promoter and the accessibility of the Rosa locus, the locus in which the DTR and YFP is located. To test the efficiency of the Cre-recombinase under the control of the CD19 promoter, CD19-Cre mice were crossed with eYFP reporter mice. This breeding verified the Cre-recombinase activity through the eYFP expression. The iDTR and the eYFP strain are constructed similar. The iDTR and the eYFP are inserted in the Rosa26 locus, where the stop cassette is positioned in front. This parallel construction of the eYFP and the iDTR mice enables to transfer the conclusions made by the analysis of CD19Cre/eYFP mice and draw back to the CD19-Cre/iDTR mice. The B-cell compartment of CD19-Cre/eYFP mice was analyzed for $eYFP^+$ cells (Fig. 9 A). In spleen, lymph nodes, peyer's patches, and peritoneal cavity, almost all $CD19^+$ were also $eYFP^+$, and the cells of the CD19-Cre control mice did not express eYFP. In bone marrow only 46% of $CD19^+$ cells were positive for eYFP. To analyze the $eYFP^-$ $CD19^+$ compartment in the bone marrow, the cells were stained for AA4.1 and CD23, and could be identified as immature B cells (Fig. 9 B). The high B cell specific

eYFP expression in the reporter mice predicted an efficient depletion of mature B cells.

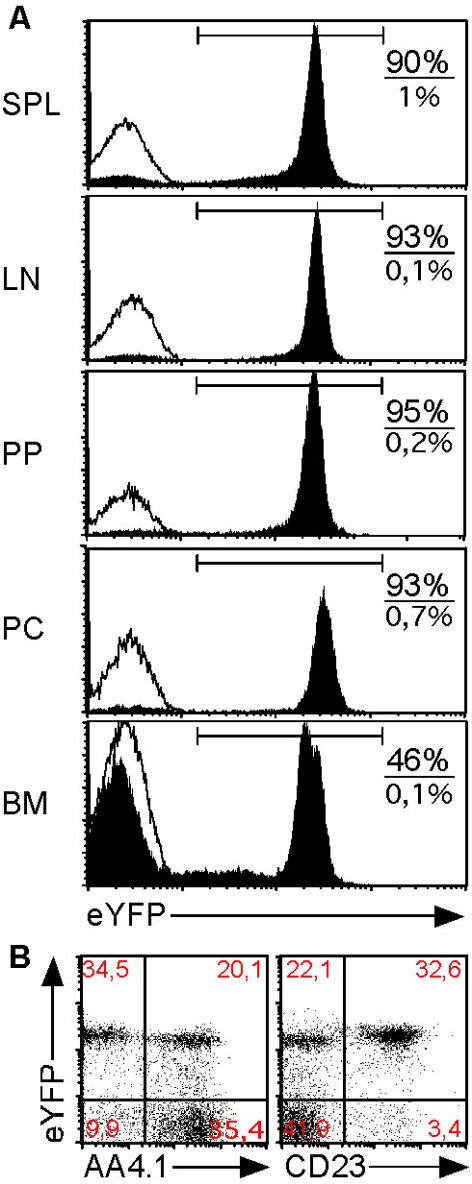

Figure 9. EYFP expression of B cells in CD19-Cre/eYFP mice. A. Analysis of CD19-Cre/eYFP and control mice (n=5) for eYFP expression in the B cell compartment of spleen, lymph nodes, payer´s patches, peritoneal cavity, and bone marrow. The filled histograms represent cells isolated from CD19-Cre/eYFP mice (percentages upper number) and the unfilled histograms represent cells isolated from control mice (percentages lower number). **B.** Analysis of the $CD19^{+}$ $eYFP^{-}$ B-cell fraction in bone marrow of CD19-Cre/eYFP mice, bone marrow cells were stained for AA4.1 and CD23.

The depletion of B cells was analyzed in CD19-Cre/iDTR mice. The mice were treated with DTx daily for four days, then spleen, lymph nodes, bone marrow, and peritoneal cavity were analyzed. After the DTx treatment in spleen 0,7% of $CD19^+$ cells were left after the depletion in contrast to 71,4% in the non-depleted mice, in lymph nodes 0,2% of 29%, in peritoneal cavity 0,3% of 47,5% and in bone marrow 8,7% of 55,4% were left (Fig. 10). The efficiency of depletion was very high in all tested organs except for the bone marrow, which was expected regarding the eYFP expression in bone marrow of CD19-Cre/eYFP mice (Fig. 9).

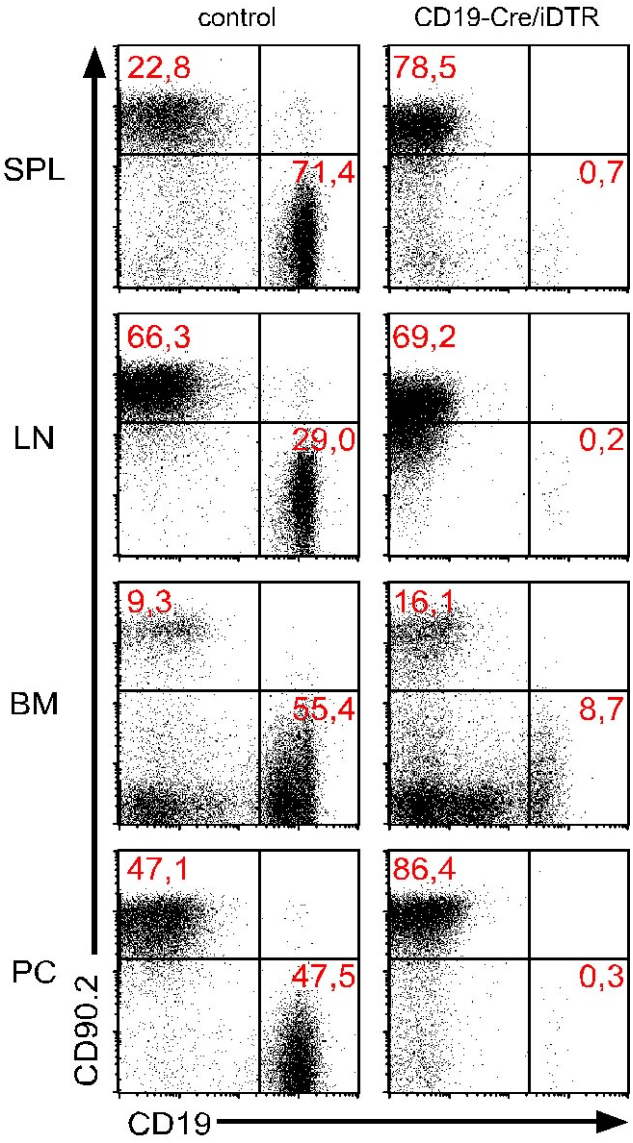

Figure 10. DTx mediated depletion of B cells in CD19-Cre/iDTR mice. Control and CD19-Cre/iDTR mice (n=5) were treated daily for four days with a dose of 25ng/g bodyweight of DTx. The decrease of B cells in comparison to T cells is shown in spleen, lymph nodes, bone marrow, and peritoneal cavity. Shown are representative dot blots of one mouse of five mice per group of three independent experiments.

To further differentiate the efficiency of depletion in various B cell compartments, we compared different B cell subsets of CD19-Cre/iDTR and control mice (Fig.: 11). The total cell number of splenic B cells was significantly decreased regarding marginal zone, follicular as well as mature and immature B cells (Fig.: 11 A). B cells are differentiated in different subpopulations regarding the developmental stage and function. In spleen several of these B cell subpopulations are found in parallel, but the subpopulations are depleted varying efficiently. A direct comparison of the remaining B cell populations showed an increase in the population of marginal zone B cells from 8,8% to 15,6% in depleted mice in relation to follicular B cells, which were decreased from 79,8% to 54,8% in depleted mice (Fig. 11 B). Mature residual B cells were stronger reduced compared to immature B cells, the percentage of mature IgD^+, IgM^+ B cells decreased from 83,3% to 55,2%, in contrast the percentage of the immature IgD^-, IgM^+ B cells, which increased from 6,7% to 17,7% (Fig. 11 C). Transitional B cells ($AA4.1^+$) were harder to deplete, this population increased from 20,5% to 31,9% (Fig. 11 D). In the peritoneal cavity, all B cell subpopulations were equally depleted (Fig. 11 E and G), except for the B-1 B cells, represented by the $CD5^+$ population in Figure 11 E, which were depleted nearly completely. The bone marrow illustrated a big difference in depletion efficiency between the B cell subsets. The IgM^- B cell compartment, consisting of pre-pro-, pro-, and pre-B cells was hardly depleted, resulting in an increase from 43,4% to 73,3%, which could be explained by the fact that CD19 expression is just starting in this B cell compartment. The next developmental stage, the immature B cells were partially depleted, demonstrated by a decrease in the percentage from 21,6% to 13,8%. The B cell population that showed the most efficient depletion in the bone marrow were the mature recirculating $B220^{high}$ B cells. In control mice, 32,1% of the B cells belonged to this population, whereas in the depleted mice only 4,5% of recirculating B cells could be detected (Fig. 11 F). Consequently, the depletion efficiency was dependent on the maturation stage of CD19 in B cells, as also demonstrated before using in CD19-Cre reporter mouse.

Further we wanted to investigate the influence of the B cell depletion on the serum immunoglobulin level. Therefore the serum of CD19-Cre/iDTR mice

treated with four daily injections of DTx was collected over a time period of six weeks. One week after the DTx treatment a decrease of total immunoglobulin was observed to a very low extent, shown by a significant decrease in Igκ, along with a low reduction in IgM and IgG1 serum immunoglobulin (Fig. 12). To investigate the influence and efficiency of B cell depletion during an immunization and especially the course of antigen specific immunoglobulin, we immunized CD19-Cre/iDTR mice and control groups intra peritoneal with NP-CG (Tesch et al., 1984). The mice were bled weekly to observe the NP-specific immunoglobulin in the serum, the bleeding was started directly before the immunization. Three weeks after the immunization at the peak of the T-cell dependent B-cell response to NP-CG, the depletion was started as described before. To check the efficiency of the depletion during the immunization, six weeks after the immunization, the remaining NP-specific plasma and memory B cells were boosted with a second NP-CG immunization, the remaining antigen-specific B cell response especially NP-specific immunoglobulin was analyzed eight days later. A decrease of the total NP-specific serum immunoglobulin was observed four weeks post immunization, meaning one week post depletion, represented in a significant decrease in the NP-specific Igκ-level, going along with a significant decrease of NP-specific IgG1 and IgM, whereas the NP-specific IgG2ab antibody level remained unaffected (Fig. 13). One week after the NP-CG boost the IgG1 level of B cell-depleted mice was significantly decreased in comparison to the control group (Fig. 13). As IgG1 is mostly synthesized and secreted by plasma cells, the decrease of IgG1 implied the depletion of plasma cells. Plasma cells loose most of their surface antigens such as CD19 by a high membrane turnover eventually including the loss of DTR. However, the used Cre-loxP system gives a genetic memory: If the stop cassette disabling the DTR transcription is excised, it stays excised and the DTR can be transcribed throughout the time. In this regard the depletion efficiency of plasma cells is highly dependent on the time point when the toxin is given. If most of the B cells are still memory B cells and not yet plasma cells, might more memory B cells are depleted due to higher surface expression of DTR in contrast to the might diminished DTR exhibition on plasma cells caused by the high

membrane turnover. The quantity of bone marrow plasma cells in the immunized and boosted mice was analyzed eight days post the NP-CG boost and cytospins of bone marrow cells were prepared. An immunohisto staining for the plasma cell marker syndecan-1 (CD138) of these cytospins showed a significant decrease in the number of plasma cells in the depleted mice (Fig. 14 A and B).

In summary, the CD19-Cre/iDTR strain is a very powerful tool to deplete B cells and partly also plasma cells. The advantage over other systems is the normal development of the whole immune system in the presence of B cells. Also the depletion of B cells can be started at any time point. Further the depletion with DTx is less expensive than the use of B cell depletion antibodies.

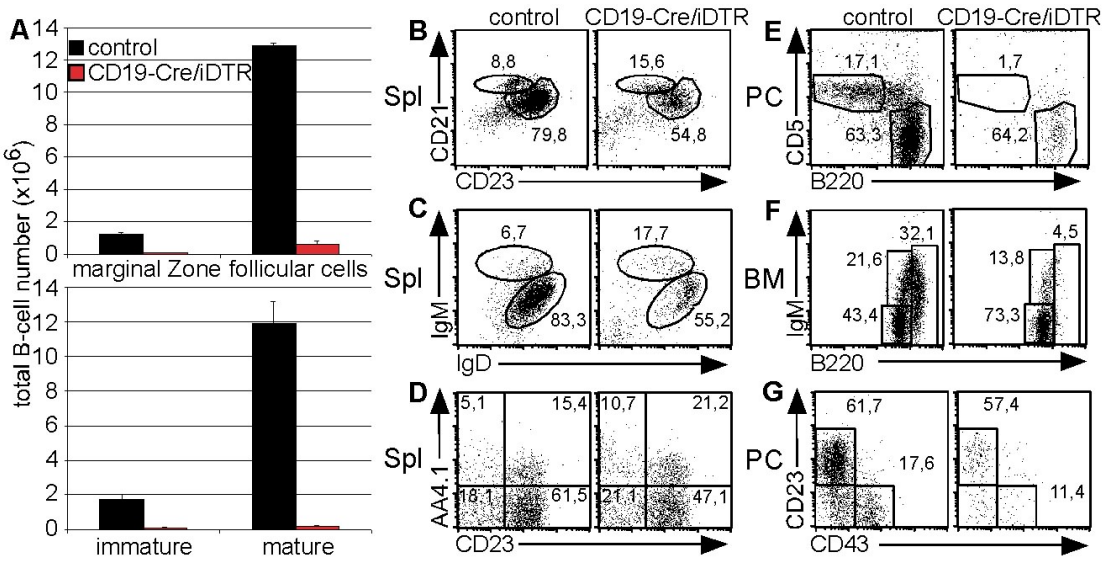

Figure 11. Efficiency of B cell depletion. A. Same experiment as shown in Figure 10. Total number of marginal zone, follicular, immature, and mature B cells in spleen. **B.** Depletion of marginal zone and follicular B cells. **C.** Depletion of mature and immature B cells in spleen. **D.** Depletion of transitional B cells in spleen. **E.** Depletion of B-1a and B-2 B cells in peritoneal cavity. **F.** Depletion of B cells in bone marrow. **G.** Depletion of resting B cells in peritoneal cavity. All gated on $CD19^+$ cells except for F, which is gated on $B220^+$ cells. Shown are representative dot blots of one mouse out of a group on five mice of three independent experiments.

Control and CD19-Cre/iDTR mice (n=5) were treated daily for four days with a dose of 25ng/g bodyweight of DTx. The decrease of B cells in comparison to T cells is shown in spleen, lymph nodes, bone marrow, and peritoneal cavity. Shown are representative dot blots of one mouse of five mice per group of three independent experiments.

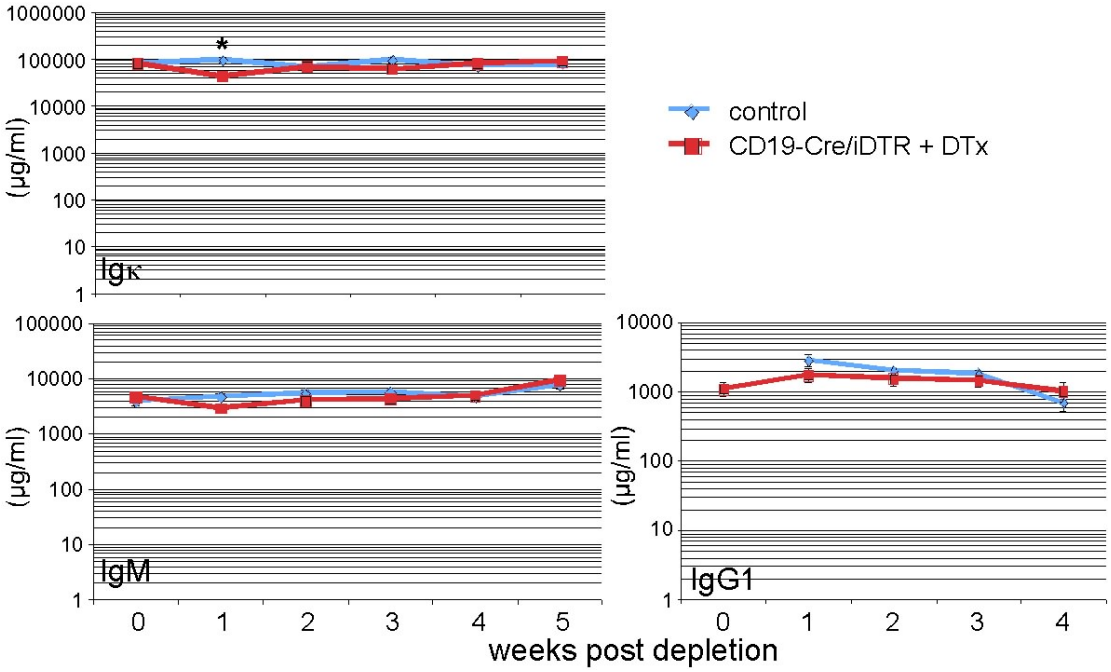

Figure 12. Serum immunoglobulin levels after B cell depletion. Two groups of CD19-Cre/iDTR mice (n=5) were treated daily for four days with a dose of 25ng/g bodyweight of DTx or PBS. Total serum immunoglobulin levels after B cells depletion were monitored over five weeks. The mice were bled once a week to collect serum. Week zero represents the steady state of serum immunoglobulin. Results are graphed as average ±SEM, and are typical for at least three experiments. Asterisks denote responses significant in comparison with PBS-treated control mice.

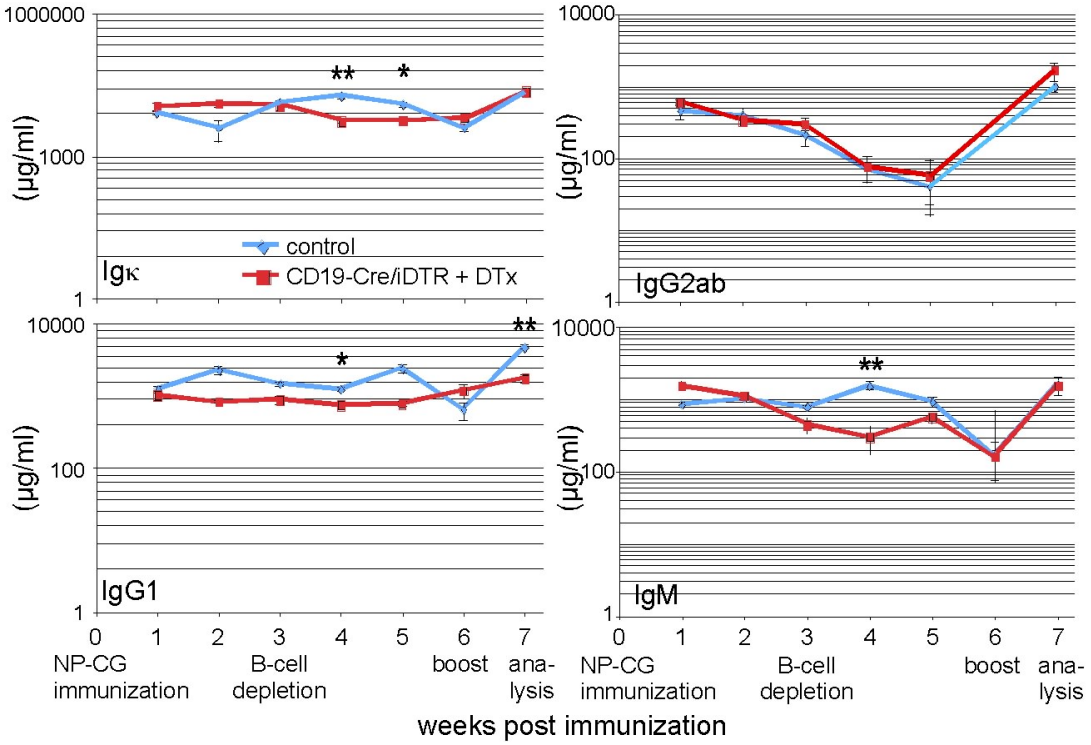

Figure 13. Effect of B cell depletion on the specific immunoglobulin levels in the course of a NP-CG immunization. Control and CD19-Cre/iDTR mice (n=5) were immunized with 50µg NP-CG and bled weekly, starting before the immunization. Three weeks post immunization mice were treated over four days with a daily dose of 25ng/g bodyweight of DTx. Six weeks post immunization the memory response of memory B cells and plasma cells was stimulated with a second immunization of NP-CG. The serum of the weekly collected blood was analyzed for NP-specific Igκ, IgG2ab, IgG1, IgG3, and IgM. Results are graphed as average ±SEM, and are typical for at least three experiments. Asterisks denote responses significant in comparison to control mice.

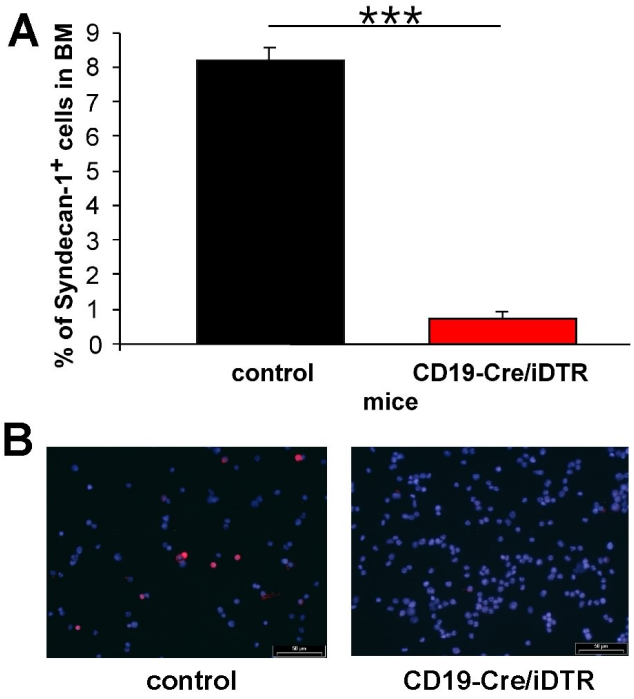

Figure 14. Syndecan-1$^+$ plasma cell of bone marrow cytospins of NP-CG immunized mice. Mice as described in Fig. 13. Bone marrow cells from NP-CG immunized and boosted mice were analyzed for plasma cells. Plasma cells are visualized in red with a surface staining of syndecan-1, all cells were stained with Hoechst (blue). **A.** The bar chart represents plasma cells found in the regarding groups (n=5). **B.** Visualization on the cytospins. The pictures show representative sections of the cytospins. Results are graphed as average ±SEM, and are typical for at least three experiments. Asterisks denote responses significant in comparison to control mice.

3.1.3.2.2 Depletion of B cells in mCMV infection leads to an impaired CD8 T cell response comparable to J_HT

The CD19-Cre/iDTR system served as a tool to answer the question, whether the low T cells response in J_HT mice is due to the total absence of B cells. The time schedule described in Figure 15 A was used for an optimal depletion in the CD19-Cre/iDTR mice. To verify the depletion efficiency in the spleen, one mouse was analyzed at the day of infection and showed almost all B cells successfully depleted (Fig. 15 B). Therefore the number of B cells in the depleted and in the J_HT mice was comparable at the day of mCMV infection with the only difference that in depleted mice B cells had been present during T cell development. Seven days post infection the virus-specific T cell response was analyzed and a diminished virus-specific CD8 T cell response,

comparable to the response in J_HT mice, was observed (Fig. 15C). The possible influence of DTx on virus replication was ruled out by an analysis of the viral load in the draining lymph node via RT-PCR for viral IE1 transcripts two days post infection in DTx-treated and non-treated CD19-Cre/iDTR and C57Bl/6 mice, which showed no differences (Fig. 16). This result confirmed, that the lacking virus-specific CD8 T cell response in J_HT mice is a direct effect of the lack of B cells during infection and not a side effect of the lifelong B cell absence.

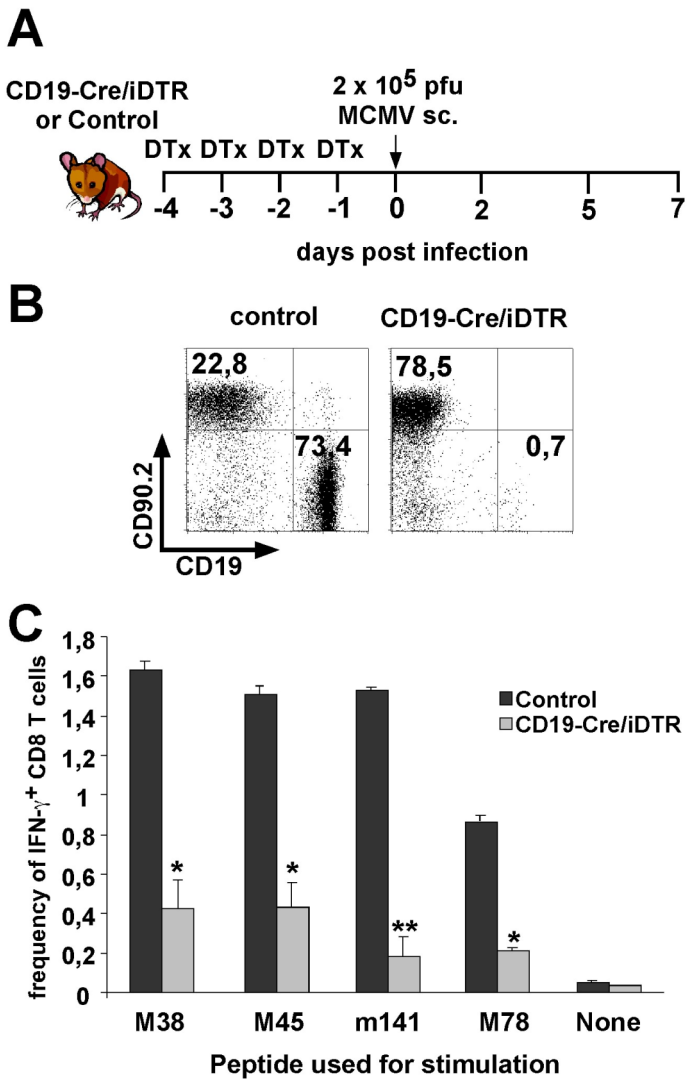

Figure 15. The livelong absence of B cells in J_HT mice is not responsible for the low virus-specific CD8 T cell response. A. CD19-Cre/iDTR and iDTR mice (n=5) were treated over four days with a daily dose of 25ng/g bodyweight of DTx according to the illustrated schedule. **B.** B cell depletion in spleen at day of infection. **C.** The depleted mice were infected with $2x10^5$ pfu mCMV-WT.Smith for seven days. The response to four epitopes was determined by intracellular staining (ICS) for IFN-γ after stimulation of splenocytes with peptide in vitro for 6h in presence of Brefeldin A. Comparison between B cell-depleted mice (CD19-Cre/iDTR) and non-depleted (control) mice. Results are graphed as average ±SEM, and are typical for at least three experiments. Asterisks denote responses significant in comparison with PBS-treated control mice.

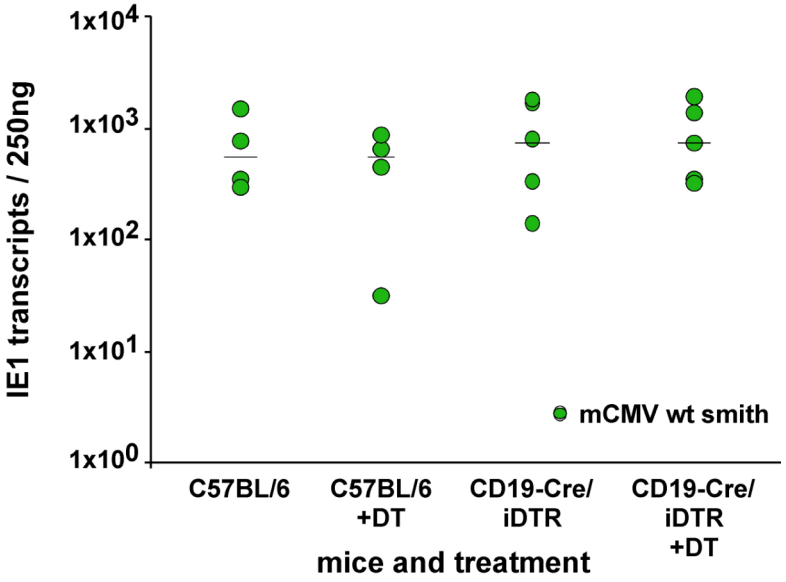

Figure 16. Influence of DTx on the mCMV replication in the draining lymph node. Four days DTx-treated and non-treated C57BL/6 and CD19-Cre/iDTR mice (n=5) were infected with $2x10^5$ pfu mCMV-WT.Smith for two days. RNA was isolated from popliteal lymph nodes and the viral load was determined via verification of viral IE1 transcripts per 250ng of RNA using RT-PCR. Each point represents the viral load of one individual popliteal lymphnode.

3.1.3.3 Depletion of dendritc cells (DCs) in mCMV infection leads to an impaired CD8 T cell response comparable to B cell depleted mice

The most prominent APCs are DCs. Therefore, it was interesting to investigate the virus-specific CD8 T cell response in the absence of this professional APC compartment. To examine this question the already described iDTR mouse strain was bred to a CD11c-Cre lineage. DTx application in the resulting CD11c-Cre/iDTR mice using the same protocol as used for the CD19-Cre/iDTR strain (Fig. 17 A) efficiently depletes DCs. The lineage marker CD11c is not as specific for DCs, as CD19 is for B cells, there are other cell types expressing CD11c, including activated CD8 T cells (Kao et al., 2007; Kim et al., 2005; Vinay and Kwon, 2010). Therefore, it was necessary to answer the question, whether the $CD11c^+$ CD8 T cell population is depleted by DTx application and whether this population belongs to the IFN-γ^+ CD8 T cells. Splenic DCs were identified with a staining for CD11c and MHCII, they were highly reduced from 6,3% to 0,3%, in DC-depleted mice (Fig. 17 B), whereas the $CD11c^+$ $CD8^+$ compartment was nearly unaffected (decreased from 0,9% to 0,7%) (Fig. 17 C). After the time point of infection

further application of DTx was avoided to prevent a depletion of virus infection activated CD11c$^+$ CD8 T cells. Seven days post infection the mice were analyzed for the CD11c$^+$ CD8 T cell population, which was decreased by half from 8,5% to 4,3% (Fig. 17 D). The IFN-γ producing CD8 T cells specific for the virus peptide M45 were similarly decreased from 3,2% to 1,6% (Fig. 17 E). There are two possible explanations for the decrease in the virus-specific CD8 T cell compartment: Firstly DCs are needed to establish a normal virus-specific CD8 T cell response or secondly the virus-specific CD8 T cells express at least for a certain time CD11c as activation marker, therefore they express DTR and are depleted by the remaining DTx.

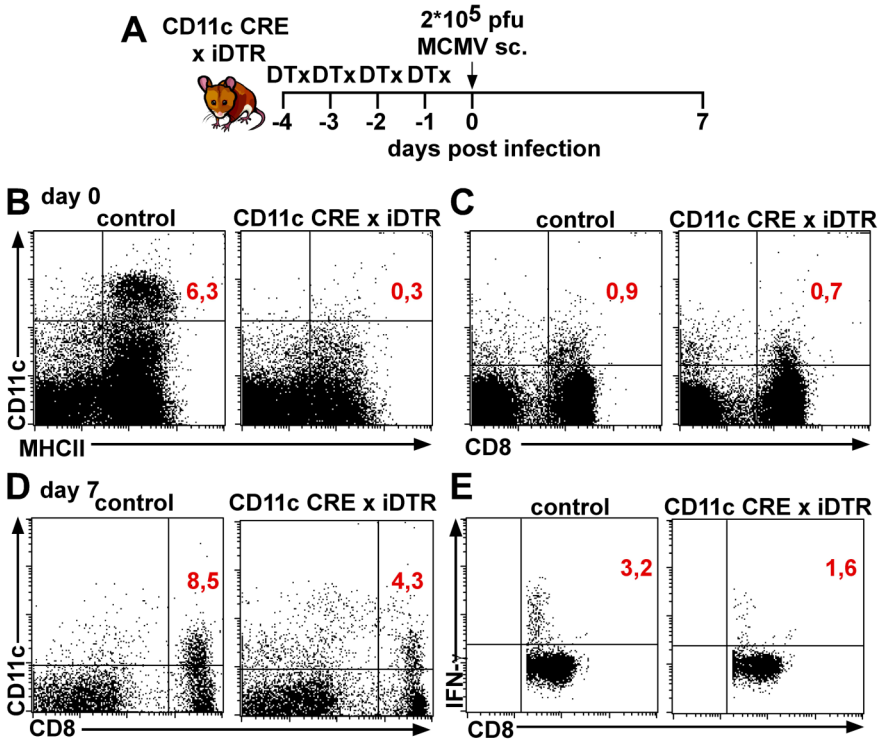

Figure 17. Depletion of DCs in mCMV infection diminishes the virus-specific CD8 T cell response. A. CD11-Cre/iDTR and iDTR mice (n=5) were treated over four days with a daily dose of 25ng/g bodyweight of DTx according to the illustrated schedule. B. DC depletion in spleen at day of infection. C. Depletion of CD11c$^+$ CD8 T cells in spleen at day of infection. D. The depleted mice were infected with 2x10^5 pfu mCMV-WT.Smith for seven days. Analysis of CD11c$^+$ CD8 T cells in spleen. E. Same experiment as E. The response to M45 was determined by ICS for IFN-γ after stimulation of splenocytes with peptide in vitro for 6h in presence of Brefeldin A. Shown are representative dot blots of one mouse out of a group of five mice of three independent experiments.

3.1.3.3.1 Activated $CD8^+$ $CD11c^+$ T cells are not depleted in CD11c-Cre/iDTR mice

Upon activation a subset of CD8 T cells start to express CD11c as a activation marker. To clarify, whether the $CD11c^+$ CD8 T cells are depleted in CD11c-Cre/iDTR mice or they just do not express the DTR, an adoptive transfer experiment was performed. CD8 T cells from CD90.1 mice were isolated and adoptively transferred to $CD90.2^+$ CD11c-Cre/iDTR mice and $CD90.2^+$ control mice. The adoptively transferred CD8 T cells had five days to home to their lymphocytic tissue before the DTx treatment was started. Five days after the transfer and four days before the infection the mice received a daily dose of DTx (Fig. 18 A). The efficiency of the DC-depletion by DTx was controlled in spleen at the day of infection in one representative mouse (Fig. 18 B). During the time of infection the mice were treated additionally two times with DTx (Fig. 18 A). The peak in the acute phase of the virus-specific CD8 T cell response in a mCMV infection is seven days post infection. That was the time point, when the virus specific IFN-γ response of CD90.1 DTR^- and CD90.2 CD8 T cells in the transferred mice was analyzed. The over-all virus-specific CD8 T cell response in CD11c-Cre/iDTR mice was significantly decreased for all tested peptides (Fig. 18 C), but did not show differences neither in the control mice nor in the experimental group of CD11c-Cre/iDTR mice in the $CD11c^+$ CD8 T cell response itemized for CD90.1 and CD90.2 (Fig. 18 D). This result implies no depletion of virus-specific CD8 T cells in CD11c-Cre/iDTR mice and showed a similar effect on the virus-specific CD8 T cell response in the depletion of either DCs or B cells.

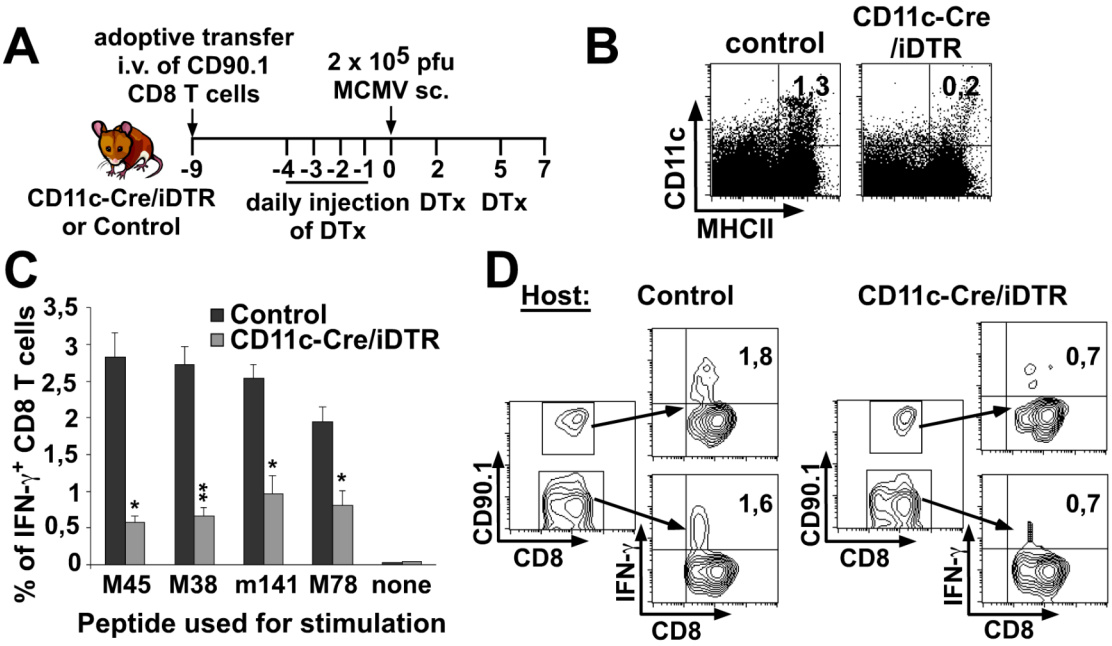

Figure 18. Activated CD8 T cells are not depleted in CD11c-Cre/iDTR mice during mCMV infection. A. Magnetic cell sorted $CD8^+$ $CD90.1^+$ T cells were adoptively transferred to $CD90.2^+$ CD11c-Cre/iDTR, iDTR and C57BL/6 mice nine days ahead of $2x10^5$ pfu mCMV wt smith intra footpad infection. Timeschedule of transfer, DTx treatment and infection. Seven days post infection the mice were analyzed. **B.** DC depletion in spleen at day of infection, after four days daily DTx treatment with a dose of 25 ng/g bodyweight in control and CD11c-Cre/iDTR mice. **C.** The depleted mice were infected with $2x10^5$ pfu mCMV-WT.Smith for seven days. The response to four epitopes was determined by intracellular staining (ICS) for IFN-γ after in vitro stimulation of splenocytes with peptide for 6h in presence of Brefeldin A. **D.** Same experiment as C. Analysis of the adoptively transferred T cells itemized for $CD90.2^+$ and $CD90.1^+$, response to m141 is shown. Asterisks denote responses significant in comparison to control mice.

3.1.3.4 Response to mCMV in the absence of B cells and DCs is impaired

Interestingly, the virus-specific CD8 T cell response was not completely absent, when B cells or DCs were lacking. In CD19-Cre/CD11c-Cre/iDTR mice we depleted both, B cells and DCs, to investigate whether the whole CD8 T cell response would be aborted. To monitor the depletion, spleen cells of depleted mice were stained for MHC class II (Fig. 19 A and B). MHC class II^+ cells were not depleted as efficiently as in the previous depletion experiments (Fig. 19 A). Further, comparatively high amounts of MHC class II-expressing cells were left at the time point of the analysis of the virus-specific CD8 T cell response (Fig. 19 B). As expected, we found a diminished virus-specific CD8 T cell response, but still up to 1% of the CD8 T cells expressed IFN-γ after stimulation with virus peptides, despite the massive reduction of B

cells and DCs.

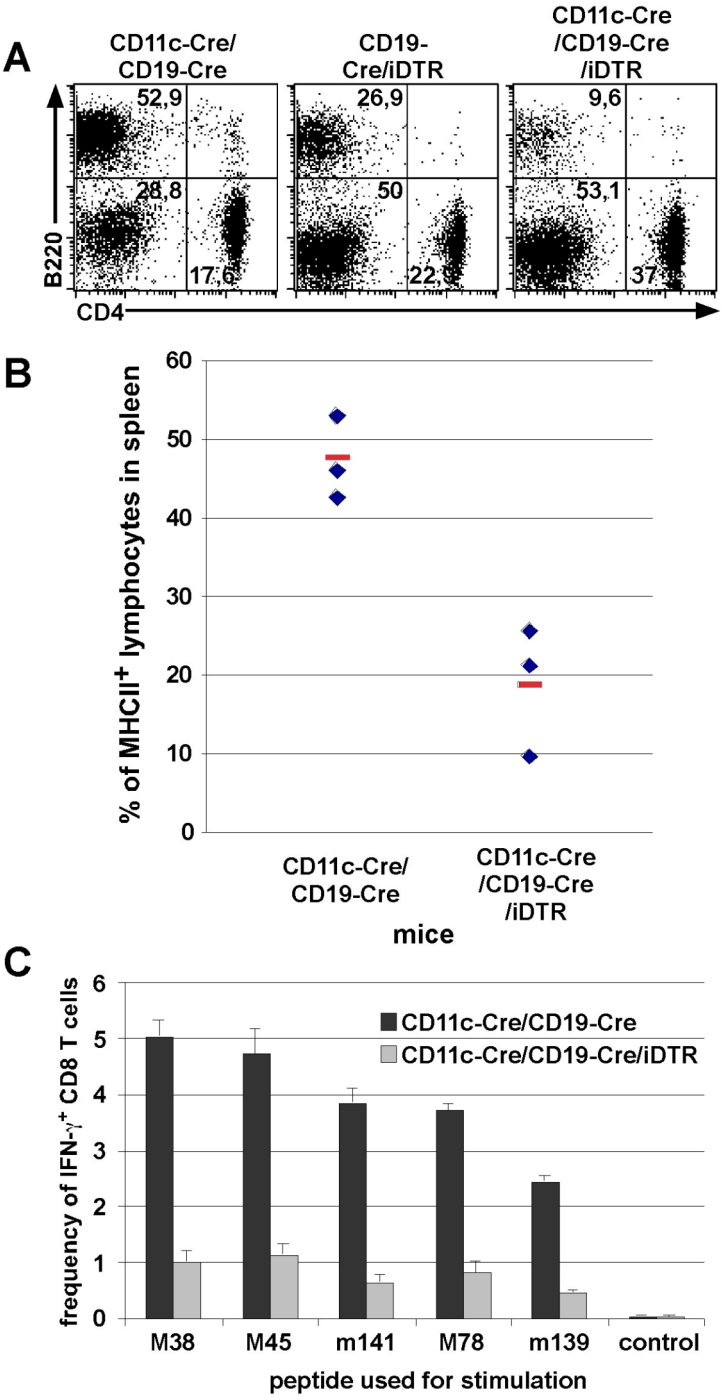

Figure 19. The depletion of B cells and DC does not prevent the whole virus specific CD8 T cell response. CD11c-Cre/CD19-Cre/iDTR and CD19-Cre/CD11c-Cre mice (n=5) were treated with DTx as described in Fig. 17. A. MHCII$^+$ cell depletion of the used mice at day of infection. B. same as A as bar graph. C. The depleted mice were infected with 2x10^5 pfu mCMV-WT.Smith for seven days. The response to four epitopes was determined by intracellular staining (ICS) for IFN-γ after in vitro stimulation of splenocytes with peptide for 6h in presence of Brefeldin A. Asterisks denote responses significant in comparison to control mice.

3.1.3.5 B Cell reconstitution partially rescued the decreased CD8 T cell response in J_HT mice

So far it was not possible to explain the phenotype of the decreased virus-specific CD8 T cell response to mCMV infection in the B cell-deficient J_HT mice. We already excluded, the absence of immunoglobulin, a defective CD8 T cell compartment, an impaired virus replication or impaired entrance of the virus into the immune system as a reason for the phenotype. One unanswered question was, whether it is possible to rescue the phenotype of the low CD8 T cell response in J_HT mice by a reconstitution with wt B cells. Thus, purified B cells were transferred intravenously into J_HT mice. One week after the transfer mice were infected intra footpad with mCMV-WT.Smith and the acute virus-specific CD8 T cell response was analyzed seven days later (Fig. 20 A). To analyze the efficiency of the transfer and to control the survival of the transferred B cells, the number of B cells in spleen was verified in addition to the virus-specific CD8 T cell response of all three groups, C57BL/6, J_HT, and B cell-reconstituted J_HT mice. The response of the reconstituted mice was neither comparable to the response of the positive control (C57BL/6) nor to the negative control (J_HT). As can be seen in Figure 20 C it was in-between C57BL/6 mice and J_HT mice (Fig. 20 B). The response to M45 and m141 was nearly the same as well as the different CD8 T cell populations in the different groups. The control wt mice were able to mount a T cell response of 1,7% specific to either M45 or m141, J_HT mice the negative control only had a specific CD8 T cell population of about 0,3% and last but not least the wt-B cell-reconstituted J_HT group had a specific CD8 T cell population of about 0,95% and therefore in-between the response of both control groups. However, the transferred B cells seem to be a problem in the reconstituted mice, the control group of C57BL/6 mice had 56,1% $B220^+$ cells in spleen, J_HT just 1,1%, mostly macrophages, a distinct cell population was not found, and in the B cell transferred J_HT mice had a population of 9,8% of $B220^+$ cells in spleen. The percentage represents one-sixth of the number found in C57BL/6 mice. Therefore, the remaining B cells in the transferred mice had a high impact on the virus-specific CD8 T cell response. It was

possible to partly rescue the phenotype of the diminished virus-specific CD8 T cell response to mCMV in J_HT mice by the transfer of B cells. The transfer protocol (Pasare and Medzhitov, 2005) was optimized in regards to time point of transfer, age of donor and recipient mice and number of transferred B cells, but a better survival of the B cells could not be achieved.

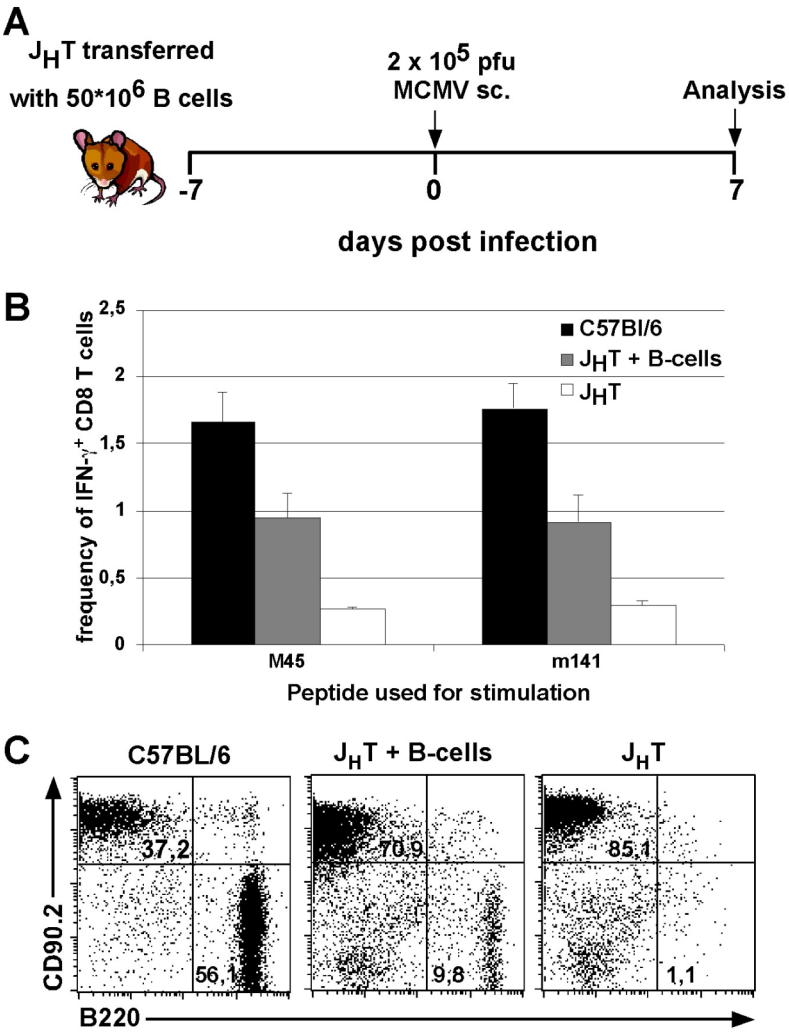

Figure 20. B cell transfer to J_HT mice to rescue the CD8 T cell response. A. Schedule of B cell transfer, infection, and analysis. B. C57BL/6, J_HT and B cell transferred J_HT mice (n=5) were infected with $2x10^5$ pfu mCMV-WT.Smith for seven days. The response to M45 and m141 was determined by intracellular staining (ICS) for IFN-γ after in vitro stimulation of splenocytes with peptide for 6h in presence of Brefeldin A. C. B cells in spleen of C57BL/6, J_HT and J_HT mice + $50x10^6$ B cells 14 days post transfer and seven days post mCMV infection.

3.1.3.6 Functional studies of B cells in mCMV using bone marrow chimeras

To investigate the detailed role of B cells in the response to mCMV, we started functional studies using bone marrow chimeras. A mixture of J_HT bone marrow and bone marrow from different knockout mice results in mice with a normal functional immune system expect for the B cells, which will carry the knockout gene of the regarding knockout mice (Fillatreau and Gray, 2003). A double knockout for $TRIF^{-/-}$ and $MyD88^{-/-}$ and a second strain deficient for $CD40^{-/-}$ were the selected strains for the bone marrow chimeras. The $TRIF^{-/-}$ /$MyD88^{-/-}$ double knockout was chosen to verify the role of toll like receptors (TLRs) expressed on B cells in mCMV infection and the $CD40^{-/-}$ knockout was chosen to investigate the role of B cells in co-stimulation and antigen presentation. The mixture of J_HT and C57BL/6 bone marrow served as positive control and the negative control consisted exclusively of J_HT bone marrow. The bone marrow was mixed in a relation of 80% to 20% and injected intravenously to sublethally irradiated C57BL/6 mice. After the transfer, the mice re-established a new immune system with the given bone marrow mixture within eight weeks. After the reconstitution of the bone marrow, the mice were infected and the virus-specific response was analyzed seven days post infection. The responding CD8 T cells were identified in an intracellular IFN-γ staining. The virus-specific CD8 T cell compartment of the TRIF and MyD88 knockout reconstituted mice was not distinguishable in size from the positive control mixed with C57BL/6 bone marrow. The CD8 T cell populations specific to m141 in both chimeras were about 0,7%, to m139 0,8% and 0,9% were specific to M38 (Fig.: 21 A). This implied a redundancy of TRIF and MyD88 in the establishment of the virus-specific CD8 T cell response. The mice reconstituted with the mixture of J_HT and $CD40^{-/-}$ knockout bone marrow were not able to mount a CD8 T cell response as good as the control mice, the response was significantly lower, here the CD8 T cell population specific to m141 was about 0,4%, to m139 0,6% and to M38 0,65% (Fig.: 21 A). This data indicates a role of CD40 on B cells for the establishment of the virus-specific CD8 T cell response in mCMV infection

(Fig. 21 B), although the response was still higher as in the B cell deficient J_HT chimeras, which mounted a CD8 T cell population specific to m141 of about 0,3%, to m139 of 0,3% and to M38 of 0,35%. The chimerism was very high as shown in Figure 21 B and C. The chimerism of the $CD40^{-/-}/J_HT$ chimeras is shown in Figure 21 B by a comparison of the CD40 expression on the surface of B cells taken from the $CD40^{-/-}/J_HT$ chimeras and B cell taken from the J_HT/C57BL/6 chimeras, a reduction in the surface expression is observable. Whereas in Fig. 21 C the lack of B cells in the J_HT/J_HT chimeras again in comparison to J_HT/C57BL/6 chimeras is shown.

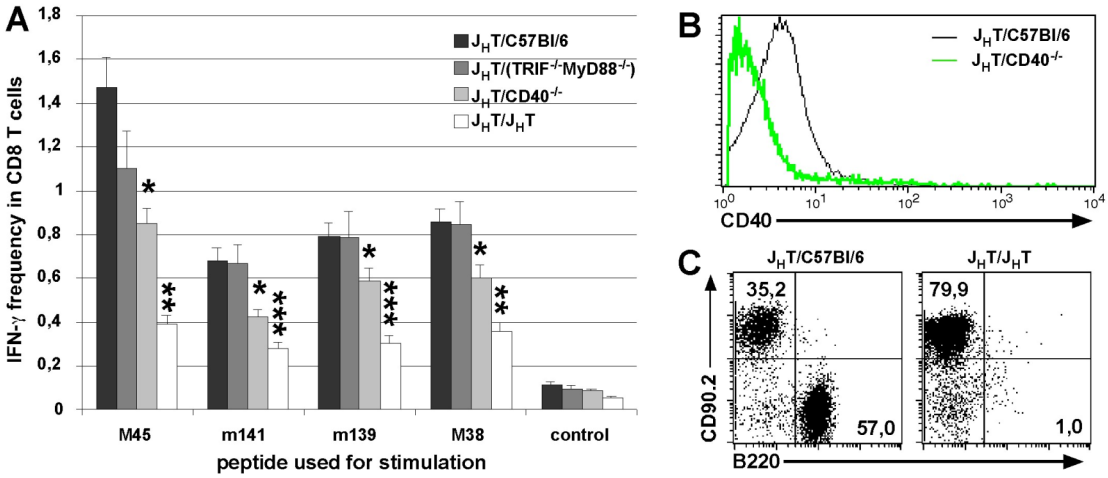

Figure 21. CD40 is involved in the diminished CD8 T cell response in J_HT mice. A. Eight weeks reconstituted BM chimeras of mixtures with 20% of C57BL/6, $TRIF^{-/-}$ /$MyD88^{-/-}$, $CD40^{-/-}$ and J_HT cells and 80% of J_HT were infected with $2x10^5$ pfu mCMV-WT.Smith for seven days. The response to four epitopes was determined by intracellular staining (ICS) for IFN-γ after in vitro stimulation of splenocytes with peptide for 6h in presence of Brefeldin A. Significances were calculated against J_HT/C57BL/6 BM chimeras. B. Chimerism of the $J_HT/CD40^{-/-}$ BM chimeras shown via staining for CD40 on splenic B cells of $J_HT/CD40^{-/-}$ and J_HT/C57BL/6 BM chimeras. C. Chimerism of the J_HT/J_HT and the J_HT/C57BL/6 chimeras shown via presence of B cells in spleen. Asterisks denote responses significant in comparison to control mice.

3.1.3.7 Normal structure of the spleen in bone marrow chimeras

The abnormal spleen structure of B cell-deficient J_HT mice, due to the absence of B cells, could be a reason for the diminished virus-specific CD8 T cell response. A normal interaction of lymphocytes in the white pulpa is not possible, because of the missing B cells. The setup of the bone marrow chimeras also gives the possibility to assess a normal structure in the spleen of the $CD40^{-/-}/J_HT$ chimeras (Fig. 22). The staining of spleen sections for CD3

(grey) and B220 (red) shows a ruptured structure of the spleen in mice reconstituted with J_HT bone marrow only. The remaining red stained cells are possibly macrophages, which are also positive for B220 as well as most of the granulocytes. The structure of $J_HT/CD40^{-/-}$ and $J_HT/C57BL/6$ reconstituted mice was comparable and showed normal distributions of B and T cells in the spleen. The intermediate response of the $J_HT/CD40^{-/-}$ described in 3.1.3.6 is due to the lack of CD40 in the B cell compartment and an involvement of a ruptured structure of the spleen can be excluded.

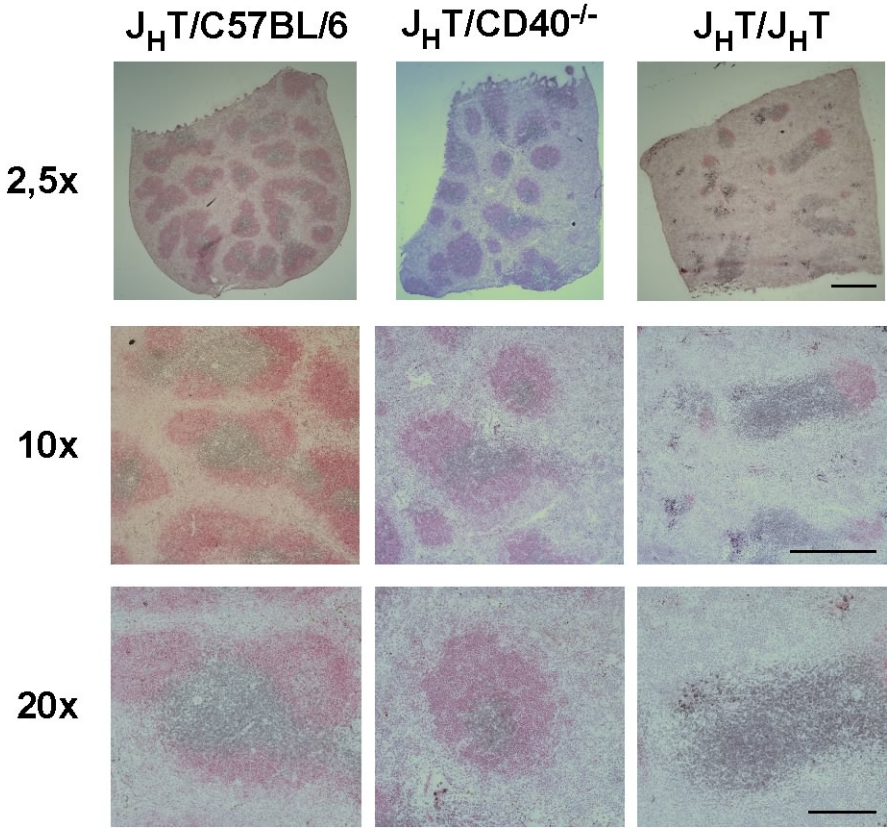

Figure 22. Spleen structure of the BM chimeras. Cryosection after eight weeks of reconstitution and seven days post infection with $2x10^5$ pfu mCMV-WT.Smith. J_HT/J_HT, $J_HT/C57BL/6$ and $J_HT/CD40^{-/-}$ section of BM chimeras in different magnifications (2,5x; 10x; and 20x) and in comparison. Staining with CD3 (grey) and B220 (red).

3.1.4 B Cell as Cytokine-Producing Cells

3.1.4.1 B cell IL-10 but not DC IL-10 has a suppressive effect on the virus-specific CD8 T cell response

When IL-10$^{fl/fl}$ mice are bred to a Cre-expressing mouse strain, the cells, in which the Cre-recombinase is active, are deficient for IL-10. To investigate the role of B cell-secreted IL-10 in mCMV infection, the IL-10$^{fl/fl}$ strain was crossed to the CD19-Cre and additionally to the CD11c-Cre mouse strain, to generate mice with IL-10 deficient DCs. The tissue specific strain for CD11c-Cre was chosen as control, to compare between two compartments of APCs, B cells and DCs. The comparison will show whether a possible phenotype is caused by the specific lack of B cell IL-10 or DC IL-10. C57BL/6, CD19-Cre/IL-10$^{fl/fl}$, and CD11c-Cre/IL-10$^{fl/fl}$ mice were infected and analyzed seven days post infection for the virus-specific CD8 T cell population in spleen and draining lymph nodes. Without B cell-secreted IL-10 the virus-specific CD8 T cell response in spleen was significantly higher. An effect of missing IL-10 of DCs was not observed (Fig. 23 A). Seven days post infection the development of plasma cells in the draining lymph node was significantly increased in the absence of B cell IL-10, control mice had 0,8% of Syndecan-1^{+} cells in contrast to CD19-Cre/IL10$^{fl/fl}$ mice with 2,3% of plasma cells. Therefore, B cell IL-10 had a suppressive effect on the virus-specific CD8 T cell response in spleen (Fig. 23 A). In the draining lymph node seven days post mCMV infection the development of plasma cells was significantly increased in the absence of B cell IL-10 (Fig. 24).

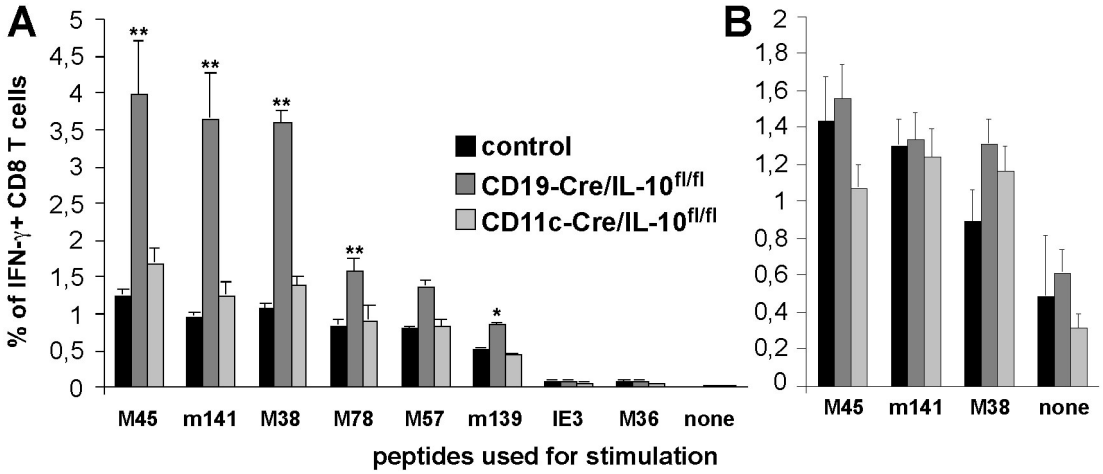

Figure 23. B cell-secreted IL-10 suppresses the virus-specific CD8 T cell response in spleen, but not in lymph nodes. A. Control, CD19-Cre/IL-19$^{fl/fl}$ and CD11c-Cre/IL-10$^{fl/fl}$ mice were infected intra footpad with 2x10^5 pfu mCMV wt smith for seven days. The response to eight epitopes was determined by intracellular staining (ICS) for IFN-γ after in vitro stimulation of splenocytes with peptide for 6h in presence of Brefeldin A. **B.** As seen in A, but lymphocytes from popliteal lymph nodes, the response to three epitopes is shown. Asterisks denote responses significant in comparison to control mice.

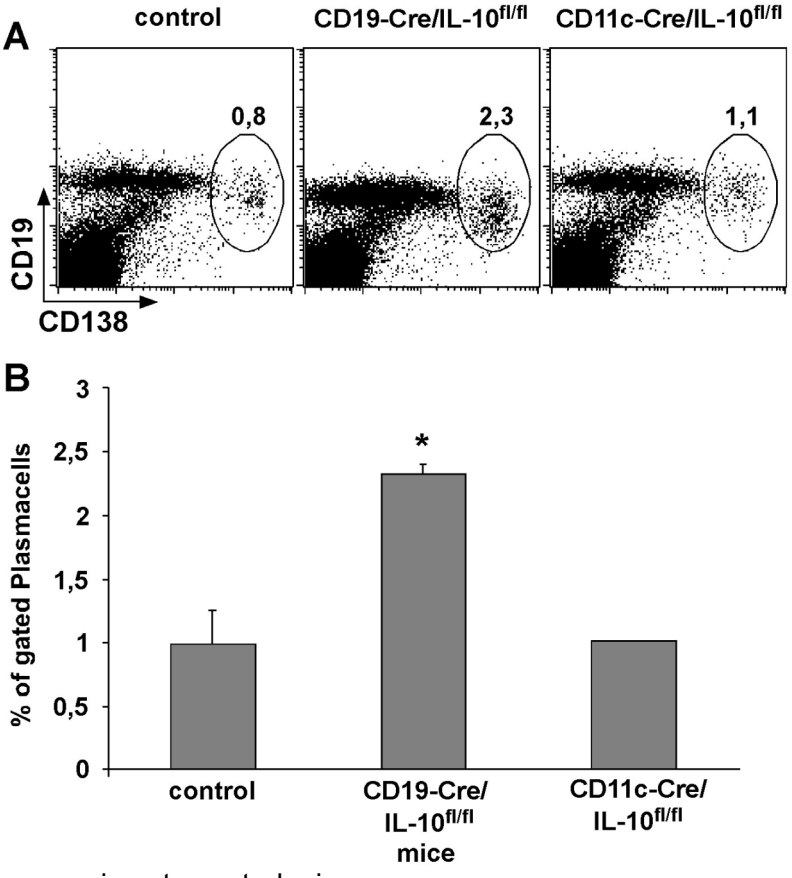

Figure 24. Percentage of plasma cells in popliteal lymph nodes of mCMV infected mice is reduced by IL-10 secreted from B cells. A. Popliteal lymph node cells of seven days intra foodpad 2x10^5 pfu mCMV-WT.Smith-infected control, CD19-Cre/IL-19$^{fl/fl}$ and CD11c-Cre/IL-10$^{fl/fl}$ mice were stained for CD138, a plasma cell marker (syndecan-1). **B.** Same Experiment as A, shown as bar graph. Asterisks denote responses significant in comparison to control mice.

3.1.5 Memory inflation in mCMV infection

Memory inflation is a phenomenon, first observed as an age dependent increase of the human Cytomegalovirus (hCMV)-specific T cell response (Khan et al., 2004; Northfield et al., 2005) and wass defined as the continuous accumulation of antiviral CD8 T cells over time. So far, it is still unknown why this population grows over time after infection. Several different hypotheses were discussed to explain this phenomenon (see introduction 1.3.10). However, investigation of this CD8 T cell population is time intensive, because these cells start to accumulate late post infection and can be identified earliest more than three month post infection. Therefore, a big advantage would be to shorten the time until populations specific for the inflationary epitopes are big enough to be identified. So far, it is necessary to wait until the chronic phase in mCMV infection starts.

In IgMi mice immunoglobulin is missing and reactivated virus is not captured by specific antibodies and directly stimulates the CD8 T cell response. We wanted to take advantage of this characteristic of the IgMi mice to investigate the inflationary epitopes in mCMV infection.

3.1.5.1 IgMi mice serve as a tool to investigate the memory inflation in a challenge model

Memory inflation was not only observed in human, but also in C57BL/6 mice (Holtappels, 2006). The five CD8 T cell epitopes, which were described by Munks to dominate the memory response of mCMV in C57BL/6 mice belong to four different genes (m139, M38, M45 and IE3). These epitopes were mostly corresponding to the epitopes inducing the highest specific CD8 T cell response in the memory response of IgMi mice found 135 days post infection, but could not be found in the memory response of the control C57BL/6 mice at that early time point (Fig. 4). Therefore, the IgMi mouse strain without immunoglobulin was used as an experimental model to investigate these special immunodominant epitopes of the memory response.

To shorten the time until the CD8 T cells specific for inflationary epitopes have established a reasonable population, we wanted to mimic reactivating virus in

a challenge system. Therefore, a newly established virus system of SIINFEKL/SIINFEKA presenting mCMV viruses was used. Either SIINFEKL or SIINFEKA is expressed under the control of the m164 promoter of mCMV. SIINFEKL is presented on the MHC class I, but SIINFEKA cannot be presented at all (Lemmermann et al., 2010). The advantage of this virus system is the possibility to distinguish between antigen presentation and secondary effects, such as cytokines, as cause for the enhanced memory CD8 T cell response. IgMi mice were infected with mCMV-SIINFEKL and six weeks post infection the group was divided into three subgroups that were subjected each to a different further treatment. Two groups were challenged with either mCMV-SIINFEKL or mCMV-SIINFEKA, whereas one group remained untreated. Seven days post challenge all groups were analyzed for the memory CD8 T cell response to the four inflationary epitopes (m139; M38, M45 and IE3), one other epitope (m141), which is immunodominant in the acute response and SIINFEKL itself. As expected, the CD8 T cell response to this epitopes was elevated, just the response to M45 was too low to identify it as inflationary epitope (Fig. 25). Cells from all groups were stimulated with SIINFEKL to verify whether the increase of the virus-specific CD8 T cell response was caused by direct antigen presentation of the peptide or by secondary factors triggered by the challenge itself. As shown in Fig. 25 a specific stimulation with SIINFEKL in the mice that were challenged with mCMV-SIINFEKL was possible, they mounted a SIINFEKL-specific CD8 T cell population about 2,3%, whereas the stimulation with SIINFEKL after the mice were challenged with mCMV-SIINFEKA was not possible. It remained on the same level of 0,9%, the same level as the untreated group The control group of non-challenged mice and mice challenged with mCMV-SIINFEKA showed a similar specific CD8 T cell population of 1% (Fig. 25). In contrast, the CD8 T cell population of mice challenged with the mCMV-SIINFEKL showed a significantly elevated population of more than 2% (Fig. 25). This result indicates, that secondary factors can be excluded as a reason for the increase in the CD8 T cell response in the challenge model. Thus, direct antigen presentation seems to be the reason for the increased response to the inflationary epitopes in IgMi mice. Another advantage of this system that was discovered by this experiment is the fact that it is possible to shorten the

time until the inflationary epitopes show an elevated CD8 T cell response.

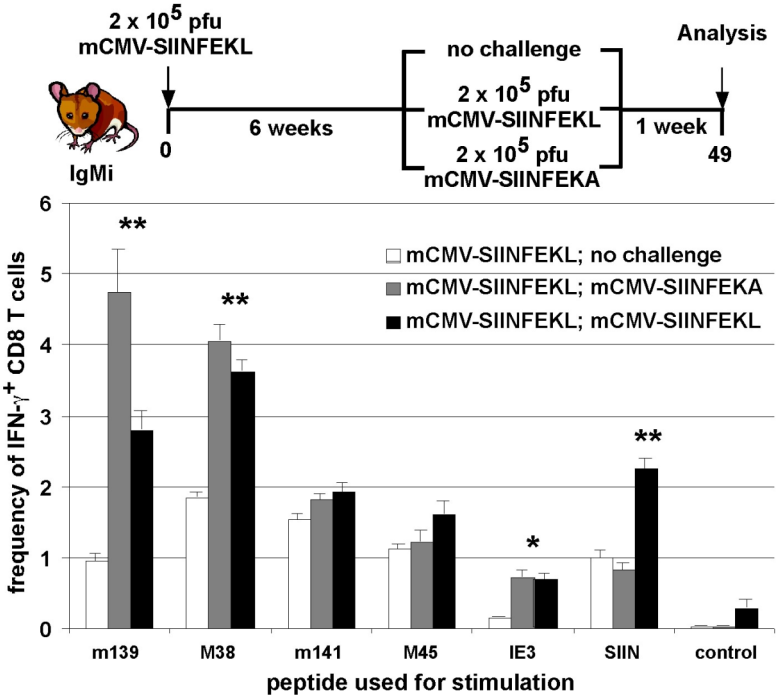

Figure 25. Challenge of IgMi mice in the mCMV-SIINFEKL/SIINFEKA system. IgMi mice (n=15) were infected with 2x10^5 pfu of mCMV-SIINFEKL intra footpad and challenged 42 days later with either 2x10^5 pfu of mCMV-SIINFEKA or mCMV-SIINFEKL intra footpad or left untreated. The response to five epitopes and SIINFEKL was determined by ICS for IFN-γ after in vitro stimulation of splenocytes with peptide for 6h in presence of Brefeldin A. Asterisks denote responses significant in comparison to control mice.

3.1.5.2 Direct antigen presentation is the origin for the increased CD8 T cell population of inflationary epitopes

A question that still need to be solved is whether the increase of the response to inflationary mCMV peptides is caused by cross priming or direct priming. MCMV-ΔvRAP-SIINFEKL an additional virus described in (Lemmermann et al., 2010) was used to answer this question. This virus is the combination of mCMV-ΔvRAP (Bohm et al., 2008b) and the mCMV-SIINFEKL. SIINFEKL is presented by this virus, and further in this virus the immune evasion genes were deleted to achieve a better presentation of mCMV peptides on MHC class I. Consequently, this virus would enhance the virus-specific CD8 T cell response, if the response is based on direct antigen presentation. Therefore,

a direct comparison of the response to mCMV-SIINFEKL and mCMV-ΔvRAP-SIINFEKL in a challenge experiment would show, whether or not the CD8 T cell response to inflationary epitopes is based on direct antigen presentation. Another advantage of the challenge system in IgMi mice is the avoidance of the immune evasion paradox described in a mCMV-ΔvRAP infection (Bohm et al., 2008a). The challenge experiment, started for all groups with the same mCMV-SIINFEKL infection, consequently the first priming of the virus-specific CD8 T cells is the same as well. The challenge response to mCMV was increased for all used peptides in comparison to mCMV-SIINFEKL (Fig. 26). The inflationary epitopes M38, m139 and IE3 show a significantly increased memory response (memory inflation), just the peptide M45 was replaced by m141 (Fig. 26). The response to SIINFEKL in mice challenged with mCMV-ΔvRAP-SIINFEKL was doubled, in the mCMV-SIINFEKL challenged about 1% T cells were responding to SIINFEKL in the other challenged group infected with the virus without immune evasion genes the response was about 2% (Fig. 26). The deletion of the immune evasion genes enhanced the virus-specific CD8 T cell population for the inflationary peptides about double and more. Taken together, the CD8 T cell population specific for the inflationary epitopes of mCMV was stimulated by direct antigen presentation

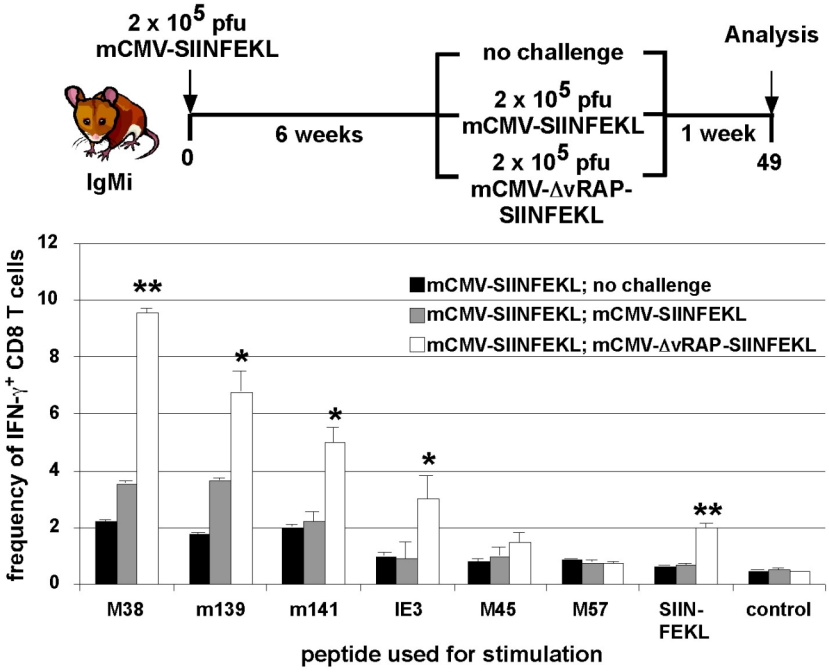

Figure 26. Challenge experiment using mCMV-SIINFEKL and mCMV-ΔvRAP-SIINFEKL. IgMi mice (n=15) were infected with 2x10^5 pfu of mCMV-SIINFEKL intra footpad and challenged 42 days later with either 2x10^5 pfu of mCMV-SIINFEKL or mCMV- -ΔvRAP-SIINFEKL intra footpad or left untreated. The response to six epitopes and SIINFEKL was determined by ICS for IFN-γ after in vitro stimulation of splenocytes with peptide for 6h in presence of Brefeldin A. Asterisks denote responses significant in comparison to control mice.

3.2 *Leishmania major*

Leishmania major is an intracellular parasite with two life cycles, the promastigote and the amastigote form. The infectious stage of metacyclic promastigote *Leishmania major* is transmitted by the bite of an infected sand fly. These metacyclic promastigote parasites infect macrophages, where they replicate and develop into the obligate intracellular amastigote stage. Via lysis of the infected macrophages the amastigote form is set free into the surrounding tissue acquiring inflammation. The amastigotes, IgG coated, are able to infect DCs, which leads to recruitment of inflammatory cells to the site of infection and the induction of IFN-γ secreting CD8 T cells. All this initiates the development of lesions. BALB/c mice establish a Th2 response upon parasite infection, whereas in C57BL/6 mice, the infection leads to a Th1

response. The major difference between these two responses is that BALB/c mice succumb the infection and are described as the susceptible strain, but C57BL/6 mice are able to clear the infection and establish a lifelong resistance.

3.2.1 IgMi and IgG1i mice on BALB/c background show smaller lesion, but a higher parasite load in *Leishmania major* infection

We were interested to clarify the role of immunoglobulin in *Leishmania major* infection and its influence on the lesion size, the parasite spread and proliferation as well as the cytokine production. Therefore, three mouse strains on BALB/c background (IgG1i, IgMi and wt BALB/c) were infected with a low dose of *Leishmania major* subcutaneously into the ear. Although, in IgMi mice the entrance of the parasites into the DCs was expected to be delayed due to the missing IgG, the onset of the lesion development occurred at the same time five weeks post infection for all tested groups (Fig. 27). In the further progression of the lesions from week seven on it got obvious, that the lesions of the IgMi mice are significantly smaller compared to the lesions of wt mice. Also the lesions of the IgG1i mice were smaller (Fig. 27).
Next, we wanted to investigate, whether the parasite burden of the ear lesions of the IgMi and the IgG1i mice was lower as well. Therefore, the experiment was repeated and the parasite load of spleen and ears was measured six and nine weeks post infection. Interestingly, IgMi mice showed the highest parasite burden at both time points and in both organs, spleen and ear (Fig. 28). At the same time point when the parasite load was measured, cells from the draining lymph node were isolated and stimulated with soluble *Leishmania* antigen (SLA) to investigate the cytokine milieu (IFN-γ, IL-10, and IL-4) specifically induced by parasite infection (Fig. 29). IFN-γ is one of the prototypic Th1 cytokines and is important for the resistance of C57BL/6 mice. The lowest level of IFN-γ was measured in IgMi mice nine weeks post infection (Fig. 29). At the same time point a difference between control and IgG1i mice was not observed (Fig. 29). Six weeks post infection, IgG1i mice showed a high IFN-γ level, but a consequence of this high level was neither

observed in parasite load nor in lesion size. Six weeks post infection the IL-4 levels were elevated in IgMi and IgG1i mice in comparison to control mice. This was reflected in an increase of the parasite load in spleen and ear (Fig 28). The last cytokine we wanted to observe was IL-10, an immunosuppressive cytokine. An elevation of the IL-10 level goes along with disease progression. The IL-10 levels of IgG1i were elevated at all measured time points. The level of IL-10 secreted in IgMi mice was highly increased six and nine weeks post infection (Fig. 29). A comparison of the IL-10 production and the parasite burden in spleens of IgMi mice suggested a correlation between these two measurements.

To investigate whether this phenotype of the IgMi mice was directly caused by the absence of immunoglobulin, a reconstitution experiment was performed. Therefore, wt mice were compared to IgMi mice were either treated with IMS or left untreated to investigate, whether the phenotype in IgMi mice is reversible by immunoglobulin reconstitution. Nine weeks post *Leishmania major* infection the parasite load in ear and spleen was measured as well as the cytokines produced of the draining lymph node lymphocytes, which were with SLA. Interestingly, serum reconstituted IgMi mice had smaller lesions compared to untreated IgMi mice, whereas wt BALB/c mice showed the largest ear lesions (Fig. 30 A). The IMS treatment was efficient to reduce the lesion size in the ear and correspondingly to this, also the parasite burden in the ear was decreased and even lower as observed in the non-treated wt mice nine weeks post infection (Fig. 30 B). The parasite load in spleen was increased for both groups of IgMi mice despite the IMS treatment (Fig. 30 B). The cytokine levels were not significantly influenced by the treatment with IMS and just IL-10 was inhibited by the IMS treatment in IgMi mice (Fig. 30 C). These results show, that the immunoglobulin treatment was sufficient to narrow the parasite burden of the ear lesion as well as the lesion size itself.

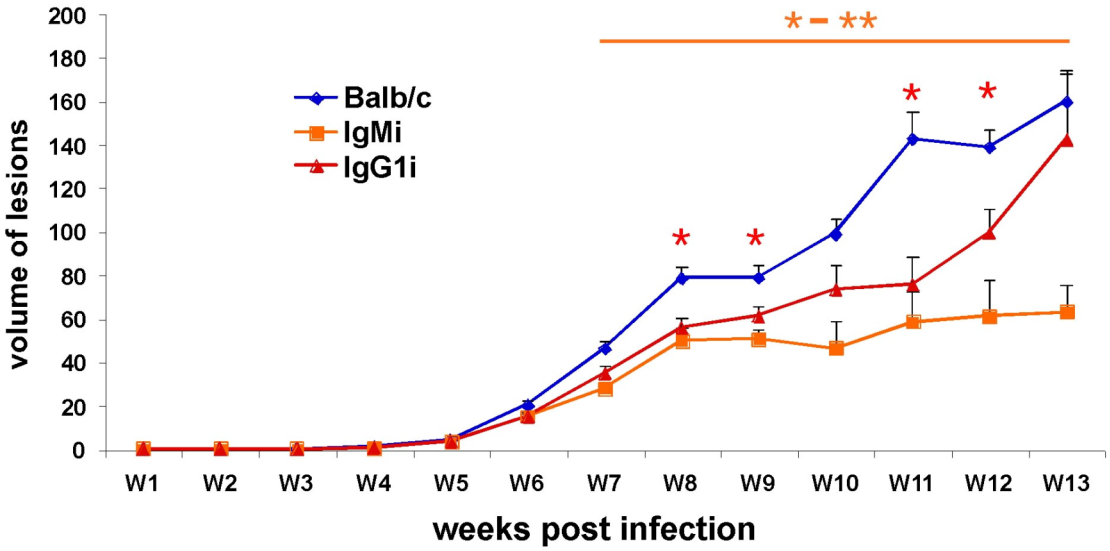

Figure 27. The course of ear lesions in *Leishmania major* low dose infection. Wt, IgMi, and IgG1i on BALB/c background were infected with a subcutaneous low dose *Leishmania major* injection into the ear. The development of ear lesions was observed for 13 weeks by measuring the lesion volume.

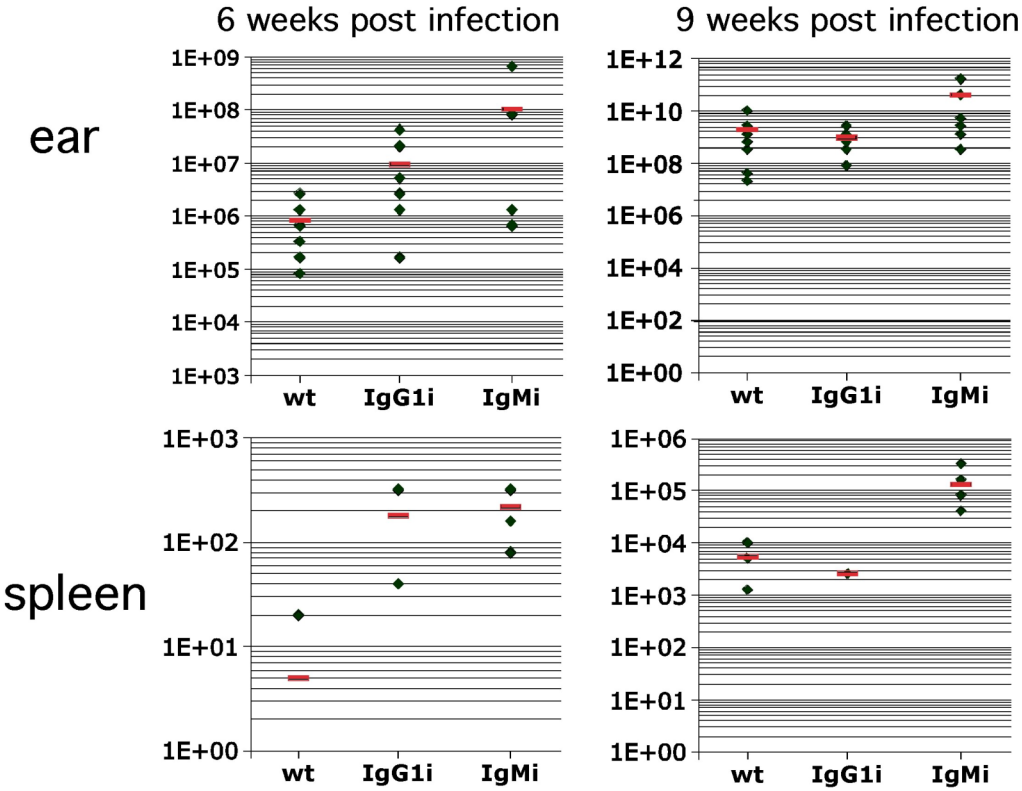

Figure 28. Parasite load in ear and spleen six and nine weeks post infection. BALB/c, IgG1i and IgMi mice were infected subcutaneous with a low dose of *Leishmania major* into the ear. After six weeks half of the infected mice were analyzed for parasite load (n=5 per group). Nine weeks post infection the remaining mice were analyzed. The parasite load is shown in a logarithmic scale.

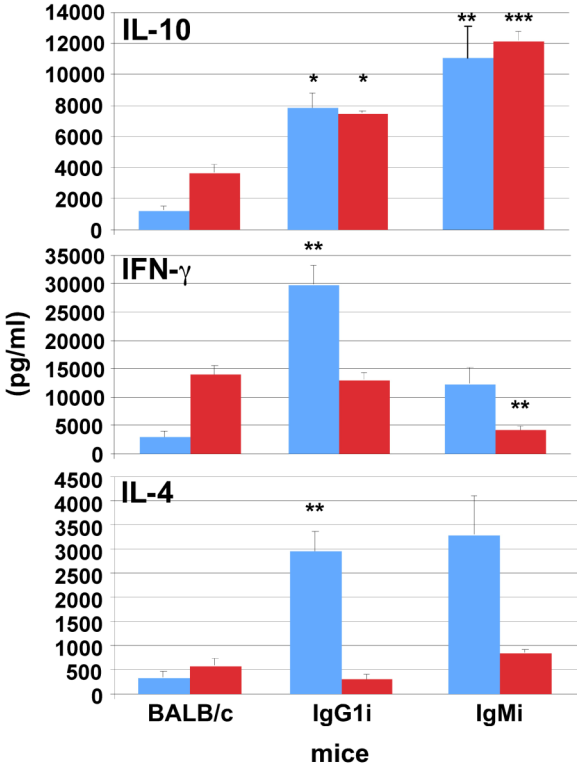

Figure 29. Cytokine expression in draining lymph nodes. Wildtype, IgG1i and IgMi mice on BALB/c background were infected subcutaneous with low dose of *Leishmania major* into the ear. Six (blue bars) and nine (red bars) weeks post infection the draining lymph nodes were taken and the resulting cells were restimulated with SLA. The supernatant was analyzed for IL-10, IFN-γ and IL-4.

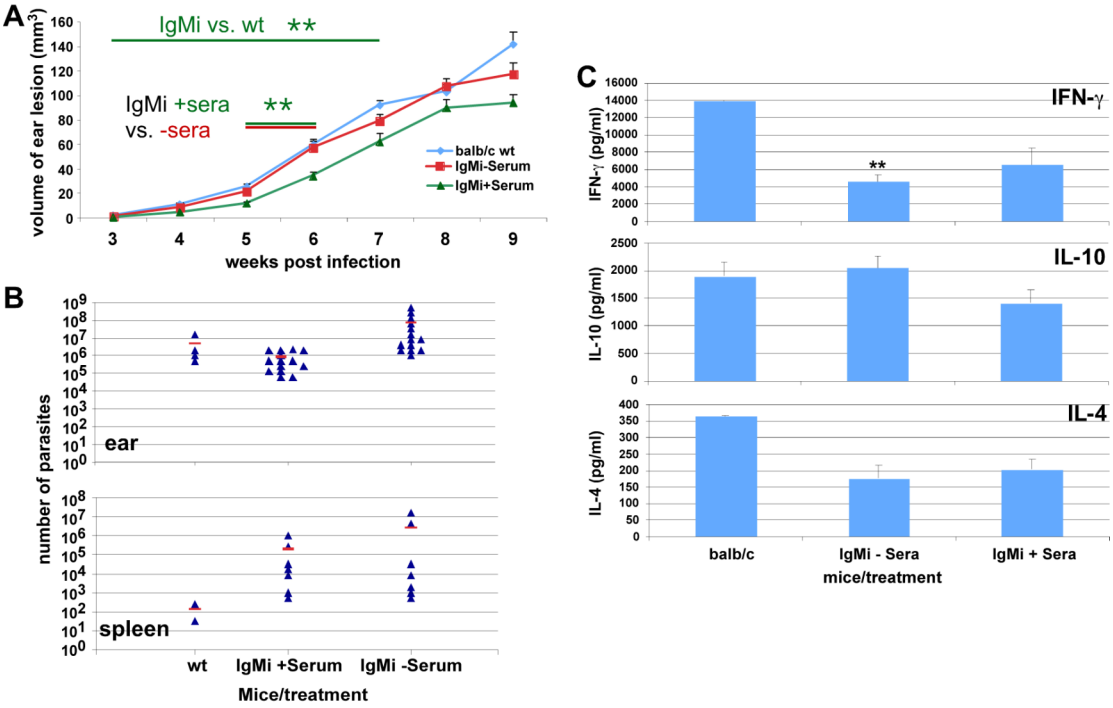

Figure 30. Serum reconstitution of IgMi mice. A. Development of the ear lesions volume in wt and two groups of IgMi mice, untreated and serum treated, on BALB/c background over nine weeks post low dose *Leishmania major* infection. **B.** Parasite load of ears and spleen nine weeks post low dose *Leishmania major* infection. **C.** Cytokine expression levels of IL-10, IFN-γ and IL-4 of lymphocytes taken from draining lymph nodes and restimulated with SLA.

3.2.2 Serum reconstitution of IgMi and IgG1i mice on C57BL/6 background in *Leishmania major* infection

B cell-deficient J_HT, IgMi, IgG1i and wt mice -all on C57BL/6 background- were infected subcutaneous with low dose *Leishmania major* and the development of the ear lesion was observed. In comparison to wt mice the ear lesions in IgMi mice showed the biggest lesions, in contrast to the IgG1i mice, which had smaller lesions this significant differences were observed from four weeks until ten weeks post infection (Fig. 31).

To investigate whether the reason for this differences are the different immunoglobulin distributions in blood of these mice, IgG1i, IgMi and C57BL/6 mice were reconstituted with normal mouse serum (NMS) and immunized mouse serum (IMS) a control group left untreated, the groups of C57BL/6 mice were just untreated and treated with IMS. The course of disease

between the groups varied in the reconstitution experiments, but the treatment with IMS and NMS had in all cases a very positive effect on the transgenic mouse groups in three independent experiments, whereas the effect of the IMS treatment in C57BL/6 mice was negligible (Fig. 32). Further, the effect of the IMS and NMS treatment on the parasite load needed to be investigated, to show that in smaller lesions less parasites are present. The parasite load was measured seven weeks post infection at the assumed peak of disease and reflected well a correlation between the lesion size and the parasite load in the ear. Therefore, the NMS and the IMS treatment diminished the number of parasites in the ear in all cases. An even better effect was observed in spleen, here the NMS treatment of IgG1i mice and the IMS treatment of IgMi mice had a higher efficiency resulting in a diminished number of parasites (Fig. 33 A). Both untreated groups of the transgenic mice showed an elevated level of parasites in comparison to the control mice and in both cases this difference was significantly higher in spleen. Another interesting phenomenon was monitored following the spleen size of the infected mice. IgMi mice showed under uninfected conditions a splenomegaly. This phenomenon was milder in the context of the reconstitution in a *Leishmania major* infection. In the IMS-treated mice the spleen weighed almost the same as infected untreated C57BL/6 mice (Fig. 33 B). The difference of spleen weight in untreated and IMS treated IgMi mice is highly significant and also reflected the lower parasite load.

To investigate the cytokine levels of IL-2, IL-4, IL-10, IL-17 and IFN-γ cells taken from the draining lymph node were stimulated with SLA. A highly increased IL-10 secretion in IgMi mice was observed, which could be decreased to the level of control mice with the serum treatment. The serum treatment had also a very positive effect on the IL-4 level, which was significantly decreased in wild type and IgMi mice (Fig. 34). This reflects the importance of immunoglobulin for the establishment of a Th1 response. The secretion of IFN-γ showed again a direction to a more pronounced Th1 response in dependency on serum treatment (Fig. 34). In C57BL/6 and IgG1i the NMS and IMS treatment increased the IFN-γ secretion in contrast to a reduction in IgMi mice to the level of treated C57BL/6 mice. The antibody

treatment increased the IL-17 level in C57BL/6 and IgG1i mice, but in IgMi mice hardly any IL-17 secretion was detectable. Interestingly the IL-2 secretion seemed to be inhibited in the transgenic mice by the serum treatment, just in C57BL/6 mice the level was increased after the treatment (Fig. 34). A general basis to explain the change of the secreted interleukins in the context of serum reconstitution could not be found. The serum treatment had the same effect on IL-10, IL-4, IL-17 and IFN-γ secretion in C57BL/6 and IgG1i mice, IgMi mice reacted similar regarding IL-10 and IL-4 secretion. Subsequently, antibodies have a big impact on the course of disease, but the effects depend on the immune system the antibodies are added to. An immune system with normal (C57BL/6) or impaired but present antibodies (IgG1i) reacts different in a comparison to an immune system without antibodies (IgMi).

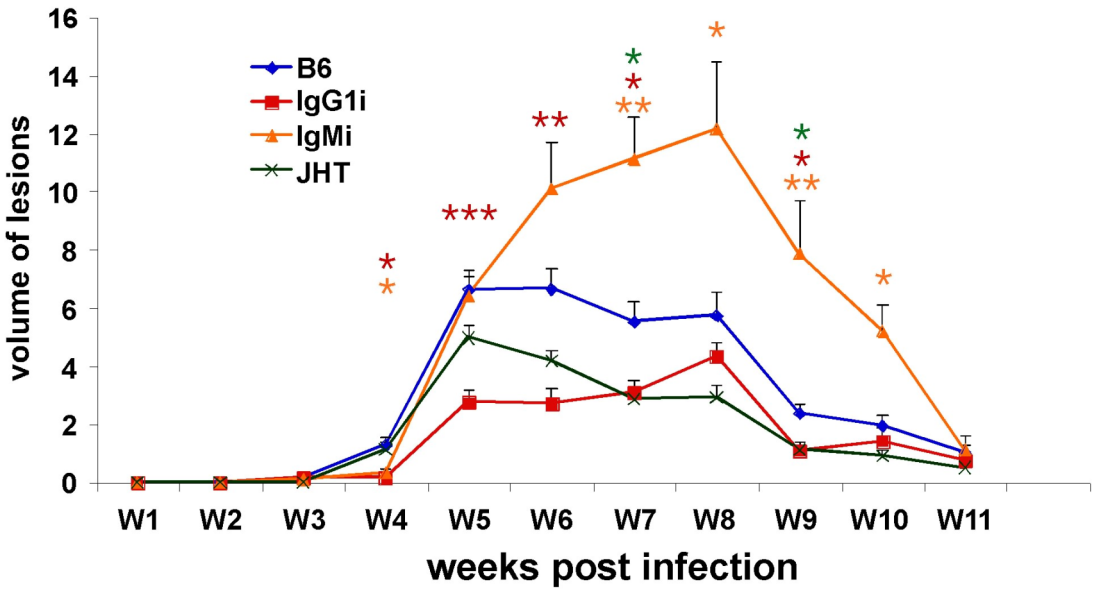

Figure 31. Development of ear lesions on C57BL/6 background in the course of a low dose *Leishmania major* infection. Wildtype, IgG1i, IgMi and J_HT all on C57BL/6 background were infected with low dose *Leishmania major* subcutaneous into the ear. The volume of the establishing lesions was measured weekly for 11 weeks.

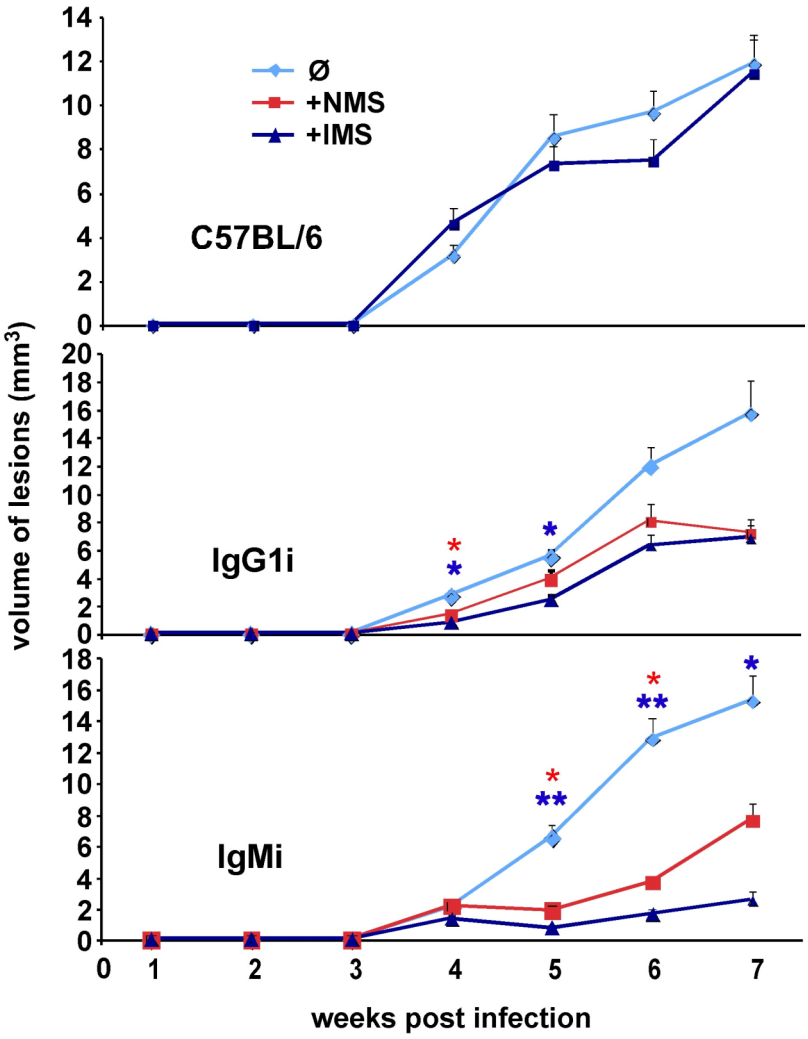

Figure 32. Development of ear lesion during serum reconstitution. C57BL/6, IgG1i and IgMi mice were treated with IMS and IgG1i and IgMi additional with NMS over seven weeks of low dose *Leishmania major* infection. The serum reconstitution started one week ahead of infection. The volume of the ear lesions was observed weekly over seven weeks.

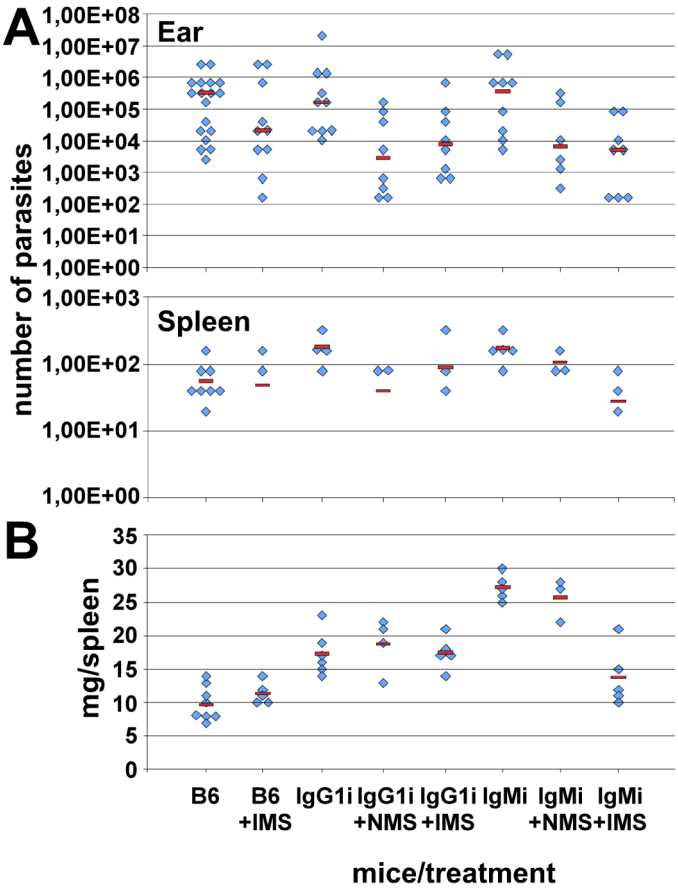

Figure 33. Parasite load and spleen weight in dependency on serum immunoglobulin. A. Ears and spleens from C57BL/6, IgG1i and IgMi treated with nothing, NMS or IMS were taken and the parasites were isolated and the number calculated. **B.** The weight of spleens was measured and is shown in mg/spleen.

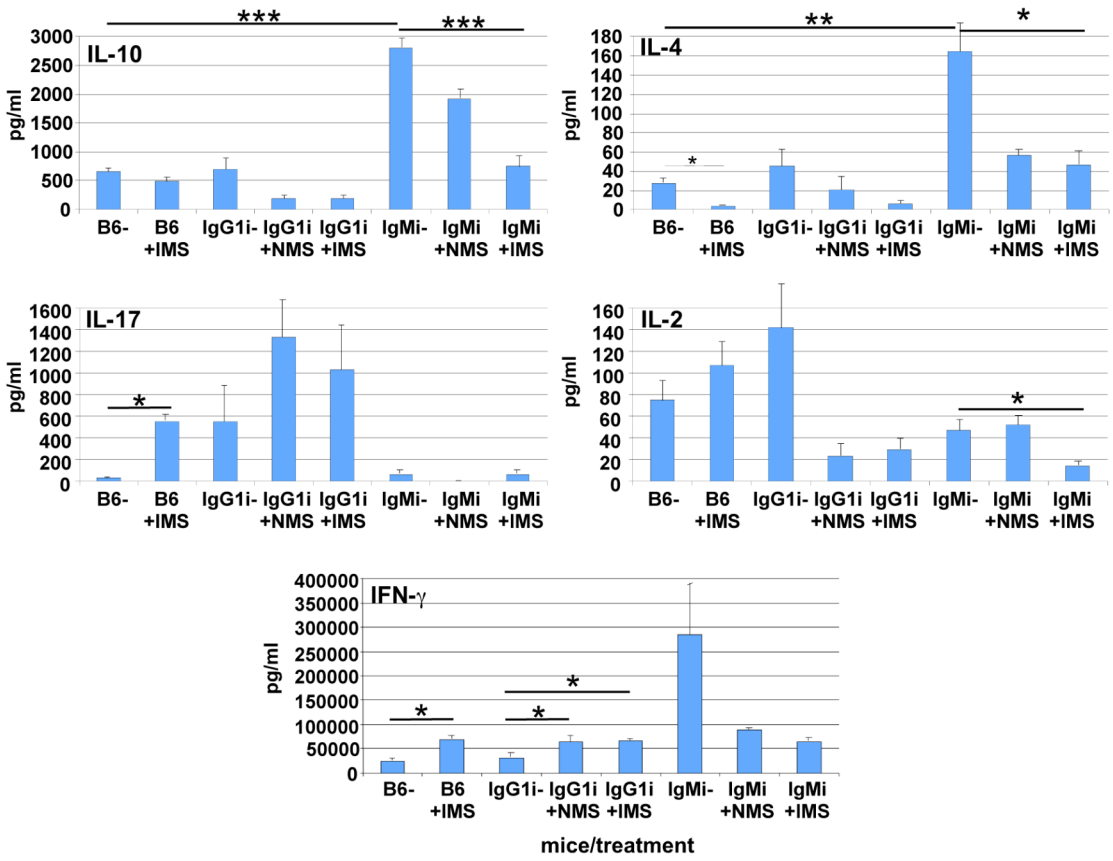

Figure 34. Cytokine expression in draining lymph nodes seven weeks post infection. C57BL/6, IgG1i and IgMi mice were treated with IMS and NMS and infected with low dose *Leishmania major* subcutaneous into the ear. Seven weeks post infection the draining lymph nodes were taken and the resulting cells were stimulated with soluble *Leishmania* antigen (SLA). The supernatant was analyzed for the presence of IL-10, IL-17, IL-4, IL-2 and IFN-γ.

4. Discussion:

4.1 B cell deficiency in mCMV infection

In 1981 Shanley et al. showed a protective role of mCMV-specific antibodies in mCMV infection (Shanley et al., 1981). Ten years later, Farrell and Shellam investigated monoclonal antibodies specific to structural proteins of mCMV, proved their protective function, and showed a correlation between mCMV-specific antibodies and the neutralization *in vitro* and *in vivo* (Farrell and Shellam, 1991). In 1994, using a strain deficient for B cells (μMT mice) Jonjic and coworkers showed that neither B cells nor antibodies were essential for the resolution of primary mCMV infection (Jonjic et al., 1994). We confirmed this by *in vivo* imaging using J_HT mice, another B cell-deficient mouse strain (Gu et al., 1993). Surprisingly we additionally found a diminished virus-specific CD8 T cell response in these mice, although the virus infection was cleared seven days post infection.

The CD8 T cell response is described to be very important and is already used in hCMV therapy. Infected immuno-compromised patients are treated with virus-specific CD8 T cells (Reddehase, 2002). As shown in the J_HT bone marrow chimeras, the splenic structure was altered completely by the absence of B cells and this could be a reason for the diminished CD8 T cell response as well. Normal antigen presentation in the spleen by B cells or dendritic cells (DCs) to T cells in the white pulp is impaired. Also the T cell compartment in B cell-deficient mice is influenced by the absence of B cells (Sun et al., 2008), which could also be a reason for the diminished virus-specific CD8 T cell response. Sun et al. observed a reduction of T_{regs} in μMT mice, which suggests that also other T cell compartments might be altered in the absence of B cells. We used CD19-Cre/iDTR mice, to exclude the possibility of an altered T cell development in J_HT mice due to the life long absence of B cells. In CD19-Cre/iDTR mice the CD8 T cell compartment

developed normally as in C57BL/6 mice. The virus-specific CD8 T cell response in B cell-depleted CD19-Cre/iDTR mice showed the same decrease as observed in J_HT mice, this ruled out the possibility of an altered CD8 T cell compartment to be responsible for the decreased response in J_HT mice.

4.1.1 Importance of B cell-CD40 for the virus-specific CD8 T cell response

To finally exclude the different spleen structure as cause for the diminished virus-specific CD8 T cell response, we formed BM chimeras containing mixtures of bone marrow from J_HT and $CD40^{-/-}$ mice. The resulting mice had a B cell compartment established from the $CD40^{-/-}$ mice, and therefore a normal splenic structure. These mixed BM chimeras showed a significant reduction in the virus-specific CD8 T cell response compared to the control mice (BM chimeras containing mixtures of J_HT and C57BL/6 bone marrow). This implies a relevance of CD40 on B cells for the diminished CD8 T cell response. Upon CD40L stimulation of B cells, the costimulatory molecule B7.1 is upregulated (Cook et al., 2009). The function of B7.1 is one target of mCMV to evade the immune system in infected DCs, the translocation of B7.1 to the surface is inhibited by the viral gene product of m138 (Mintern et al., 2006). Further, it was described that the complete loss of B7 leads to a diminished CD8 T cell response (Cook et al., 2009). This reflected our phenotype in J_HT mice and the BM chimeras with a B cell compartment deficient for CD40. The big advantage of B cells in the B7.1 costimulation is that B cells are not directly infected by mCMV. Therefore the viral protein m138 is not expressed in B cells, and the translocation of B7.1 is not inhibited, as it is the case in DCs, which are infected by and hosting mCMV. B7.1 is expressed on activated B cells and provides a costimulatory signal necessary for T cell activation and survival (Fiumara and Younes, 2001). It binds to CD28 to prime T cells against antigens presented by MHC class I on antigen presenting cells. B7.1 belongs to the B7 family of peripheral membrane proteins, the second important member of this family is B7.2. Both proteins interact with CD28 on a T cell for costimulation. mCMV established a mechanism to prevent the

localization of B7.2 on the surface of DCs. The product of the gene m147.5 inhibits the localization of B7.2 on the surface of DCs (Loewendorf et al., 2004). B cells themselves are not infected by mCMV, therefore we suggested a role for B cells in cross presentation. To verify this hypothesis, experiments with BM chimeras containing mixtures of J_HT and $CD80^{-/-}/CD86^{-/-}$ double knockout bone marrow are ongoing. Compared to the strict B cell-deficient BM chimeras, the BM chimeras with the CD40-deficient B cell compartment showed a higher virus-specific CD8 T cell response, which was still significantly lower than the response of the control BM chimeras. The reason for the elevated response of the BM chimeras with the CD40-deficient B cell compartment could be due to the normal spleen structure or any other secondary effect.

4.2 Memory inflation

As already mentioned antibodies play a role in protection during mCMV infection. It was shown that the transfer of memory B cells to immuno-deficient mice infected with mCMV rescued these mice from the lethal disease course (Klenovsek et al., 2007). In the IgMi strain that lacks antibodies, but contains B cells, we observed an altered CD8 T cell response in the memory phase, when compared to control C57BL/6 mice. The response to four epitopes was significantly elevated. Normally, the memory response to mCMV infection shows four different patterns (Munks et al., 2006a). One pattern represents the central memory phenotype. The three other patterns have one fact in common: the CD8 T cell compartment specific to these epitopes increases with time. This phenomenon is called memory inflation and was first described by Holtappels (Holtappels et al., 2000; Karrer et al., 2003). The first memory pattern next to the central memory was represented the response specific for peptide m139, characterized by a rapid expansion of the CD8 T cell compartment followed by a contraction, and was ending in a memory inflation. In the second pattern the contraction was missing, the CD8 T cell response specific to M38 increased with time, and directly ended up in memory inflation after the first expansion. The third and last pattern next to central memory did not show a first rapid expansion, the CD8 T cell compartment specific to IE3

was established with time, and consequently showed only memory inflation (Munks et al., 2006a).

The enhanced virus-specific CD8 T cell compartment in IgMi mice, which is found 135 days post infection, could be induced by the missing antibody-protection. Therefore we hypothesized that normal serum antibodies directly capture reactivated virus, these serum antibodies are lacking in IgMi mice. Thus, a direct reactivation of the memory response of the adaptive CD8 T cell compartment was induced and responsible for the elevated CD8 T cell memory response. We took advantage of this phenomenon in IgMi mice and established a protocol for a challenge system with mCMV to investigate these inflationary epitopes. The advantage was that memory inflation in IgMi mice could be observed after much shorter time than described by Munks (Munks et al., 2006a). Using the virus system of mCMV-SIINFEKL and mCMV-SIINFEKA (Lemmermann et al., 2010), we could prove that the elevated CD8 T cell response was indeed induced by the challenge. Munks and his colleagues were not able to show that viral interference with antigen presentation alters the chronic CD8 T cell immunodominance in mCMV infection by using a virus with deleted genes of m4; m6, and m152 (Munks et al., 2007). In our challenge system, we had the big advantage that the established memory response was based on the normal mCMV response. We challenged the mice with mCMV-ΔvRAP-SIINFEKL and mCMV-SIINFEKL and the outcome gave evidence for the hypothesis that direct antigen presentation by MHC class I is needed to stimulate the CD8 T cell response specific for the inflationary epitopes and that viral interference at least alters the chronic CD8 T cell immunodominance in mCMV.

4.3 IL-10 in mCMV infection

IL-10 plays an important role in immuno-regulation in different model systems such as infections, autoimmune diseases, as well as allergic models. But so far the cellular source of IL-10 is rather unknown. On the one hand IL-10 was shown to have suppressive functions, on macrophages for example it suppresses activation and also secretion of cytokines and therefore indirectly

suppresses the stimulation of T cells and NK cells. On the other hand IL-10 was reported to have a stimulatory effect on the proliferation and differentiation of B cells, mast cells, as well as immature and mature T cells (Ho and Moore, 1994). In mCMV infection, using CD19-Cre/IL-10$^{fl/fl}$ mice, we could show, that IL-10 secreted by B cells had a high impact on the CD8 T cell response in a suppressive manner. In contrast, using CD11c-Cre/IL-10$^{fl/fl}$ mice, IL-10 secreted by DCs had no influence on the virus-specific CD8 T cell response. It was also possible to show that B cell-secreted IL-10 influenced B cell development. In absence of B cell-secreted IL-10 in mCMV infection, the percentage of plasma cells in the peripheral lymph nodes was elevated. This result indicated a suppression of the development of plasma cells by B cell-secreted IL-10. The elevation could be also due to the increased virus-specific CD8 T cell response and an overall increased virus-specific response of the whole immune system.

4.4 Depletion of B cells in the CD19-Cre/iDTR system

These days, B cell depletion is a quite common therapy of diverse diseases, mostly of autoimmune or malignant diseases. In 1988, the first attempt to deplete B cells was done with anti-IgM antibodies (Cerny et al., 1988). This depletion method as well as new methods, including Rituximab the most prominent anti-CD20 B cell depleting antibody in clinical therapies (Kessel et al., 2008), have a higher efficiency in depletion, but are left with high antibody titers after depletion of B cells. Memory B cells and plasma cells secrete antibodies, but plasma cells stop the expression of surface antigens and hardly any surface antigens are left on plasma cells. Therefore, the depletion with B cell targeting antibodies is not sufficient to ensure the depletion of antibodies. The system we used to deplete antibodies was the CD19-Cre/iDTR system, a mouse model in which B cells express the simian diphtheria toxin receptor (DTR). Diphtheria toxin (DTx) application to these mice depletes the B cells. This system is based on a Cre-recombinase expressed under control of the CD19 promoter in combination with the DTR inserted in the ubiquitous Rosa locus. A loxP-site flanked Stop cassette

inhibits the expression of the DTR. The Stop cassette is excised by Cre-recombinase activity, which is found in CD19-expressing B cells and the DTR is expressed. This system serves as a "genetic memory", because if one recombination occurs, the genome stays recombined and the DTR can be transcribed (Waisman et al., 2008). The consequence of this 'memory' is that also plasma cells, which stop CD19 expression, still express the DTR. The antibody titers following DTx application were hardly influenced by the absence of B cells, which indicated that plasma cells were not or hardly depleted, but the histology of bone marrow cytospins showed a significant reduction in plasma cells even if they were not completely depleted. Here the half-life of antibodies played a role together with the recovery of the B cell compartment. Another possibility to explain the remaining antibodies would be a high turnover of the plasma cell membrane and a low expression of the DTR. Unfortunately it was impossible to mimic a B cell-deficient mouse, antibodies were still remaining in this system.

4.5 Role of antibodies in *Leishmania major* infection

In a *Leishmania major* infection of wild type (wt) mice, the volume of the lesion normally corresponds to the parasite burden: The bigger the lesions, the higher the parasite burden.

The analysis of *Leishmania*-infected IgMi and IgG1i mice on BALB/c background resulted in a contrary outcome. The lesions of the IgMi mice showed that this strain suffered less from the *Leishmania major* infection. The lesions were significantly smaller in comparison to IgG1i and wt mice, but the parasite load in ear and spleen showed that these mice had a higher parasite burden in spleen and ears throughout the whole course of disease. Also IgG1i mice were shown to have smaller lesions than wt mice, but a higher parasite burden. This result gave evidence to the assumption that the volume of the lesions was not only dependent on the number of parasites. Infiltrating inflammatory cells at the site of infection may also contribute to the volume. Further studies of the infiltrating cells in IgMi and IgG1i mice will show whether the abnormal B cell compartment in these two mouse strains

influences the recruitment of inflammatory cells to the side of infection.

The role and involvement of antibodies in the course of leishmaniasis seemed to be a multiple one. Antibodies prevent the spread of the parasite from the lesion side shown by parasite load, and this was emphasized by the lowered level of IL-10 and IL-4 level. Less IL-10 secretion meant lower parasite proliferation, and a lower IL-4 level showed a better direction to Th1 in BALB/c mice. Without antibodies in the serum, the untreated IgMi mice on C57BL/6 background showed an extremely high IFN-γ level, but the serum treatment reduced the levels back to normal. One reason for the normalized IFN-γ level may was the lower parasite load and therefore lower inflammation in spleen. Therefore, antibodies are important to control a cutaneous leishmaniasis. As shown in IgMi mice, without antibodies, the infection had a tendency to become systemic indicated by the elevated parasite load in spleen.

5. Summary

This thesis focuses on the role of B cells in mCMV and *Leishmania major* infection. B cells are an essential component of the adaptive immune system and play a key role in the humoral immune response. In mCMV infection we analyzed the influence of B cells on the virus-specific CD8 T cell response, in detail the role of B cells as IL-10 secreting cells, as source of immunoglobulin (Ig) and as antigen presenting cells. In *Leishmania major* infection we investigated the role of Ig in Th1 and Th2 directed disease.

We found in mCMV infection that the B cell secreted IL-10 suppresses effectively the acute virus-specific CD8 T cell response, while the IL-10 secreted by dendritic cell has no obvious effect. Ig has no effect in the acute virus-specific CD8 T cell response, but in memory response Ig is essential. If Ig is missing the CD8 T cell population remains high in memory response 135 days post infection. The complete absence of B cells dramatically reduces the acute virus-specific CD8 T cell response, while B cell reconstitution just partially rescues this dramatic reduction. A comparison of this reduction in a B cell free organism to an organism with depleted dendritic cells gave a similar result. To exclude a malfunction of the CD8 T cells in the B cell deficient mice, the decreased virus-specific CD8 T cell population was confirmed in a B cell depletion model. Further, bone marrow chimeras with a B cell compartment deficient for $CD40^{-/-}$ showed a decrease of the virus-specific response and an involvement of CD40 on B cells. Taken together these results suggest a role for B cells in antigen presentation during mCMV infection.

Further we took advantage of the altered mCMV specific CD8 T cell memory response in mice without Ig to investigate the memory inflation of CD8 T cells specific for distinct mCMV specifc peptides. Using a SIINFEKL-presenting virus system, we were able to shorten the time until the memory inflation occurs and show that direct presentation stimulates the memory inflation.

In *Leishmania major* infection, Ig of Th2 balanced BALB/c mice has a central role in preventing a systemic infection, although the ear lesions are smaller in IgMi mice without specific Ig. Here the parasite loads of ears and spleen are elevated, and an IMS-reconstitution does not affect the parasite load. In contrast in Th1 balanced C57BL/6 mice, reconstitution of IgMi mice with serum of either untreated or immunized mice decreased the parasite load of spleen and ear, further IMS treatment reduces the size of the spleen and the cytokine-levels of IL-10, IL-4, IL-2 and IFN-γ to a level comparable to wt mice.

6. Zusammenfassung

In der vorliegenden Arbeit wird die Rolle von B Zellen in mCMV und in *Leishmania major* Infektion im Mausmodell untersucht. B Zellen sind eine essentielle Komponente des adaptiven Immunsystems und übernehmen eine Schlüsselrolle in der humoralen Immunantwort. In der mCMV Infektion wurde der Einfluß von B Zellen auf die virus-spezifische CD8 T Zellantwort analysiert. Dabei wurden unterschiedliche Aspekte der B Zellen, Einfluss auf die Immunantwort zu nehmen, wie die Sekretion von IL-10, als Antikörperquelle und als Antigen präsentierende Zellen, untersucht. Bei der *Leishmania major*-Infektion lag der Fokus auf der Untersuchung des Einflusses von Antikörpern auf die Th1 und die Th2 Antwort.

In mCMV Infektionen konnte gezeigt werden, dass von B Zellen sekretiertes IL-10 die virus-spezifische CD8 T Zellantwort supprimiert, im Gegensatz zu IL-10 sekretiert von dendritischen Zellen, das die virus-spezifische CD8 T Zellantwort nicht merklich beeinflusst. Die von B Zellen sekretierten Antikörper haben nur in der ‚*memory*'-Antwort das Gewicht die virus-spezifische CD8 T Zellantwort zu beeinflussen; fehlen Antikörper, verbleibt die virus-spezifische CD8 T Zellantwort 135 Tage nach Infektion auf dem Niveau der akuten Antwort. Das komplette Fehlen von B Zellen hingegen führt zu einer enormen Verringerung in der akuten CD8 T Zell Antwort, die durch Rekonstitution mit B Zellen nur partiell aufgehoben werden konnte. Ein Vergleich der Immunantwort in einem B Zell-defizienten Organismus mit einem dendritische Zellen depletierten Organismus zeigte dieselbe verminderte Immunantwort. Durch einen weiteren Vergleich eines B Zell defizienten Organismus mit einem B Zell depletierten Organismus konnte eine Beeinträchtigung der CD8 T Zellen in dem B Zell defizienten Organismus ausgeschlossen werden. Knochenmarkschimären mit einer B Zell Population ohne CD40 Expression zeigten, dass CD40 auf B Zellen einen Einfluss auf die virus-spezifische CD8 T Zellantwort hat, die in Abwesenheit von CD40 verringert ist. Diese Ergebnisse implizieren eine Rolle von B Zellen als Antigen präsentierende Zellen hin.

Des Weiteren wurde die veränderte virus-spezifische CD8 T Zellantwort in Abwesenheit von Antikörpern benutzt, um ein Phänomen der ‚*memory*'-Antwort zu untersuchen, ‚*memory inflation*' genannt. Die durchgeführten Untersuchungen führten zu einem experimentellen System, in dem die Zeitspanne bis zum erkennbaren Auftreten der ‚*memory inflation*' verkürzt werden konnte. Es konnte auch nachgewiesen werden, dass die für die ‚*memory inflation*' charakteristische virus-spezifische CD8 T Zell Population

durch direkte Präsentation stimuliert wird.
In *Leishmania major* Infektion hängt die Immunantwort vom genetischen Hintergrund der Mäuse ab. BALB/c Mäuse antworten mit einer Th2 Antwort, in der Antikörper eine zentrale Rolle bei der Prävention vor einer systemischen Infektion spielen. Während in C57BL/6 Mäuse der *Leishmania major* Infektion mit einer Th1 Antwort begegnet wurde: Durch die Rekonstituierung von Mäusen ohne Antikörpern mit Serum von bereits *Leishmania major* infizierten Mäusen oder unimmunisierten Mäuse konnte eine starke Verbesserung erzielt werden, resultierend waren die Parasitenlasten in der Milz und den Ohren nach der Rekonstituierung vergleichbar mit den gemessenen in Wildtyp Mäusen, das Gleiche galt für die Größe der Milz sowie den Zytokinen IL-10, IL-4, IL-2 und IFN-γ.

7. References

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8. Acknowledgments

9. Versicherung

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Mainz, im April 2011 Filiz Demircik

10. Lebenslauf