Toll-like receptor 9 mediated tolerance in murine systemic lupus erythematosus

vorgelegt von Diplom-Biologin

Carolin Schön

aus Mühldorf am Inn

Von der Fakultät III,

Institut für Biotechnologie der Technischen Universität Berlin

zur Erlangung des akademischen Grades

Doktor der Naturwissenschaften

Dr. rer. nat.

genehmigte Dissertation

Promotionsausschuss:

Vorsitz: Prof. Dr. Leif-Alexander Garbe

Gutachter: Prof. Dr. rer. nat. Roland Lauster

Gutachter: Prof. Dr. rer. nat. Marc Ehlers

Tag der wissenschaftlichen Aussprache: 17.12.2010

Berlin 2011

D 83

„Am Anfang jeder Forschung steht das Staunen.
Plötzlich fällt einem etwas auf.“

Wolfgang Wickler

Für meine Eltern.

Abstract

The autoimmune disease *Systemic Lupus Erythematosus (SLE)* is associated with high titers of inflammatory serum IgG autoantibodies, which accumulate in the kidney, where they recruit inflammatory immune cells such as macrophages, inducing nephritis. Autoreactive B cells have been shown to be activated by B cell receptor (BCR) and innate Toll-like receptor co-signaling. Thus, Toll-like receptor 9 (TLR9), which specifically binds DNA, is associated with the generation of pathogenic DNA-reactive IgG antibodies. However, the role of TLR9 in the development of *SLE* remains controversial. In various lupus mouse models loss of TLR9 abolishes the generation of anti-nucleosome serum IgG autoantibodies but at the same time exacerbates lupus disease. Hence, besides the induction of autoimmunity, TLR9 also regulates tolerance to self-antigens. In this study we used mice deficient for the IgG inhibitory receptor FcγRIIB (FcγRIIB$^{-/-}$) to investigate the role of TLR9 in murine lupus. We show that loss of TLR9 in FcγRIIB$^{-/-}$ mice is associated with low levels of IgG antibodies specific to nucleosomes, but at the same time with exaggerated IgG antibody responses specific to other self-antigens, such as DNA, RNA associated ribonucleoproteins (RNP) and self-IgG. TLR9 deficiency in FcγRIIB$^{-/-}$ mice furthermore results in the activation and accumulation of pro-inflammatory T helper cells, most notably Th1 and Th17 cells and highly increased mortality rates. Inconsistent with raised IgG serum autoantibody responses, levels of self-reactive IgM serum antibodies are significantly reduced in the absence of TLR9. Contrary to high affinity IgG serum autoantibodies, self-reactive IgM serum antibodies are associated with protection and not inflammation in several autoimmune disorders. Indeed, reconstitution of IgM levels in FcγRIIB$^{-/-}$ mice lacking TLR9 (FcγRIIB$^{-/-}$TLR9$^{-/-}$) with recombinant monoclonal self-reactive IgM antibodies was sufficient to reduce the frequency of pro-inflammatory Th17 cells and to ameliorate lupus disease. We could ascribe low levels of protective self-reactive IgM serum antibodies in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice to low peritoneal B-1b cell frequencies. This work clearly demonstrates that TLR9 stimulation *in vitro* and *in vivo* leads to the activation and proliferation of innate-like FcγRIIB deficient B-1b cells, promoting the production of protective self-reactive IgM antibodies. Similarily to the application of monoclonal self-reactive IgM antibodies, the transfer of TLR9-expressing peritoneal B-1b cells from FcγRIIB-deficient mice prevents the accumulation of pro-inflammatory Th17 cells and lupus disease in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice. Taken together, these data provide evidence for the function of self-reactive IgM antibodies produced by peritoneal B-1b cells as mediators of TLR9-dependent tolerance and thus, may be implicated in TLR9 ligand-dependent therapies and B cell depletion protocols as a treatment of SLE. In particular, these results should encourage the generation of human monoclonal self-reactive IgM antibodies that may be beneficial for the treatment of SLE and other autoimmune disorders.

Zusammenfassung

Die Autoimmunerkrankung *Sytemischer Lupus Erythematodes (SLE)* ist mit der Entstehung selbstreaktiver Antikörper verbunden, die sich in Form von Immunkomplexen in der Niere ablagern. Autoreaktive B Zellen werden während des Krankheitsverlaufes durch die Kostimulierung des B Zellrezeptors und verschiedener *Toll- like* Rezeptoren (TLR) aktiviert. Dabei bindet TLR9 spezifisch an bakterielle DNA und wurde deshalb lange Zeit mit der Bildung von pathogenen anti-DNA IgG Autoantikörpern in Verbindung gebracht. Allerdings ist die Funktion von TLR9 in der Entstehung von SLE immer noch sehr umstritten. In verschiedenen Lupus Mausmodellen verhindert die Deletion von TLR9 die Generierung von IgG anti-Nukleosomen Antikörpern, gleichzeitig aber verschlimmert der Verlust von TLR9 das Krankheitsbild. Dies unterstreicht die wichtige Funktion von TLR9 in der Toleranzregulation gegenüber Selbstantigenen. In dieser Arbeit wurden FcγRIIB defiziente (FcγRIIB$^{-/-}$) Mäuse verwendet, um die Rolle von TLR9 in murinem *SLE* zu untersuchen. Es konnte gezeigt werden, dass der Verlust von TLR9 in FcγRIIB$^{-/-}$ Mäusen einerseits die Entstehung von anti-Nukleosomen IgG Antikörpern verhindert, andererseits aber die IgG Autoantikörperantwort spezifisch für zahlreiche andere Selbstantigene wie DNA, RNA assoziierte Ribonukleoproteine (RNP) und Selbst-IgG stark erhöht. Das Fehlen von TLR9 führt ausserdem zur Aktivierung und Akkumulation proinflammatorischer T Helfer Zellen, vorallem Th1 und Th17 Zellen, und zu einer stark verminderten Überlebensrate. Es konnte weiterhin gezeigt werden, dass anders als bei IgG Autoantikörpern, die Abwesenheit von TLR9 zu einer drastischen Verringerung der selbstreaktiven IgM Antikörper führt. Im Gegensatz zu hochaffinen IgG Autoantikörpern, werden selbstreaktive IgM Antikörper nicht mit Entzündungsreaktionen sondern mit dem Schutz vor Autoimmunität assoziiert. Durch die Rekonstitution der Serum IgM Titer in TLR9 defizienten FcγRIIB$^{-/-}$ (FcγRIIB$^{-/-}$TLR9$^{-/-}$) Mäusen mit monoklonalen selbstreaktiven IgM Antikörpern konnte die Bildung proinflammatorischer Th17 Zellen unterdrückt und der Ausbruch von SLE verhindert werden. Niedrige Titer selbstreaktiver IgM Antikörper in FcγRIIB$^{-/-}$TLR9$^{-/-}$ Mäusen konnten eindeutig auf stark verringerte peritoneale B-1b Zellzahlen zurückgeführt werden. Diese Arbeit zeigt, dass TLR9 Stimulierung *in vitro* und *in vivo* unweigerlich zur Aktivierung und Proliferation von FcγRIIB$^{-/-}$ B-1b Zellen führt und dabei die Produktion von protektivem selbstreaktivem IgM induziert. Ähnlich wie der direkte Transfer von monoklonalen selbstreaktiven IgM Antikörpern, führt auch der Transfer von FcγRIIB$^{-/-}$TLR9$^{+/+}$ B-1b Zellen in FcγRIIB$^{-/-}$TLR9$^{-/-}$ Mäuse zur Reduktion von Th17 Zellen und verminderten Sterberaten. Zusammengefasst zeigen die in dieser Arbeit generierten Daten, dass TLR9 Stimulierung in B-1b Zellen zur Produktion von selbstreaktiven IgM Antikörpern führt, welche die Toleranz gegenüber Selbstantigenen aufrecht erhalten. Humane monoklonale selbstreaktive IgM Antikörper können daher eine neue Möglichkeit der Therapie von SLE und anderen Autoimmunerkrankungen darstellen.

Diese Arbeit entstand im Deutschen Rheumaforschungszentrum (DRFZ) in Berlin, einem Mitglied der Leibniz-Gemeinschaft, im *Laboratory of Tolerance and Autoimmunity* unter der Leitung von Prof. Dr. Marc Ehlers. Die vorliegende Dissertation wurde in der Zeit von Dezember 2007 bis Dezember 2010 angefertigt.

Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich vorliegende Dissertation in allen Teilen selbständig verfasst habe. Genutzte Hilfsmittel wurden vollständig angegeben. Bei der Anfertigung wurde keine Hilfe Dritter in Anspruch genommen. Veröffentlichungen von Teilen der vorliegenden Dissertation sind von mir nicht vorgenommen worden. Diese Dissertation wurde bisher nicht für eine Prüfung oder Promotion zur Beurteilung eingereicht. Ich versichere, dass ich die vorstehenden Angaben nach bestem Wissen vollständig und der Wahrheit entsprechend gemacht habe.

Carolin Schön Berlin, 19. November 2010

Table of Contents

Table of Figures

List of Tables

Abbreviations

ADCC	*antibody dependent cell mediated cytotoxicity*
ANA	*anti nuclear antigens*
APC	*allophycocyanin*
APC	*antigen presenting cell*
APS	*ammonium persulphate*
BCR	*B cell receptor*
BSA	*Bovine serum albumin*
BsIWI	*restriciton enzyme*
C1q	*early complement subunit*
C5a	*protein fragment released from complement component 5*
C57BL/6	*wild type mouse model*
CCL	*chemokine ligand, inducing effector cell chemotaxis*
CCR	*chemokine receptor*
CD	*cluster of differentiation*
CLP	*common lymphoid progenitor cell*
CpG ODN	*unmethylated sequences of DNA*
CRP	*c-reactive protein*
CSR	*class switch recombination*
CTL	*cytotoxic T lymphocyte*
CXCR	*chemokine receptor*
Cy5	*carboxymethylindocyanin-5*
DC	*dendritic cell*
DMEM	*Dulbecco/Vogt modified Eagle's minimal essential medium*
DNA	*desoxyribonucleic acid*
ds	*double strand*
EDTA	*ethylenediaminetetraacetic acid*

ELISA	*Enzyme-linked immunosorbent assay*
EtBr	*ethidium bromide*
Fab	*antibody variable region; fragment antigen binding*
FACS	*fluorescence activated cell sorting*
Fc	*antibody constant region; fragment crystallizable*
FcR	*Fc-receptor*
FcγR	*Fcγ-receptor*
FcγRIIB	*Fcγ-receptor IIB*
FCS	*fetal calf serum*
FDC	*follicular dendritic cell*
FITC	*fluoresceinisothiocyanat*
FO	*follicular B cell*
FSC	*forward scatter*
GC	*germinal center*
Hek293T	*human embryonic kidney cells withT-antigen*
HindIII	restriction enzyme
HLA	*human leukocyte antigen*
HRP	*horse radish peroxidase*
HSC	*hematopoetic stem cell*
IC	*immune complex*
IEC	*intestinal epithelial cells*
IFNγ	*interferone γ*
Ig	*immunglobulin*
IgH	*BCR heavy chain*
IgL	*BCR light chain*
IL	*interleukin*
i.p.	*intra peritoneal*
IRF	*interferone regulatory factor*
IMDM	*Iscove's Modified Dulbecco's Medium*
ITAM	*immunoreceptor tyrosine-based activation motif*
ITIM	*immunoreceptor tyrosine-based inhibitory motif*
IvIg	*intravenous immuno globulin*
La	*protein binding to RNA*
LN	*lymph node*

lpr . . . . . *lymphoproliferation gene*
LPC . . . . . *lymphoid progenitor cell*
LPS . . . . . *lipopolysaccharide*
MacI . . . . . *surface marker*
MBL . . . . . *mannose binding lectine*
MCP . . . . . *monocyte chemotactic protein*
MHC . . . . . *major histocompatibility complex*
MIP . . . . . *macrophage inflammatory protein*
MRL . . . . . *mouse model*
MyD88 . . . . . *myeloid differentiation primary response gene 88*
MZ . . . . . *marginal zone*
NF-κB . . . . . *nuclear factor κ B*
NLR . . . . . *nucleotide-binding oligomerization domain-like receptor*
nuc . . . . . *nucleosomes*
NK . . . . . *natural killer*
NZB . . . . . *mouse model, New Zealand Black*
NZW . . . . . *mouse model, New Zealand White*
OD . . . . . *optical density*
PAMPs . . . . . *pathogen associated molecular patterns*
PAGE . . . . . *polyacrylamide gel electrophoresis*
PBS . . . . . *phosphate buffered saline*
PC . . . . . *plasma cell*
PCR . . . . . *polymerase chain reaction*
Pe . . . . . *phycoerythrin*
PEI . . . . . *polyethylene imide*
PerCP . . . . . *peridin-Chlorophyll-Protein*
PU + ... *proteinuria positiv*
PU - . . . . . *proteinuria negative*
RIIB$^{-/-}$ . . . . . *mouse model deficient in FcγRIIB*
RIIB$^{-/-}$TLR9$^{-/-}$ . . . . . *mouse model deficient in FcγRIIB and TLR9*
pH . . . . . *potentiometric hydrogen ion concentration*
PMA . . . . . *phorbol-12-myristat-13-acetic acid*
PRR . . . . . *pattern recognition receptors*
PS . . . . . *penicillin/streptomycin*

RF	*rheumatoid factor*
RNA	*ribonucleic acid*
RNP	*Ribonucleoprotein*
Ro	*ribonclеic complex*
RPMI	*Roswell Park Memorial Institute medium*
SalI	*restriction enzyme*
SEM	*standard error of the mean*
SDS	*sodium lauryl sulfate*
SLE	*systemic lupus erythematosus*
Sm	*Smith-antigene*
SPF	*specific pathogen free*
ss	*single strand*
SSC	*side scatter*
TAE	*tris base, acetic acid, EDTA*
Taq	*DNA-polymerase Thermus aquaticus*
TCR	*T cell receptor*
TD	*T dependent*
TGF-β	*transformal growth factor β*
Th	*T helper*
TI	*T independent*
TE	*Tris/EDTA*
TLR	*Toll-like receptor*
TNF-β	*tumor necrosis factor β*
TMB	*Tetramethylbenzidine*
Treg	*regulatory T cell*
TRIF	*(TIR)-Domain-containing adapter inducing interferone β*
V	*variable*
yaa	*y linked autoimmune accelerator*

1 Introduction

1.1 The Immune System

The immune system of vertebrates is a highly adaptive system capable of discriminating between self and non-self structures. It has been evolved to defend the organism against invading pathogens like parasites, fungi, bacteria and viruses and is based on complex molecular and cellular interactions between different components and cell types of predominantly hematopoietic origin. By specific mechanisms the immune system assures that only pathogenic antigens and not self-structures are eliminated. Thus, defects in specific immune responses, which foster the recognition of endogenous self-structures, are the main reasons for autoimmunity. In principle, the immune system is composed of two major subdivisions, the innate or non-specific immune system and the adaptive or specific immune system.

1.1.1 The innate and adaptive immune system and their functions

The term "innate" immunity comprises a variety of cells and mechanisms that defend the host in early stages of infection by unspecific immune reactions (Kimbrell and Beutler 2001). Only minutes after the infiltration of pathogens the machinery of the innate immune system is initiated *(Fig 1.1).* Innate immune cells, such as mast cells, are present in high numbers in the skin and mucosa of the respiratory, gastrointestinal and genitourinary tract, where they are in prime position to recognize invading pathogens. When activated, mast cells rapidly release characteristic granules, rich in histamine and heparin, chemokines and chemotactic cytokines. Histamine dilates blood vessels, causing the characteristic signs of inflammation, and recruits neutrophils and macrophages.

Macrophages and neutrophils are the first cells to arrive at the site of infection (Godaly, Bergsten et al. 2001; Segal 2005). They can easily move out of the blood vessels and migrate to the tissues. Neutrophils are essential for resistance to bacterial and fungal infections due to their production of a variety of toxic products like antimicrobial peptides and oxidizing agents including hydrogen peroxide, free oxygen radicals and hypochlorite. As their name implies, macrophages are capable of recognizing foreign antigens and perform their engulfment via phagocytosis while patroling the sub-endothelial tissues in pursuit of invading pathogens (Mogensen 1979; Aderem and Underhill 1999). Their activation leads to the secretion of a range of inflammatory cytokines and chemokines provoking the recruitment of other immune effector cells. Cytokine secretion and antigen-presentation by macrophages are crucial for the induction of the adaptive part of the immune system (Luster 2002). Recruitment and activation of cells referred to as natural killer cells (NK cells) is promoted by anti-viral effector molecules called interferons and macrophage-derived cytokines. NK cells recognize tumor cells or virus-infected cells by low levels of the expressed surface protein MHCI (major histocompatibility complex) and directly bind to compromised host cells and enforce the lysis of infected cells by release of perforine and granzyme (Biron, Nguyen et al. 1999; Boyington and Sun 2002). Eosinophils and basophils, known as granulozytes, also participate in the first line of immune defence. Thereby these cells secret a range of highly toxic proteins and free radicals that are effective in killing bacteria and parasites. Besides the innate effector cells, another important effector arm of the innate immune system is displayed by the complement system. The complement system consists of various small plasma proteins and glycoproteins, which are predominantly synthesized by liver hepatocytes. When stimulated, the complement system mediates clearance of pathogens by direct lysis or engulfment by macrophages. Three different pathways are capable of activating the complement cascade. The classical complement pathway typically requires antigen-antibody complexes for activation, and is therefore considered as part of the adaptive immune system. The mannose-binding lectin pathway (MBLectin pathway) is initiated by binding of mannose-binding lectin (MBL) to mannose containing carbohydrates or glycoproteins on bacteria or viruses whereas the alternative pathway is triggered by direct binding of complement components to the cell surface of pathogens which activates the cascade. As part of the innate immune

system, elements of the complement cascade can be found in species earlier than vertebrates (Tomlinson 1993; Dunkelberger and Song 2010).

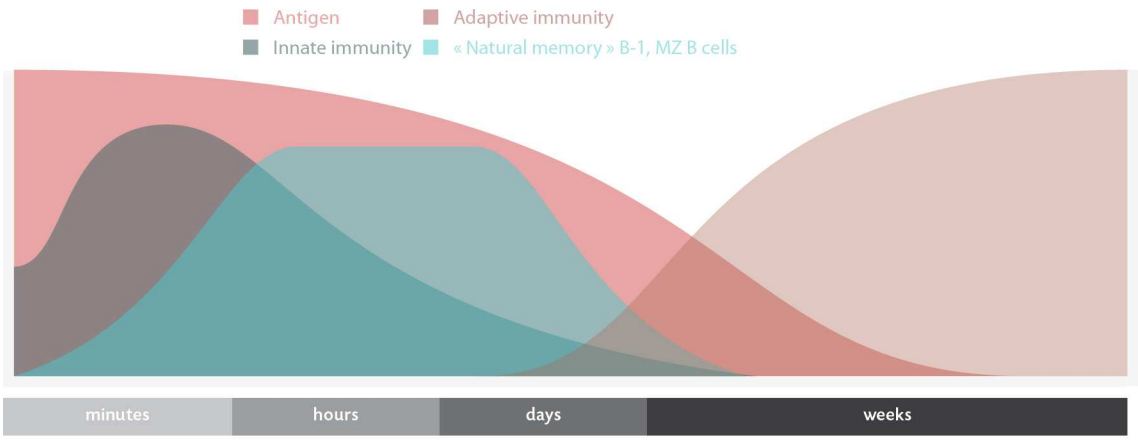

Figure 1.1 Immune response to antigen. Scheme of host response to antigenic challenge. Quantitatively, the majority of antigens is removed very early by innate immunity. Only if antigens cannot be eliminated, the adaptive immune system is activated. Bridging the two are components of the "natural immune memory"compartment, which ensure an optimal early transition and antigen clearance. Adapted from (Martin and Kearney 2001)

Quantitatively, the majority of pathogenic antigens is removed very early by innate immunity. Only when the innate immune mechanisms are insufficient to clear invading pathogens or respectively the pathogens evade the innate immune system, the highly specific adaptive immune response is induced, which takes from days up to weeks to fully develop (McHeyzer-Williams, McHeyzer-Williams et al. 2000) *(Fig 1.1)*. Adaptive immune responses are most notably mediated by B and T cells, which carry specific antigen-binding receptors on their surface: the T cell antigen receptor (TCR) and the B cell antigen receptor (BCR). TCR and BCR provide the recognition of specific non-conserved structures on pathogens and induce specific defense startegies for the protection from invading pathogens. The highly diverse repertoire of B and T cell receptors is based on the rearrangment of germline encoded variable gene segments during different developmental stages.

In the course of an infection professional antigen presenting cells (APC), such as dendritic cells (DC) efficiently internalise antigen by phagocytosis or receptor-mediated endocytosis on the site of infection and subsequently migrate to the secondary lymphoid organs, where they present processed antigenic protein fragments to naïve B and T cells. Thereby APCs serve as a link between innate and

adaptive immune systems (Banchereau, Briere et al. 2000). Dependent on their effector function activated T cells either directly kill infected cells (cytotoxic T cells) or support the activation of B cells and other immune cells (T helper cells) (Mak 1995). Depending on the type of pathogen and the cytokines secreted by antigen-presenting cells, distinct T cell responses are induced. Upon activation, B-cells are able to secrete soluble antigen-receptor molecules, the antibodies or immunoglobulins, which are the major effector molecules of the humoral immune response. Besides its complex defense functions, the adaptive immune system is able to develop a so-called immunological memory, which provides long-lasting protection against repetitive infection with the same microorganism (McHeyzer-Williams, McHeyzer-Williams et al. 2000). Another feature of the adaptive immunity is the capacity of immune cells to distinguish between self-structures and unwanted foreign invaders and thus mostly guarantees a balance between protection from pathogens and self-tolerance.

In addition to 'classic' innate and adaptive immune mechanisms, several components of the B cell lineage participate during the early stages of an immune response, hence, bridging innate and adaptive immune responses *(Fig 1.1).* Innate like B-1 and MZ B cells exhibit outstanding functions in producing natural IgM antibodies, which efficiently remove pathogens. These two peripheral B cell subsets are said to contribute to the so-called 'natural memory' (Martin and Kearney 2000), as they resemble memory B cells in several functional characteristics but contrary to follicular (FO) B cells, innate-like B-1 cells and MZ B cells do not generate immunological memory.

1.1.2 Receptors of innate immune cells - The *Toll-like* receptors (TLR)

Cells of the innate immune system are not provided with specific receptors, like adaptive immune cells. Elimination of invading pathogens is rather unspecific and is based on the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) on the surface or inside the cells of the innate immune system. So-called NLRs (nucleotide-binding oligomerization domain-like receptors) for instance are capable of binding infiltrated pathogens in the cytosol (Franchi, McDonald et al. 2006). Another example for PRRs are C-type lectines on the surface of innate immune cells such as the mannan binding lectin (MBL) (Turner and

Hamvas 2000), mannose receptors on macrophages, special lectines on dendritic cells and most notably the Toll like receptors (Takeda and Akira 2005; Buzas, Gyorgy et al. 2006).

Mammalian TLRs comprise a large family consisting of at least 11 members whereas TLR1–9 are conserved between human and mouse. Originally they were discovered in the fruitfly *Drosophila melanogaster* (Lemaitre, Nicolas et al. 1996). All eleven known TLRs recognize different functional components (PAMPs) of viruses, bacteria and funghi such as LPS (TLR 4), flagella (TLR 5), bacterial DNA (TLR 9) or RNA (TLR7,8) (Barton and Medzhitov 2002; Takeda and Akira 2005) and are localized either on the cell surface or in endosomes inside the cell *(Fig 1.2)*.

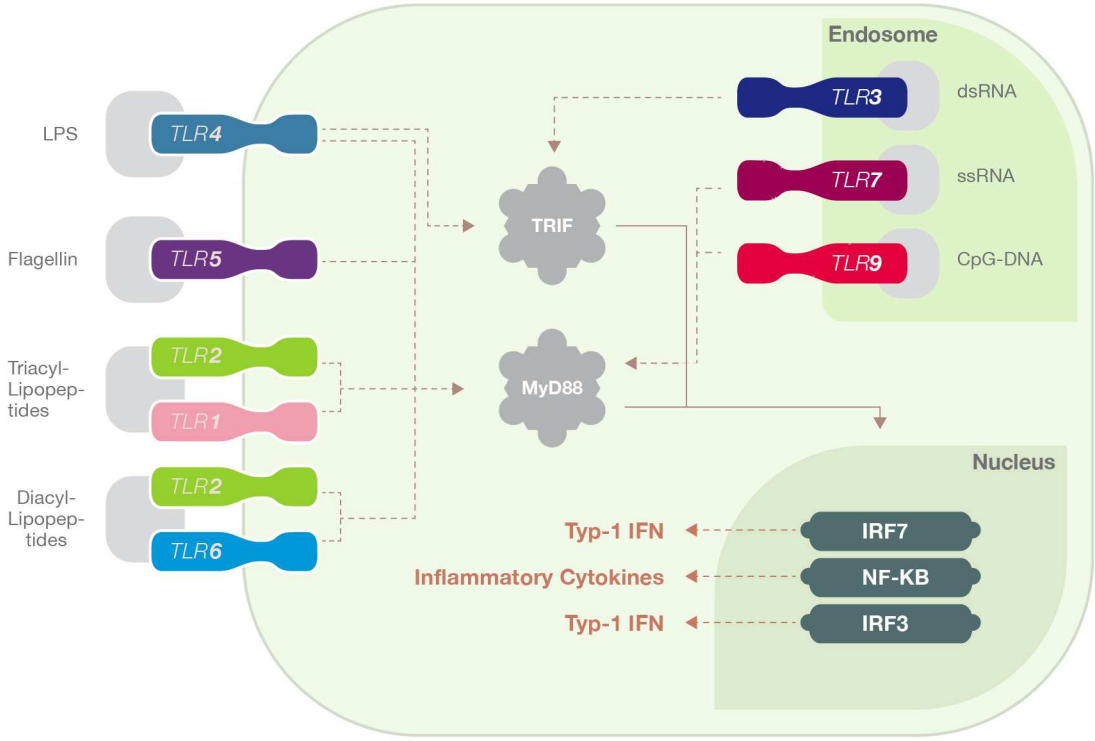

Figure 1.2 Toll-like receptors (TLRs), their stimulation and signal transduction. Schematic illustration of various TLRs. TLRs recognizing cell surface proteins of pathogens are located on the surface of immune cells, whereas TLRs binding to pathogenic nucleotide structures are expressed in the endosomes. Every TLR exhibits a cytosolic domain, which enables the binding of the adaptor proteins MyD88 or TRIF. MyD88 and TRIF mediate signal transduction by transferring signals to the nucleus. There the transcription factor NF-kB and the interferone regulatory factors IRF3 and IRF7 are activated and consequently inflammatory cytokines and interferones are produced. Adapted from (Takeda and Akira 2005)

After binding to a molecular pattern of a pathogen, TLRs give rise to intracellular signal cascades via adaptor protein molecules. Except TLR3 all TLRs use the adaptor molecule MyD88 for signal transduction. Activation of TLR3 and also TLR4 initiates signal transduction via the adaptor protein TRIF. Signal transduction upon activation of TLRs results in the expression of transcription factors, like NF-κB and IRF, which leads to the production of type I interferones and inflammatory cytokines.

Besides their function as receptors in the innate part of the immune system, TLRs also serve as important co-stimulators in the activation process of B and T cells.

All receptors of innate immune cells are germline encoded and therefore are characterized by defined specificities to PAMPs. This feature allows on the one hand prompt immune responses without previous exposure to pathogens, but on the other hand innate immune cells cannot protect the organism from pathogens without PAMPs. Hence, the adaptive immune system comes into play.

1.1.3 T lymphocytes and their functions in adaptive immunity

T lymphocytes (T cells) are one of the two major fractions of lymphocytes mediating adaptive immunity. The development of T cells is initiated in the thymus after a lymphoid progenitor cell of hematopoietic origin migrates from the bone marrow to the thymus (Carlyle and Zuniga-Pflucker 1998; Carpenter and Bosselut 2010). Antigen-recognition by T cells is carried out via the T cell receptor (TCR), which recognizes antigens, in form of a complex of a foreign peptide bound to a MHC (MHCI or MHCII) molecule on antigen presenting cells (APCs). The TCR heterodimer is composed of two transmembrane glycoprotein chains, each with a constant and variable region. During their development in the thymus, T cells undergo rearrangement of the genes for the TCR α, β, γ, and δ chains which contributes to the huge repertoire of α/β or γ/δ TCRs found on T cells (Kisielow and von Boehmer 1995). The TCR heterodimer is associated with a complex of four protein chains, the CD3 complex, and a homodimer of ζ-chains. These protein chains exhibit immunoreceptor tyrosine-based activation motifs (ITAM) and hence are capable of mediating signal transduction upon T cell activation. T cells in general can be subdivided into two major classes distinguished by the expression of the cell surface proteins CD4 and CD8: the CD8 T cells, referred to as cytotoxic “killer” T

cells, and the CD4 T helper cells (Seder and Ahmed 2003). The proteins CD4 and CD8 thereby function as TCR co-receptors, CD8 co-interacting with MHCI and CD4 co-interacting with MHCII (Mosmann, Cherwinski et al. 1986; Williams and Bevan 2007). Upon binding of TCR and CD8 to an antigenic peptide presented by MHC class I molecules on antigen-presenting cells, cytotoxic T cells (CTL) are activated and kill target cells either by the release of cytotoxic granules containing perforine and granzymes or by inducing target cells to undergo apoptosis via Fas-Fas ligand interactions (cellular immunity) (Harty, Tvinnereim et al. 2000; Barry and Bleackley 2002). Upon stimulation by their cognate antigenic peptide presented on MHC class II molecules, naïve CD4 T helper cells differentiate into effector helper CD4 T cells of specialized phenotypes depending on respective additional activating signals (Mosmann, Cherwinski et al. 1986; Abbas, Murphy et al. 1996). This commitment is governed by the cytokine content of the microenvironment in which antigen presentation proceeds *(Fig 1.3)*. In the presence of IL-12, IL-4, or TGF-β T cells differentiate into Th1, Th2, or Tregs, respectively, whereas the presence of both TGF-β and IL-6 in mice or TGF-β ,IL-6, IL-1β and IL-23 in humans forces the commitment to the Th17 phenotype (Louten, Boniface et al. 2009; Strom and Koulmanda 2009; Wan 2010; Zhu, Yamane et al. 2010). Different CD4 T helper cell phenotypes and functions are mainly distiguished by their cytokine production. Th1 cells produce IFNγ, IL-2, and TNF-β, which activate macrophages and are responsible for cell-mediated immunity and phagocyte-dependent protective responses. Th1 cells are furthermore known to activate B cells and foster the generation of IgG2a antibodies (Snapper and Paul 1987). Th2 cells produce IL-4, IL-5, IL-10, and IL-13, which are responsible for strong antibody production by activated B cells, eosinophil activation, and inhibition of several macrophage functions, thus providing phagocyte-independent protective responses (Romagnani 1999). Tregs produce TGF-β and IL-10 and thus mediate immune suppression and promote tolerance whereas the newly identified Th17 lineage is characterized by the production of IL-17. Besides the protection against extracellular pathogens, Th17 cells play an essential pro-inflammatory role in autoimmunity (Dong 2006; Dong 2008; Jin, Zhang et al. 2008) .

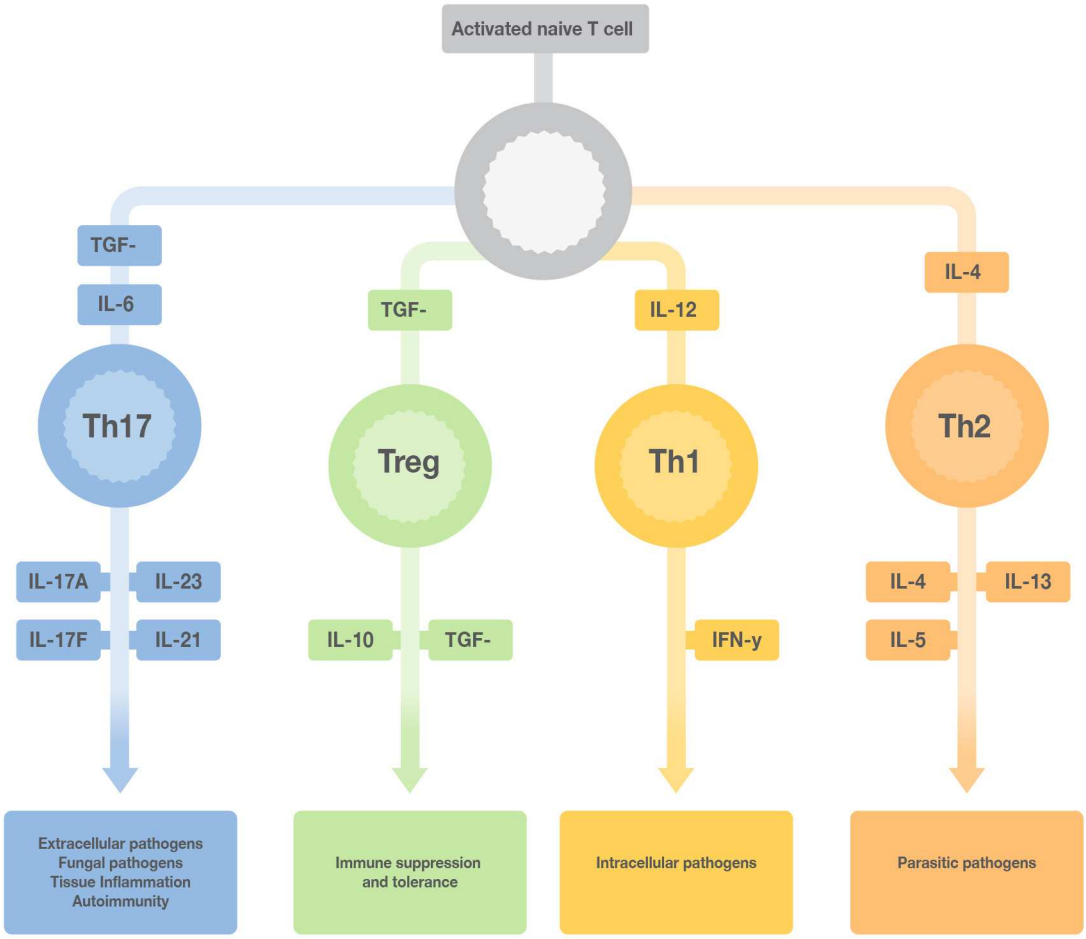

Figure 1.3 Development and functions of distinct T helper cell subsets. Different cytokine profiles are responsible for the differentiation of activated naïve T cells into the four distinct Th cell subsets (Th1, Th2, Tregs and Th17). Depending on their effector cytokines distinct T helper cell subsets exhibit different functions in mediating immune responses. Adapted from (Dong 2008)

1.1.4 B lymphocytes and their functions in adaptive immunity

B lymphocytes (B cells) represent the second fundamental part of the adaptive immune system and are characterized by their membrane bound receptor, the B cell receptor (BCR), which recognizes and binds to three-dimensional structures (epitopes) of soluble antigens. The BCR complex is composed of the Y-shaped membrane-bound immunglobulin, consisting of two identical heavy chains (IgH) and two identical light chains (IgL) and the associated invariant proteins Igα and Igβ. With its N-terminal variable regions, the membrane bound immunglobulin functions as antigen recognition and binding site whereas each of the invariant protein chains Igα and Igβ exhibits a single immunoreceptor tyrosine-based

activation motif (ITAM) and promotes the signal transduction, resulting in the triggering of various downstream activation pathways. Vast numbers of BCR specificities are required for the recognition of diverse antigens. These are reached during the different phases of B cell development. Due to unique gene segment rearrangements (somatic recombination) of the H and L chain every single B cell gains certain specificity. Beyond their widely known role in mediating humoral immune response, B cells can also secrete cytokines and have the potential to present antigens to naïve T cells.

1.1.4.1 Peripheral B cell subsets – Follicular B cells, MZ B cells and B-1 cells

Naïve B cells are generally divided into three subsets: Follicular B cells, marginal zone B cells and B-1 B cells (Allman and Pillai 2008) *(Table 1.1).*

Follicular B cells migrate through blood and lymph to B cell areas in lymph nodes, Peyer`s Patches and spleen. In the well established "follicular niches" B cells present engulfed T-dependent (TD) antigens to activated antigen-specific T cells resulting in the proliferation and differentiation of follicular B cells into short-or long-lived plasma cells and memory cells (Pereira, Kelly et al. 2010).

Marginal Zone B cells (MZ B cells) are considered to-be innate like cells that can be induced to differentiate into short-lived plasma cells in the absence of BCR ligation. Although these cells have been described as being sessile outside the marginal sinus (Martin and Kearney 1999), it has been demonstrated recently that they possess the ability to transport antigen immune complexes from the marginal sinus to follicular B cells and follicular DCs in the splenic follicles (Ferguson, Youd et al. 2004; Cinamon, Zachariah et al. 2008). MZ B cells express high levels of CD21 (Complement Receptor-2), which is probably involved in the capturing of immune complexes. The immune responses mediated by MZ B cells are mostly directed against antigens of blood-borne pathogens and are primarily T cell independent (TI).

Another peripheral B cell population termed "innate-like" is the B-1 cell subset, which is the dominant B cell subset seeding the peritoneal and pleural cavities (Fagarasan, Watanabe et al. 2000). In general, B-1 cells can be distinguished from

follicular or B-2 cells by their expression of the surface marker MacI or low levels of IgD and are typically subdivided into $CD5^+$ $B220^{dull}$ B-1a cells and $CD5^-$ $B220^+$ B-1b (Stall, Adams et al. 1992). The BCR of peritoneal B-1 cells is known to be polyreactive, meaning that it has low affinities for many different antigens, including self- antigens and common bacterial polysaccharides. Because different individual mice have been shown to frequently exhibit the same heavy and light chain Ig rearrangement, it has been postulated that there is a conserved B-1 cell repertoire (Seidl, MacKenzie et al. 1997).

Table 1.1: Characteristic features of distinct peripheral B-cell subsets

	B-1a	B-1b	MZ	FO
CD19	+	+	+	+
B220	+	++	++	++
IgM	++	++	+++	+
IgD	+/–	+/–	+/– to ++	++
CD5	+	–	–	–
MacI	+	+	–	–
CD21	+	+	+++	++
Location	Spleen/PerC	PerC	Spleen	Spleen/LN
Development	mainly fetal	fetal and adult	fetal and adult	mainly adult
BCR-diversity	restricted	restricted	restricted	diverse
Response	T-independent	T-independent	T-independent	T-dependent
Natural antibody	+	+	+	–

Adapted from (Hardy 2006)

B-1 cells contribute to the generation of IgM responses either by participating in a variety of antigen stimulated responses upon activation with T-independent antigens such as phosphorylcholin, which is an antigen on the surface of many pathogenic bacteria (Masmoudi, Mota-Santos et al. 1990; Kantor 1991; Herzenberg 2000) or by the spontaneous production of innate "natural IgM antibodies" (Baumgarth, Herman et al. 1999; Manson, Mauri et al. 2005). In contrast to B-1a cells, secretion of antibodies by B-1b cells seems to require antigen-mediated activation (Alugupalli, Leong et al. 2004; Alugupalli and Gerstein 2005; Haas, Poe et al. 2005; Montecino-Rodriguez and Dorshkind 2006). Thus, B-1b cells have recently been shown to be critical in producing adaptive pneumococcal polysaccharide antibodies (Haas, Poe et al. 2005). As already mentioned, antibodies produced by B-1 cells are almost exclusively of the IgM isotype.

In vivo circulating IgM antibodies exist in general as pentamers and occasionally as hexamers (Randall, Brewer et al. 1992) , and thus they are highly efficient in binding pathogens and activating the complement pathway (Ehrenstein, O'Keefe et al. 1998; Baumgarth, Chen et al. 2000). As B-1 cells are said to be responsible for the maintenance of IgM antibody levels that exists prior to infection, they are essential for linking the innate and adaptive part of the immune system *(Fig 1.1)* (Martin and Kearney 2000).

1.1.4.2 Distinct development of peripheral B cell subsets

In the year 2006 a unified model of the developmental stages of all known peripheral B cell subsets has been publicated (Hardy 2006) *(Fig 1.4).* Progenitors for B-1 cells preferentially develop during fetal life, whereas B-2 cells or follicular B-cells originate in hematopoietic stem cells (HSCs) in adult bone marrow (Dorshkind and Montecino-Rodriguez 2007). B cells produced by fetal liver and a minor fraction of in bone-marrow generated B cells must be selected by self-Ag recognition, whereupon they enter the distinctive B-1 cell pool. Positive selection via self-antigen recognition and strong BCR-signaling is essential for B-1 cell maturation. In contrast, most bone marrow B cells experience deleterious consequences upon self-antigen recognition at the newly formed stage, undergoing apoptosis or remaining blocked in a short-lived unresponsive stage or revising their BCR through light chain editing to eliminate the self-reactivity (negative selection). Expression of typical polyreactive B-1 type BCRs by these cells is usually non-productive. Cells with a reactivity intermediate between B-1a fetaltype and B-2 follicular-type BCRs may promote entry into the B-1b and MZ B cell pools, suggesting a hierarchy of selection. Recent adoptive transfers showed that bone marrow cells definitely are sufficient to generate B-1b cells, whereas B-1a cells exclusively are generated in the fetal liver (Alugupalli, Leong et al. 2004). Once developed and matured, B-1a cells populating the peritoneal cavity show self-renewing capacities.

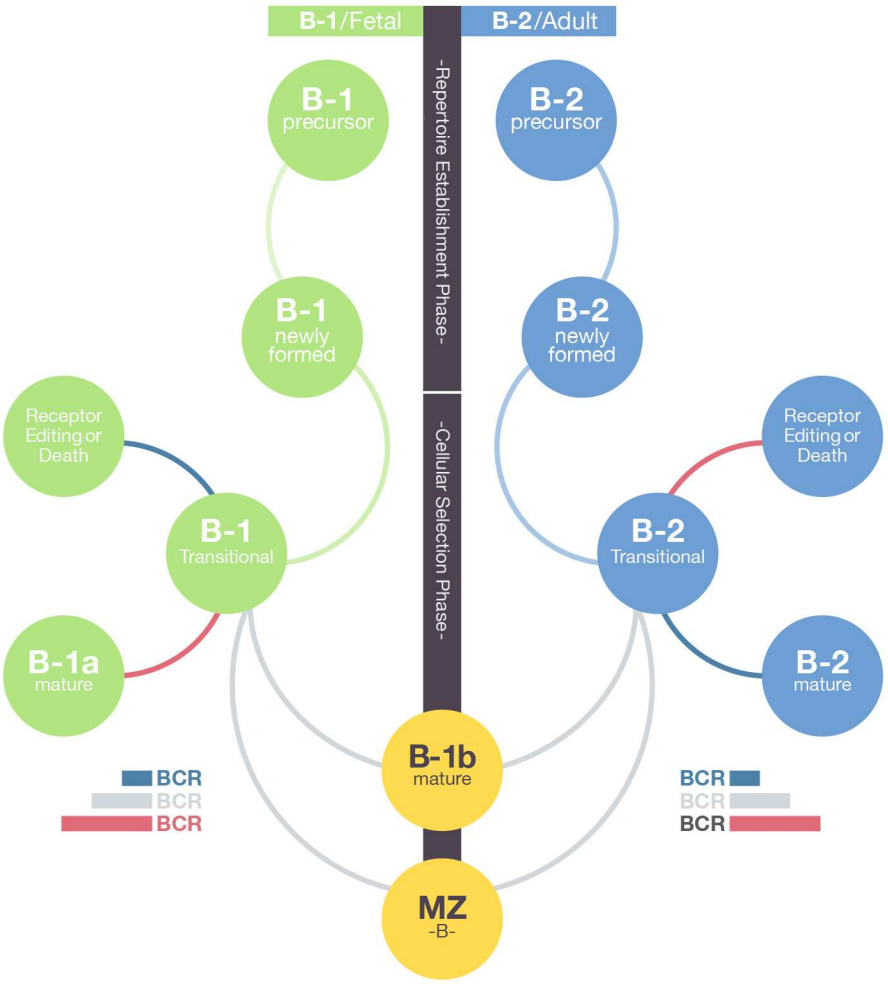

Figure 1.4 Unified model for B cell development. B-1 cell progenitors arise from fetal liver cells whereas B-2 cell or FO B cell progenitors are generated in the adult bone marrow. The development of B-1a cells requires positiv selection by self-antigen recognition and strong BCR-signaling (marked red). In contrast, development of B-2 or FO B cells is dependent on negative selection by low BCR-signaling (marked blue). Cells with a reactivity intermediate (marked grey) between B-1a fetaltype and B-2 follicular-type BCRs enter the B-1b and MZ B cell pools. Adapted from (Hardy 2006; Hardy 2006).

1.1.4.3 T cell dependent (TD) and T cell independent (TI) B cell activation

The recognition of antigens by the B cell receptor is not sufficient to activate naïve B cells. Either T cell dependent or T cell independent co-stimulation is necessary to initiate B cell proliferation, differentiation and Ig production. Protein antigens are T cell dependent (TD), which means that B cells recognizing these antigens require the help of CD4 T helper cells for maximal antibody production (McHeyzer-Williams, Malherbe et al. 2006). After binding to an antigen, BCR-antigen complexes

are internalized by the B cell, and are processed and presented on MHCII molecules to an activated T cell with the same specificity, respectively. Subsequently, CD40 on B cells binds to CD40L on activated T cells, which induces the secretion of B cell stimulatory cytokines (Jaiswal and Croft 1997). The interaction between CD40 and CD40L and the stimulation by cytokines induce the proliferation of the B cells (Burstein and Abbas 1991; Ruprecht and Lanzavecchia 2006). After activation, B cells can either migrate to extrafollicular sites and directly differentiate into short-lived plasma cells (Allen, Okada et al. 2007) or pass through a germinal center (GC) reaction in the lymphoid organs. Inside the germinal centers co-stimulatory signals of T cells initiate clonal expansion of activated B cells and class switch recombination is enforced (Klein and Dalla-Favera 2008). Another property of B cells passing through a germinal center reaction are somatic hypermutations in their BCR variable regions, altering or rather amplifying the affinity to their antigens (Jacob, Kelsoe et al. 1991; Berek 1993). Positive selection via antigen-recognizing on follicular dendritic cells (FDC) terminates GC reactions and selected B cells differentiate into long lived plasma or memory cells (Sze, Toellner et al. 2000; Allen, Okada et al. 2007; Klein and Dalla-Favera 2008).

Many bacterial and viral pathogens express antigens on their surface, which are able to activate B cells in the absence of CD4 T cell help. These antigens are classified as T cell-independent (TI) antigens. TI antigens fall into two major categories: The first category, referred to as TI type 2 are large antigens, such as polysaccharides exhibiting repeated antigenic epitopes which extensively cross-link multiple BCRs (El Shikh, El Sayed et al. 2009) and thereby activate B cells. The second category comprises the TI type 1 non-protein antigens containg TLR ligands such as lipopolysaccharides (LPS) or CpG. These antigens provide polyclonal activation after stimulation of TLR co-receptors. While LPS primarily stimulates TLR 2 and TLR 4, CpG mainly triggers TLR 7 and TLR 9 (Lanzavecchia and Sallusto 2007). All three different subsets of peripheral B cells express similar constellations of Toll like receptors (TLR). Follicular B cells can be induced to proliferate upon TLR stimulation, but unlike MZ B cells and peritoneal B-1 cells, follicular B cells lack the intrinsic ability to differentiate into antibody secreting plasma cells if stimulated only by TLR ligands (Allman and Pillai 2008). Hence, follicular or B-2 B cells mostly are activated dependent on CD4 T cell help, whereas activation of the innate like MZ B cells and peritoneal B-1 B cells is performed T cell independently.

1.1.4.4 Antibodies – the effector molecules of humoral immune responses

Differentiation of activated B cells into plasma cells ensures the generation of the humoral effector molecules, the immunoglobulins or antibodies. In general, antibodies are composed of a constant (Fc) and a variable (V) region. The variable region forms the highly specific antigen binding site, whereas the effector function of antibodies is mediated by their constant part. As IgM antibodies can be expressed without class switching, they are the first antibodies to be produced in a humoral immune response. Class switch recombination during germinal center reactions initiates the production of antibodies of other isotypes, such as IgGs, IgA or IgE. During class switch recombination different cytokines produced by T helper cells induce the switching to different antibody classes, for instance, as shown in mouse experiments, IL-4 promotes the switch to murine IgG1 and IgE and suppresses the production of IgG2a whereas TGF-β induces IgG2b and IgA and inhibits the switch to IgG3 and IgM and IFNγ favors the development of IgG2a antibodies (Snapper and Paul 1987; Stavnezer 1996; Stavnezer 1996). Antibodies exhibit various effector functions, while promoting the elimination of pathogens. In general, antibodies are capable of recognizing and opsonizing the invading pathogens, which results in the engulfment of pathogens by macrophages. Additionally, bound to their antigens, antibodies activate the complement cascade and promote antibody-dependent cell-mediated cytotoxicity (ADCC) via the activation of NK cells (Chan and Carter 2010). Among these effector capacities, every antibody isotype exhibits specialized functions. *In vivo*, IgM antibodies form polymeres, primarily pentamers, being linked with a protein called J-chain. Due to their pentameric structure, IgM antibodies are predestinated to activate the complement system whereas human IgG2 or IgA is not able to induce the complement cascade (Bjornson and Detmers 1995). IgE for instance is necessary to sensitize mast cells and IgG1 and IgG2 are the best isotypes for opsonization and clearance of pathogens (Ward and Ghetie 1995).

The effector function of antibodies is mainly dependent on the receptors on innate or adaptive target cells, which bind to their Fc regions (Fc receptors) (Nimmerjahn and Ravetch 2008; Nimmerjahn and Ravetch 2008). The family of Fc receptors recognizes antibodies of one or a few closely related isotypes through a recognition domain located on the α-chain of the Fc receptor. Until today, four classes of murine Fcγ-receptors, binding to IgGs (Nimmerjahn and Ravetch 2006), are known

as well as one Fc α/μ-receptor (bindung to IgA and IgM) (Shibuya and Honda 2006) and one Fcε-receptor (binding to IgE). The human Fc receptor system is more complex. Binding of antibody immune complexes to Fc receptors on target cells defines the course of the immune reaction. Most Fc receptors carry associated ITAM motifs, transducing pro-inflammatory activation signals. Binding of antibody-ICs to activating FcRs initiates signaling pathways starting with the tyrosine phosphorylation of the ITAMs by kinases of the SRC family. ITAM-phosphorylation and further signal transduction to downstream targets leads to the activation of effector cells, such as neutrophils, mast cells, macrophages or dendritic cells (Brownlie, Lawlor et al. 2008; Nimmerjahn and Ravetch 2008). Just one Fc receptor, the murine Fcγ receptor IIB and its human analogue, carries an ITIM motif, transmitting negative signals. B cells exclusively express the Fcγ receptor IIB. Complexed IgG triggers ITIMs and BCRs simultanously resulting in the interference of ITIM mediated signals with downstream pathways necessary for B cell activating. Accordingly, FcγRIIB functions as an important negative feedback regulator of the activating signals that are transmitted by the BCR (Nimmerjahn and Ravetch 2008).

Thus, immune complex binding to activating and inhibitory Fc receptors initiates the elimination of pathogens but simultanously controls the intensitiy of humoral immune response.

Antibodies, referred to as “natural antibodies” are an outstanding part of the humoral immune response. Natural antibodies are mainly of the IgM isotype and predominantly produced by peritoneal B-1 cells in the absence of apparent stimulation by specific foreign antigens, as “germ-free” mice also produce natural antibodies. Due to germline encoded V gene segments, natural antibodies show low affinities but broad specificites to both foreign and self structures (Boes 2000). As these antibodies are polyreactive they can bind to a particular antigen or pathogens, even if the host has never been exposed to it (Casali and Schettino 1996). Mice lacking natural antibodies showed an increased susceptibility to influenza infections and an increased mortality (Baumgarth, Herman et al. 2000). Because of their ability to recognize a variety of exogenous antigens, including those on bacteria and viruses, natural antibodies play a major role in the primary line of defense against infections. Thereby natural IgM may effect neutralisation of foreign organisms without necessarily recruiting the adaptive immune response by activation of the

complement cascade (Brown, Hussell et al. 2002). The participation of natural IgM in autoimmunity has been a subject of speculation for some time, but there is recent evidence that demonstrates a protective role of self-reactive IgM in autoimmune diseases (Manson, Mauri et al. 2005).

1.2 Autoimmunity

It is essential that all mentioned defence mechanisms are activated just upon the encounter with pathogenic structures. Therefore the immune system must be able to distinguish between self and non-self patterns (Goodnow 1996). During the adaptive lymphocyte maturation, central and peripheral tolerance mechanisms prevent the formation of self-reactive antigen-receptors (Goodnow, Adelstein et al. 1990). Self-antigen recognition by immature adaptive lymphocytes in bone-marrow or thymus results in either their deletion or in the revision of their receptor through L chain editing (Nemazee and Buerki 1989; Nemazee and Burki 1989). Mature lymphocyes encountering self-antigens once they have left the central lymphoid organs either undergo apoptosis or are pushed in the state of peripheral anergy due to weak signaling without co-stimulation (Nossal and Pike 1980).

Due to genetic dispositions and/or environmental factors the mechanisms eliminating or inhibiting self-reactive adaptive lymphocytes may sometimes not take effect, resulting in the development of self-reactive antibodies or self-reactive T effector cells and subsequently leads to the development of autoimmunity (Kotzin 1996). In the process of autoimmunity self-reactive antibodies and autoreactive T effector cells start to fight against the host`s own antigens, cells and tissues leading to tissue destruction and respectively to the manifestation of an autoimmmune disease. Autoimmune diseases can be broadly divided into systemic and organ-specific autoimmune disorders, depending on the principal pathologic features of each disease. Systemic autoimmune diseases include for example *SLE, Sjögren's syndrome, scleroderma and rheumatoid arthritis*. IgG autoantibodies specific to antigens, which are not tissue specific are associated with these disorders. Hence, organ-specific autoimmune diseases, such as *diabetes mellitus type 1* or *autoimmune haemolytic anaemia* are associated with tissue specific immune reactions.

Until today, the cause of autoimmune diseases is still not clear. Thus, nearly no causal therapies are available and patients still are treated symptomatically with anti-inflammatory and immune suppressive substances, such as glucocorticoids, high doses of intravenous IgG purified from human donors (IVIG) or the TNF-α blocker Infliximab, which is however associated with numerous side effects such as infections. Therefore, the development of therapies influencing the disease pattern specifically is fundamental.

1.2.1 Systemic Lupus Erythematosus (SLE)

SLE is a fairly heterogeneous disease predominantly affecting young women of reproductive age (Bertsias, Salmon et al. 2010) and its prevalence in northern Europe comprehends 40 cases per 100.000 persons (Rahman and Isenberg 2008). SLE can affect various organs such as joints, liver, heart and the nervous system, but kidney and skin are the organs studied the most. In many cases, SLE patients suffer from the classic *butterfly rash*, a characteristic facial erythema. The diagnosis of SLE can however be elusive as the course of disease shows great heterogeneity. As prognostic markers mainly autoreactive antibody titers are determined using serological tests. 80% of SLE patients are postive for anti-nuclear antibodies (ANA), measured by immune flourescence stainings. Nuclear antigens include DNA and RNA autoantigens as well as the ribonucleotide complex Ro and the RNA-binding protein La. Furthermore, antibody specificities to a subunit of the complement cascade, C1q , Fc parts of IgG antibodies (RF) and different phospholipids denotated as Smith-antigens (Sm) have been observed in SLE patients (Lefkowith and Gilkeson 1996). Anti- double stranded DNA antibodies are highly specific for SLE and directly reflect disease activity. As many SLE patients develop nephritis, another test routinely performed in suspected SLE is the renal function.

Besides environmental factors like UV-radiation, virus infections or drug-abuse, genetic predispositions are mentioned to cause SLE (Cooper, Gilbert et al. 2008). Genes linked to the development of SLE are for instance the CRP (C-reactive protein) gene, genes of the major histocompatibility complex, particularily HLA-A1, B8 and DR3, as well as genes of early complement components and the FcγRIIB gene (Rahman and Isenberg 2008). The typical course of disease in SLE patients includes

two different phases *(Fig 1.5):* The pre-clinical phase is characterized by the development of autoantibodies specific to nuclear antigens, whereas the clinical phase starts with the damage of the first organs and involves time periods of flares and intercepting periods of remission. Due to the continous treatment of SLE symptoms with systemic immune suppressive drugs, co-morbidities namely infections, artheriosclerosis and malignancies occur (Bertsias, Salmon et al. 2010).

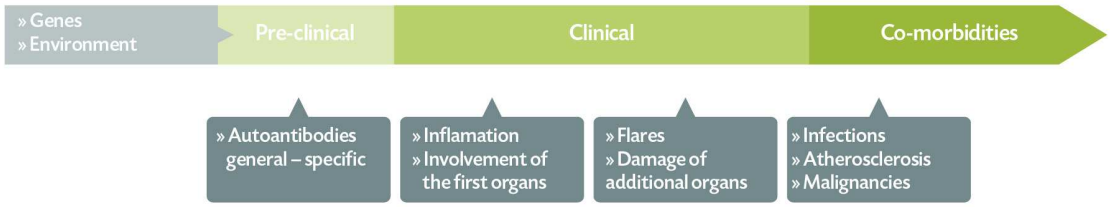

Figure 1.5 Course of disease in human systemic lupus erythematosus (SLE). SLE disease starts with a preclinical phase associated with the development of antibodies specific to nuclear antigens. The following clinical phase is characterized by organ inflammation and damage, involving time periods of active flares and remission. Late damage, namely infections, atherosclerosis and malignancies, is usually related to immunosuppressive therapy. Adapted from (Bertsias, Salmon et al. 2010)

1.2.2 The FcγRIIB$^{-/-}$ murine model of lupus

Analysis of the development of SLE using different mouse models contributes to the clarification of pathogenic mechanisms and particularily to the generation of future therapies. Mouse strains used for these analyses are exclusively models which show lupus-like glomerulonephritis due to the development of anti-nuclear IgG autoantibodies *(Table 1.2).*

The mouse model used in this work is based on the *knock out* of the only known inhibitory Fcγ receptor (FcγRIIB) in C57BL/6 wild type mice (Takai, Ono et al. 1996; Bolland and Ravetch 2000). Due to the missing of FcγRIIB in these mice, the negative regulation of several immune cell types such as dendritic cells and B cells is disturbed, leading to the development of autoreactive activated B cells and respective plasma cells, which continously produce autoreactive antibodies. Thus, FcγRIIB$^{-/-}$ mice are characterized by the production of high titers of anti-ds DNA and ANA IgG2c (IgG2a, haplotype b) and IgG2b autoantibodies. In general, FcγRIIB$^{-/-}$ mice start to produce autoreactive antibodies with the age of around 5 months. These

autoantibodies accumulate in the kidney, resulting in fatal glomerulonephritis. FcγRIIB$^{-/-}$ mice show spontaneous disease manifestation, which means, that not every FcγRIIB$^{-/-}$ mouse is affected.

Table 1.2: Common murine models of SLE

Name	Characterization
NZBxNZW F1	F1 generation of New Zealand Black (NZB) and New Zealand White (NZW)
Mrl-*Fas^{lpr}*	MRL mice homozygous for the allele of lymphoproliferation of *Fas (lpr)*
BXSB	*yaa* gene on the Y chromosome *(y linked autoimmune acceleretor)*
FcγRIIB$^{-/-}$	FcγRIIB$^{-/-}$ *knock out* on C57BL/6 background

1.2.3 IgG and IgM autoantibodies and SLE

More than 50 years ago, autoantibodies were eluted from kidney biopsis from SLE patients for the first time (Koffler, Schur et al. 1967). Today it is known that in approximately 60–80% of SLE patients, anti-dsDNA antibodies can be found, depending on the test used for detection: *Crithidia luciliae* immuno fluorescence, Farr assay or ELISA (Forger, Matthias et al. 2004; Bertsias, Salmon et al. 2010). The high affinities of these autoreactive antibodies indicate the involvement of germinal center reactions, somatic hypermutations and therefore activation by T cells in their development (Kotzin 1996). So far people assume that antibody immune complexes which accumulate in the kidney mediate tissue destruction by either activating the complement cascade or directly activating inflammatory effector cells via the binding to Fcγ receptors (Yung and Chan 2008). In humans autoantibodies of the IgG1 and IgG3 isotypes are associated with SLE, as these isotypes show the highest affinities to activating Fcγ receptors. In mice the analog isotypes IgG2b and IgG2c are linked with organ damage (Giorgini, Brown et al. 2008).

In contrast to the pathogenic role of autoreactive IgG in the development of SLE and glomerulonephritis, autoreactive IgM antibodies do not correlate with disease activity or specific clinical symptoms. Unlike IgG autoantibodies, self-reactive IgM antibodies mostly exhibit low affinities to their antigens. Clinical studies even ascribe a protective role to autoreactive IgM as SLE patients with a ratio of autoreactive IgG/IgM beneath 0.8 are unlikely to be affected by glomerulonephritis

(Forger, Matthias et al. 2004). In the NZB/NZW F1 mouse model, prophylactic and therapeutic application of monoclonal DNA-reactive IgM antibodies delayed disease onset (Werwitzke, Trick et al. 2005). The idea of a protecting role of autoreactive IgM in SLE is also supported by the study of a murine *knock out* model of lupus (MRL/*lpr*) that does not secrete IgM. Lacking IgM antibodies, MRL/*lpr* mice showed accelerated development of IgG autoantibodies against dsDNA and histones, more severe glomerulonephritis, more abundant glomerular immune complex deposition, and a shortened lifespan (Boes, Schmidt et al. 2000). However, it is still not clear, how self-reactive IgM antibodies influence lupus disease and which cells contribute to their generation.

1.2.4 B-1 cells and SLE

Antibodies produced by peritoneal B-1 cells are mainly poly- and self-reactive.Thus, besides their capacity in contributing to the first line of defense against pathogens, a role of B-1 cells in autoimmune pathogenesis has been postulated. As already mentioned, the B-1 cell population is typically subdivided into $CD5^+$ $B220^{dull}$ B-1a cells and $CD5^-$ $B220^+$ B-1b (Stall, Adams et al. 1992). Most of the well-known studies, concerning B-1 cells and autoimmunity have targeted either the whole B-1 population or specifically B-1a cells, whereas the function of B-1b cells is nearly unidentified (Duan and Morel 2006). Whereas the few available studies link B-1b cells with the mediation of tolerance (De Lorenzo, Brito et al. 2007; Shimizu, Kawahara et al. 2007), there are indeed examples, in both human and mice, of an association between autoimmune diseases like SLE and B-1a cells (Dauphinee, Tovar et al. 1988). The most compelling evidence for the involvement of B-1a cells in murine lupus, is that the deletion of the B-1a population by hypotonic shock reduces disease severity in NZB/NZW F1 mice (Murakami, Yoshioka et al. 1995). But not consistent with these studies, B-1a cells in FAS-deficient models do not contribute to autoantibody production and disease progression (Reap, Sobel et al. 1993). Furthermore over-expression of IL-5 in the NZB/NZW F1 model greatly increases the number of B-1a cells, but significantly reduces IgM and IgG anti-dsDNA antibody production and incidence of nephritis (Wen, Zhang et al. 2004). Recently it has been postulated that B-1a cell homing to the peritoneal cavity in aged lupus prone mice is abberant, and thus B-1a cells migrate to target organs like the

kidney (Ishikawa and Matsushima 2007) where they switch to IgG producing cells and presumably secrete autoantibodies and contribute to the local and systemic pathogenesis (Enghard, Humrich et al. 2010). However, the definite role of B-1a cells in the generation of autoantibodies in SLE is still not clear. Furthermore, it remains to be investigated whether B-1b cells are involved in the development of SLE.

1.2.5 T helper 17 cells and SLE

Extensive work has uncovered the existence of a novel T helper cell effector, the Th17 subset, whose deregulation plays a crucial role in the development of a wide range of autoimmune disorders such as *rheumatoid arthritis* and *multiple sclerosis* (Pernis 2009). In general, Th17 responses are critical for mucosal and epithelial host defense against extracellular bacteria, fungi and also viruses and parasites (van de Veerdonk, Gresnigt et al. 2009). Th17 cells are thereby characterized by the production of the cytokine IL-17, which is important for neutrophil recruitment and infiltration and their pro-inflammatory phenotype is additionally aggravated by the generation of IL-21, IL-22 and IL-23.

Recent evidence indicates that Th17 cells also play a role in the pathogenesis of SLE. SLE patients exhibit higher serum levels of IL-17 and IL-23 than healthy controls and the frequency of IL-17-producing T cells is increased in peripheral blood of SLE patients, correlating with active flares (Crispin, Oukka et al. 2008; Garrett-Sinha, John et al. 2008; Wong, Lit et al. 2008). Furthermore, IL-6 expression, which is necessary for the differentiation of naïve T cells into the Th17 subset, seems to be increased in SLE patients (Dong, Ye et al. 2003; Decker, Kotter et al. 2006).

In line with the observations in SLE patients, several murine models of SLE indicate a pathogenic role of Th17 cells (Nalbandian, Crispin et al. 2009) *(Table 1.3).* For instance, BXD2 mice show high IL-17 levels in serum as well as increased numbers of IL-17$^+$ cells in the spleen. IL-17 overexpression in these mice results in enhanced disease progression whereas the blocking of IL-17 receptor signaling reduced its intensity (Hsu, Yang et al. 2008). Besides their effector function in promoting inflammation through the secretion of pro-inflammatory cytokines like IL-17, Th17 cells also seem to have stimulatory effects on B cells via IL-21 production, inducing germinal center formation and autoantibody production (Hsu, Yang et al. 2008; Mitsdoerffer, Lee et al. 2010). Thus, the exact place of Th17 cells within the

mechanisms that lead to SLE remains unclear. Further investigation of Th17 cell regulation in SLE is essential as Th17 cells display an interesting target for the treatment of lupus disease.

Table 1.3: IL-17 in murine models of SLE

Murine model	Experimental evidence and outcomes	References
MRL/*lpr*	Enhanced IL-17-mediated tissue injury after ischaemia reperfusion	(Edgerton, Crispin et al. 2009)
BXD2	Increased numbers of IL-17-producing T cells provide help to B cells and stimulate spleen germinal center formation; IL-17 over-expression enhanced disease; IL-17R blockade reduced its intensity.	(Hsu, Zhou et al. 2006)
FcγRIIB$^{-/-}$	Increased numbers of IL-17 producing T cells in peripheral blood, LNs and spleens of diseased mice	M.Mertes, unpublished data

1.2.6 Toll-like receptor 9 and SLE

As mentioned before Toll-like receptors (TLR) are a family of pattern recognition receptors (PRRs). TLRs are widely expressed in the innate and also adaptive immune system. Stimulation of TLR by pathogen-associated molecular patterns (PAMPs) is an important prerequisite for either the induction of innate immune responses to eliminate infiltrated pathogens or the activation of B cells (Leadbetter, Rifkin et al. 2002; Ruprecht and Lanzavecchia 2006). Activation of autoreactive B cells via TLR co-stimulation favors the development of antibodies specific to various antigenic structures, as TLR antigen recognition is diverse. Co-stimulation with TLR7 for instance results in the development of antibodies specific to RNA associated antigens (Pisitkun, Deane et al. 2006) whereas TLR9 binds to CpG-rich DNA containing antigens such as nucleosomes, inducing the production of antibodies specific to DNA (Leadbetter, Rifkin et al. 2002; Christensen, Shupe et al. 2006; Ehlers, Fukuyama et al. 2006; Fischer and Ehlers 2008).

However, loss of TLR9 is associated with immune complex deposition in the kidney, diminished renal function and enhanced disease progression in virtually all lupus

prone mouse models indicating a tolerogenic role of TLR9 in SLE (Lartigue, Courville et al. 2006; Wu and Peng 2006). As the lack of MyD88 signaling blocks renal disease and autoantibody production in general, diverse speculations on the involvement of other TLR in pathogenic autoantibody production circulate (Ehlers and Ravetch 2007). Here TLR7 is considered as the most promising candidate, as TLR7 deficiency has been shown to decrease markers of T and B cell activation and the mortality rate in lupus-prone MRL-*Fas*^{lpr} mice (Christensen and Shlomchik 2007; Ehlers and Ravetch 2007; Santiago-Raber, Dunand-Sauthier et al. 2010). In this regard, TLR9 seems to keep TLR7 mediated pathogenicity in balance. Otherwise, recent investigations demonstrate that TLR9 mediated tolerance in B cells is dependent on the production of IL-10 (Lampropoulou, Hoehlig et al. 2008). However, precise mechanisms mediating exacerbated clinical disease in lupus-prone mice deficient in TLR9 are unclear.

1.3 Aims of this thesis

Spontaneous models of murine lupus have been characterized by the development of pathogenic antibodies specific to nuclear antigens and their subsequent accumulation in target organs like renal tissues resulting in fatal glomerulonephritis. Autoreactive B cells have been shown to be activated by the co-stimulation of BCR and innate Toll-like receptors. Thus, Toll-like receptor 9 (TLR9), which specifically binds to bacterial DNA, was associated with the generation of pathogenic DNA-reactive IgG antibodies for a long time. However, the lack of Toll-like receptor 9 in various murine models of lupus evokes the impairment of lupus disease. Hence, TLR9 signaling is not only capable of inducing autoimmunity but might also regulate tolerance to self-antigens. The tolerance mechanism induced by TLR9 stimulation in murine lupus is a widely discussed topic and it still remains to be answered how TLR9 mediates tolerance.

The purpose of this study was to determine the role of TLR9 stimulation and signaling in the murine FcγRIIB$^{-/-}$ lupus mouse model. Thereby, the impact of TLR9 signaling on self-reactive IgG and IgM antibody generation, pro-inflammatory T cell responses and the B-1 cell population should be investigated.

In particular, we aimed to identify liable cells and effector molecules hiding behind the TLR9 mediated tolerance mechanism in the FcγRIIB$^{-/-}$ lupus mouse model.

2 Materials and Methods

2.1 Mice

C57BL/6 mice were purchased from Charles River Laboratories. FcγRIIB$^{-/-}$ mice have been described previously (Bolland and Ravetch 2000; Ehlers, Fukuyama et al. 2006). TLR9$^{-/-}$ (Hemmi, Takeuchi et al. 2000) and µs (Ehrenstein, O'Keefe et al. 1998) mice were crossed with FcγRIIB$^{-/-}$ mice to produce FcγRIIB$^{-/-}$TLR9$^{-/-}$ and FcγRIIB$^{-/-}$ µs double-deficient mice. TCRβ$^{-/-}$ mice were purchased from the Jackson Laboratories (#003288) and crossed with FcγRIIB$^{-/-}$ mice to produce FcγRIIB$^{-/-}$ TCRβ$^{-/-}$ mice. All mice were backcrossed for at least eight generations to the C57BL/6 background. Mice were bred and maintained in accordance with institutional guidelines. Adequate animal housing, under SPF conditions, took place at the animal facility of the DRFZ or the MPI for Infectionbiology in Berlin, Marienfelde. The feeding took place *ad libitum.*

2.2 Materials

2.2.1 Technical devices

Used technical devices are listed in table *2.1*.

2.2.2 Consumables

All used consumables are summed up in table *2.2*.

2.2.3 Chemicals

All chemicals used in this work are listed in table *2.3*.

Table 2.1: Technical Devices

Device	Name	Supplier
Centrifuge	Biofuge	Heraeus
Centrifuge	Biofuge fresco	Heraeus
Centrifuge	Multifuge 3 L-R	Heraeus
Cytometer	FACS Calibur	BD Biosciences
Confocal microscope	LSM710	Carl Zeiss AG
Dri-Block Heater	DB-2A	Techne
Electrophoresis System	A2 Gator OWL	VWR
Fluorescence Reader	Tecan SpektraFluor	Tecan
Incubator	Function Line	Heraeus
Microscope	DM 4000B	Leica
Microscope	DM IRE2	Leica
Microtom	Kryostat Microm HM 500 OM	Microm
Pipet	Pipet Discovery	Abimed
Pipet	Pipet Boy	Eppendorf
Shaker	MACS Tube Rotator	Miltenyi Biotech
Shaker	Thermomixer comfort	Eppendorf
Shaker	Vortex genie 2	Scientific Industries
Spektrophotometer	NanoDrop 1000	Thermo Fisher Scientific
Spektrophotometer	VersaMax	Bucher
Thermocycler	T3	Biometra
UV Transilluminator	Vilber Lourmat	Vilber
Vertical gel electrophoresis	Protean3	Biorad

Table 2.2: Consumables

Name	Supplier
30µm Pre-Separation filters	Miltenyi Biotec
FACS tubes	Sarstedt
96well plates (flat bottom)	Costar
96well plates (V bottom)	Costar
0.2ml PCR tubes	Peqlab
1.5ml tubes	Sarstedt
Serum tubes	Greiner
Urin teststrips	Macherey & Nagel
Cryomold Standard histocassettes	Tissue-Tek
Microscope Slides	Menzel-Gläser
Super Pap Pen	Beckman Coulter
20 µm filters	B. Braun
1 ml single use syringes	B. Braun
27G 3/4 disposable needles	B. Braun
Bio-spin chromatography column	BioRad
50 kDa centrifugal filter devices	Millipore

Table 2.3: Chemicals

Name	Supplier	Name	Supplier
Acetone	Roth	$MgCl_2$	Sigma-Aldrich
Acryl-bisacrylamide	Sigma-Aldrich	Na_2HPO_4	Sigma-Aldrich
APS	Carl-Roth	$NaCH_2COOH$	Sigma-Aldrich
Agarose	Invitrogen	NaCl	Sigma-Aldrich
BSA	Sigma-Aldrich	NaH_2PO_4	Sigma-Aldrich
Carbonate-Bicarbonate	Fluka	$NaHCO_3$	Sigma-Aldrich
CNBr-Sepharose	GE-Healthcare	NH_4Cl	Sigma-Aldrich
Cytofix/Cytoperm	BD Biosciences	OCT Medium	Tissue Tek
EDTA	Sigma-Aldrich	PCR-nucleotides	Invitrogen
Acetic Acid	Roth	PCR-Buffer	Genexpress
Ethanol	Roth	Perm/Wash	BD Biosciences
Ethidium-Bromide	Roth	Protein G Sepharose	GE-Healthcare
Fluoromount G	SouthernBiotec	PEI	Sigma-Aldrich
Gelatine	Sigma-Aldrich	SDS	Merck
H_2SO_4	Roth	TEMED	Invitrogen
HCl	Roth	TMB	BD Biosciences
Isopropanol	Roth	Tris-HCl	Sigma-Aldrich
KCl	Sigma-Aldrich	Tween20	Roth
$KHCO_3$	Roth		

2.2.4 Buffers, solutions and media

All used buffers, solutions and media are listed in table *2.4.*

2.2.5 Kits

The kits listed in table *2.5* were applied according to the manufacturer's instructions.

2.2.6 Enzymes, TLR agonists and other biological derivatives

All used enzymes, TLR agonists and other biological derivatives are summed up in table *2.6.*

Table 2.4: Buffers, solutions and media

Name	Composition
Acetic Washing buffer	0,1 M $NaCH_2COOH$, 0,5 M NaCl, pH 4,0
Block/ wash buffer	PBS; 3% BSA; 1mM EDTA, 0.1% gelatine
Coating buffer	0.05 M Carbonate-bicarbonate, pH 9.6
Coomassie destaining solution	5% methanol, 5% acetic acid
Coomassie staining solution	1% Coomassie brilliant blue R250 (Serva), 50% methanol, 10% acetic acid
Coupling buffer	0.1 M $NaHCO_3$, 0.5 M NaCl
Elution buffer	0.1 M glycine
PBS	137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, pH 7.2
PBS/ BSA	PBS, 0.5% BSA
Protein sample buffer (4x)	240 mM Tris/HCl pH 6.8, 8% SDS, 5% 2-mercaptoethanol, 0.04% bromphenol blue; 40% Glycerol
Lysis buffer	0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2
Neutral washing buffer	0.1 M Tris/HCl, 0.5 M NaCl, pH 8.0
Phosphate buffer	20 mM Na_2HPO_4, 20 mM NaH_2PO_4, pH 7.0
SDS-PAGE buffer (10x)	250 mM Tris, 1.92 M glycine, 1% SDS, pH 8.3
SDS resolving gel buffer	1.5 M Tris/HCl pH 8.8, 0.4% SDS
SDS stacking gel buffer	1 M Tris/HCl pH 6.8, 0.8% SDS
DMEM GlutaMAX-I (Gibco/Invitrogen)	DMEM Glutamax, 10% FCS; 20 µM 2-mercaptoethanol,100 U/l Penicillin, 100 U/l Streptomycin
RPMI GlutaMAX-I (Gibco/Invitrogen)	RPMI 1640 Glutamax, 10% FCS, 20 µM 2-mercaptoethanol, 100 U/l Penicillin, 100 U/l Streptomycin
LB Media Capsules	purchased from MPBIO
TAE (50*x*)	2 M Tris-HCl, 57% Acetic Acid, 100 mM EDTA, pH 8,0
TE-Puffer	10 mM Tris-HCl pH 8,0, 1 mM EDTA pH 8,0
Proteinase K buffer	100 mM Tris-HCl pH 8,5, 5 mM EDTA pH 8,0, 0,2% SDS, 200 mM NaCl

Table 2.5: Kits

Name	Supplier
anti-Nucleosome-ELISA	Orgentec
ANA-detect-ELISA	Orgentec
anti- RNP- ELISA	Orgentec
IL6 ELISA kit	BD Bioscience
IL10 ELISA kit	BD Bioscience
NucleoBond Xtra Maxi	Macherey-Nagel
QIAprep Spin Miniprep-Kit	Quiagen

Table 2.6: Enzymes, TLR agonists and other biological derivatives

Name	Supplier
e.coli Top10 cells	Invitrogen
Penicillin	Seromed
Streptomycin	Seromed
Ampicillin	Sigma
Taq	Genexpress
T4 DNA-Ligase	NEB
Sal I	NEB
BsiW I	NEB
Hind III	NEB
CIP	NEB
Proteinase K	Sigma
FCS	Sigma
Primatone RL	MP Biomedicals
Imiquimod	Invivogen
CpG ODN 1826	Invivogen
e.coli DNA	Sigma-Aldrich
LPS	Sigma-Aldrich
Insulin	Sigma-Aldrich
Heparin	Biochrome KG
PMA	Sigma-Aldrich
Ionomycin	Sigma-Aldrich
Brefeldin A	Sigma-Aldrich
Trypsin	Sigma-Aldrich
Reference Sera	Bethyl
Kaleidoscope protein marker	BioRad

2.2.7 Cells

The only cell line used in this work was a human embryonic kidney cell line stably expressing the large T-antigen of SV40 (HEK 293T) which was supplied by the DRFZ House Facility.

2.2.8 Primer

All primers used for genotyping or cloning of monoclonal antibodies are listed in table *2.7* and *2.8*.

Table 2.7: Primer sequences for genotyping

Name	Sequence 5` → 3`
R2b-wt-fw	ATC TTC CAA AGG CTG TGG TC
R2b-ko-rev	CTC GTG CTT TAC GGT ATC GCC
R2b-rev	TTG ACT GTG GCC TTA AAC GTG TAG
TLR9-fw	CAT GGC CTG GTG GAC TGC AA
TLR9-wt-rev	TGA AGA GAA CGC GCA GG
TLR9-ko-rev	ATC GCC TTC TAT CGC CTT CTT GAC GAG
actin-fw	GAA CCC TAA GGC CAA CCG T
actin-rev	CAC GCA CGA TTT CCC TCT C
µS-wt-fw	TCTGCCTTCACCACAGAAG
µS-wt-rev	TAGCATGGTCAATAGCAGG

Table 2.8: Primer sequences for cloning of monoclonal antibodies

Name	Sequence 5` → 3`
Murine IgM HC constant region fw	CCT CGC GTC GAC GTC CTT CCC AAA TGT CTT CCC CCT
Murine IgM HC constant region rev	TTA TTC GGC GTA CGC GTC AATA GCA GGT GCC GCC TGT G
Murine kappa LC constant region fw	CCT CGC CGT ACG GAT GCT GCA CCA ACT GTA TCC ATC
Murine kappa LC constant region rev	TTA TTC GGA AGC TTT CAA CAC TCA TTC CTG TTG AAG

2.2.9 Plasmids

The plasmids that were used are listed in table *2.9*.

Table 2.9: Plasmids

Name	Reference
ED38 heavy and light chain	(Meffre, Schaefer et al. 2004)
mgO53 heavy and light chain	(Wardemann, Yurasov et al. 2003)
1RIIgc7 heavy and light chain	Tiller et al. 2010, JEM in press

2.2.10 Antibodies

All used antibodies were either produced by the DRFZ in-house facility or purchased from commercial suppliers and are listed in table *2.10* and *2.11*.

Table 2.10: Conjugated Antibodies

Specificity	Clone	Conjugate	Supplier
mouse CD4	YTS191	FITC	House facility
mouse CD4	RM4-5	PerCp	BD Bioscience
mouse CD4	RM4-5	Alexa 546	House Facility
mouse CD138	N418	Pe	BD Bioscience
mouse IL17	TC11-18H10	Pe	BD Bioscience
mouse IL17	TC11-18H10	FITC	BD Bioscience
mouse FoxP3	FJK-16s	Pe	BD Bioscience
mouse IFN-g	AN18.17.24	Pe	BD Bioscience
mouse MacI	M1/70.15.11	FITC	House Facility
mouse MacI	M1/70.15.11	Cy5	House Facility
mouse macrophage marker	F4/80	Cy5	House Facilty
mouse MHCII	M5/114	Cy5	House facility
mouse IgM	M41	Cy5	House Facility
mouse CD44	IM7	FITC	House Facility
mouse CD62L	MEL14	Pe	House Facility
mouse CD21	7G6	FITC	House Facility
mouse CD23	B3/B4	Pe	House Facility
mouse CD5	19-3	Pe	BD
mouse CXCR3	220803	APC	R&D Systems
mouse IgG2c	polyclonal goat	FITC	Bethyl
mouse IgM	polyclonal goat	HRP	Bethyl
mouse IgG-Fc	polyclonal goat	HRP	Bethyl
mouse IgG2c	polyclonal goat	HRP	Bethyl
mouse IgG2b	polyclonal goat	HRP	Bethyl

Table 2.11: Unconjugated Antibodies

Specificity	Clone	Supplier
mouse CD21	7G6	House Facility
mouse IgM	M41	House facility
mouse IgM	polyclonal goat	Bethyl
mouse IgG2c	polyclonal goat	Bethyl
mouse IgG2b	polyclonal goat	Bethyl
mouse IgG-Fc	polyclonal goat	Bethyl
human total IgG	polyclonal	Preparation

2.2.11 Software

The software programs used in this work are listed in table *2.12.*

Table 2.12: Software

Name	Supplier
FlowJo	Tree Star
GraphPad Prism 4.0	GraphPad Software
ND1000	Thermo Fisher Scientific
Softmax PRO v3.0	Molecular Devices
Leica Confocal	Leica
Zen 2009	Zeiss

2.3 Methods

2.3.1 Molecular methods

2.3.1.1 DNA Modifications

Standard DNA-technics were performed as described previously (Maniatis et al. 1982). Polymerase Chain reactions were carried out according to Mullis *et al.* (Mullis, Faloona et al. 1986). *In vitro* modification of DNA samples were performed according to recommended conditions of the supplier. Linearized vectors were treated with alcalic phosphatase (CIP) to avoid religation.

2.3.1.2 Transformation and plasmid isolation

50µl of competent *E. coli* Top10 cells (in house preparation) were transformed at 42 °C with 1µl of plasmid DNA or 5µl ligation products, respectively. Subsequently, 500 µl of LB-medium without any antiobiotics was added and the culture was gently rotated at 37°C for 45 minutes. Following over-night incubation of transformed *E.coli*

cells was performed in fluid LB medium (3ml) containing 50 µg/ml ampicillin (Sigma) or in case of ligation products on LB agar plates containing 50 µg/ml ampicillin for 16h at 37°C. Single bacterial clones were picked from LB plates and transferred to fluid LB media and incubated again for 16h at 37°C. Plasmid DNA was isolated from fluid cultures using the QIAprep Spin Miniprep-Kit. For large scale plasmid purification the NucleoBond Xtra Maxi Kit was used.

2.3.1.3 Quantification of DNA

By photometric measurement of the optical densitiy at 260 and 280 nm wavelength the concentration and the purity of an aqueous nucleic acid solution can be determined. With the help of a Nanodrop (Peqlab) the extinction at 260 nm (OD_{260nm}) and 280 nm (OD_{280nm}) wavelength was measured. The TE buffer used for elution was used as reference. The quotient OD_{260nm}/OD_{280nm} is an indication for the purity of the nucleic acid solution. At high purity a value between 1.8 and 2.0 is reached. Lower values suggest a contamination with proteins.

2.3.1.4 Gelelectrophoresis

Gelelectrophoresis was used for the separation of DNA fragments. Therefore gels with 1-2% Agarose in 1x TAE buffer, containing 1 µg/ml Ethidiumbromide (EtBr) (Carl-Roth, Karlsruhe, Germany) were prepared. DNA samples were mixed with 6x DNA loading buffer (Orange G) and loaded on the gel, which was run with 5-10V/cm in 1x TAE buffer. To determine the length of the separated DNA fragments, a molecular weight marker (100 bp DNA - Ladder Plus marker; Fermentas) was run simultaneously on the gel. Separated DNA was documented by UV radiation at 312 nm wavelength and a video supported system. If necessary, DNA fragments were seized from the agarose gel and purified with the Nucleospin Extract II kit (Machery Nagel) according to the manufacturer`s recommendations.

2.3.1.5 Murine DNA Isolation

Tail biopsys were lysed for 4h at 55°C with 200µg/ml Proteinase K in 500µl Proteinase K buffer. After centrifugation 500µl Isopropanol was added. Precipitated DNA was transferred into 150µl TE buffer and incubated 10 minutes at 55°C.

2.3.1.6 Cloning of monoclonal murine IgM antibodies

The human IgG1 and kappa constant regions in the heavy and light chain expression vectors of the monoclonal self- and polyreactive antibodies ED38 (Meffre, Schaefer et al. 2004) and 1RIIgc7 (Tiller et al. 2010, JEM in press) and the control antibody mgo53 (Wardemann, Yurasov et al. 2003) were exchanged for the murine IgM heavy chain (SalI-BsiWI) and murine kappa light chain (BsiWI-HindIII) constant regions. The murine IgM heavy chain and the murine kappa light chain were amplified by PCR from a generated C57BL/6 splenic cDNA (see table *2.8*: Primer). PCR products of the expected size were sequenced to exclude incorrectness. The leader sequences of the original expression vectors were used (Wardemann, Yurasov et al. 2003).

2.3.2 Expression and Purification of antibodies

2.3.2.1 Cultivation of human embryonic kidney cells (HEK 293T)

HEK 293T Cells were cultured in DMDM high glucose medium (Invitrogen) supplemented with 10% FCS and 1% penicillin/streptomycin (PS) at 37°C and 5% CO_2 in 145 mm tissue plates. Optimal growth was achieved by passaging the cells twice a week at a confluency of 80%. Cells were trypsinised with 1ml of trypsin-EDTA (PAA) after removal of the medium and washing with 15ml 1xPBS and distributed to new culture dishes at a ratio of 1:3 – 1:10.

2.3.2.2 Production of monoclonal IgM antibodies

Recombinant monoclonal IgM antibodies were produced by polyethylenimine-mediated co-transfection of HEK 293T cells with the respective IgH and IgL chain encoding plasmid DNA (*2.3.1.6*). For transfection, cells were grown at a confluency

of 80% washed with PBS and cultured in 25ml DMEM high glucose containing 1% Primatone RL (MP Biomedicals) and 1% PS. 10µg of heavy chain and 10µg of light chain encoding plasmid DNA in 3ml PBS were mixed with polyethylenimine (PEI) (Sigma-Aldrich) in a ratio of 1:3 and incubated for 10 minutes at room temperature. Positively charged PEI forms complexes with negatively charged DNA, which can be taken up by the cells when the transfection mix is carefully added to the transfection medium on the cell culture dish. The cell medium supernatant was harvested after 4-6 days of incubation at 37°C and 5% CO_2.

2.3.2.3 Preparation of a murine IgM Purification Column

For purification of IgM antibodies an anti-murine IgM antibody (clone M41) was coupled to CnBr-activated Sepharose 4B (GE-Healthcare). In brief, the CnBr activated Sepharose was swelled in 1 mM HCl for 15 minutes. After washing with 200ml 1 mM HCl, the CnBr activated Sepharose was incubated with 1-2 mg anti-murine IgM antibody per ml Coupling Solution over night at 4°C. To block remaining unreacted groups, the CNBr-activated Sepharose, was washed with 0.1 M Tris-HCl pH8 and rotated at 4°C for 2 hr. After blocking was completed, the CnBr activated Sepharose was washed three times with alternating acidic and neutral washing buffer. The IgM Sepharose Column was stored at 4°C in 20% Ethanol in PBS.

2.3.2.4 Purification of monoclonal IgM antibodies

Harvested supernatant containing the secreted antibodies was centrifuged 10min at 4000rpm to remove cell contamination followed by sterile filtration in a Stericup® (Millipore). Clear sterile supernatant was run through the prepared murine IgM Purification Column. Binding to the coupled anti-IgM antibodies, retained IgM antibodies. After removing unspecific proteins, which have bound to the column by washing with PBS, specific IgM molecules were eluted with 0.1 M glycine pH 2.7. Collected fractions were neutralised in 1/10 volume of 1 M Tris-HCl pH 9.0 and dialysed against PBS over night at 4°C. Determination of the antibody concentration was done photometrically at 280 nm using Nanodrop spectrophotometer. If necessary, the IgM molecules were concentrated by a centrifugation device with a MWCO of 50 kDa. Concentrations were confirmed by IgM ELISA, self-reactivity was

verified by ELISA and antibody integrity was analyzed by SDS-PAGE. The IgM molecules were stored at -20°C.

2.3.3 Mouse experimental methods

2.3.3.1 Blood Sampling

Blood samples were taken by carefully scarifying the tail vein using a scalpel. To examine antibody levels and isotype distribution in serum of mice, 200µl blood was collected in a serum collection centrifugation tube (Greiner Bio-One). Tubes were centrifuged at 6000rpm for 10min. Serum was transferred to a 1,5ml tube and stored at -80°C. For further processing peripheral lymphocytes from blood samples, blood was collected in tubes containing 5µl of heparin.

2.3.3.2 Removal of murine organs and preparation of cells

Mice were sacrificed by cervical dislocation. Peritoneal wash out cells were obtained by injecting 3-6ml PBS/BSA into the peritoneal cavity. Skin and subcutaneous tissues were seperated from the fascia and the dissolved peritoneal cells were aspirated by using a syringe. After opening the abdominal cavity spleen and lymphnodes (inguinal, mesenteric and lumbal) were isolated and stored in 1 ml PBS/BSA at 4°C until further processed. For subsequent lymphocyte isolation, organs were homogenised in PBS/BSA. Cells were treated with lysis buffer to remove erythrocytes from the suspension. Lysis was stopped after 5 minutes with PBS/BSA and cells were filtrated by using a 30 µm preseparation filter.
Blood samples required repeated treatment with lysis buffer. Finally cells were washed with PBS/BSA and resuspended in the appropriate volume of PBS/BSA or medium.

2.3.3.3 Application of monoclonal IgM antibodies

200µg of the recombinant monoclonal IgM antibodies were i.p. injected twice a week into FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice starting at the age of 3.5 months. Alternatively, 200µg

of the recombinant monoclonal IgM antibodies were injected i.p. once into mice at the age of 5-6 months.

2.3.3.4 Depletion of Marginal Zone B cells

To deplete the Marginal Zone B cell population, FcγRIIB$^{-/-}$ mice were treated with 4µg of the anti-CD21 (clone 7G6) antibody. Anti- CD21 antibody was injected i.p. at day 0 and 8 (Whipple, Shanahan et al. 2004).

2.3.3.5 Transfer of peritoneal cells

Peritoneal cells were obtained as described in 2.3.3.2. Peritoneal cells were washed with PBS/BSA and 0.5-1.5 x 10^7 donor cells per recipient mouse were injected i.p every other week until the age of 9 months starting at the age of 3.5-4 months. Alternatively 1 x 10^7 total peritoneal cells were injected i.p. once into recipient mice at the age of 5-6 months.

2.3.3.6 Transfer of sorted peritoneal B-1b cells

Peritoneal cells were obtained as described in *2.3.3.2.* After washing with PBS/BSA, peritoneal cells were stained with anti murine IgM-Cy5, anti murine MacI-FITC, anti murine B220-PerCP and anti murine CD5-Pe and subsequently peritoneal IgM^{high} MacI^{int} CD5$^-$ B-1b cells were sorted using a FACS-Aria (Becton Dickinson) cell sorter. The purity obtained for positive cell sorting by FACS was higher than 95%. 1x10^6 IgM^{high} MacI^{int} B220$^+$ CD5$^-$ B-1b cells were injected i.p. into recipient mice either once at an age of 5-6 months or every other week starting at an age of 3.5-4 months.

2.3.3.7 TLR stimulation *in vivo*

Mice were injected i.p. with either 25µg Imiquimod (Invivogen) or 50µg CpG ODN 1826 (Invivogen) alone or with 25µg Imiquimod together with 50µg CpG ODN 1826. Mice were analyzed on day 7 after TLR stimulation.

2.3.3.8 Determination of Proteinuria

Proteinuria is one of the most reliable markers for murine lupus nephritis. To determine the scale of proteins in the urine of mice, urine was gained by gently pressing the back of the mice. The urine was applicated on urine test strips (Machery&Nagel). According to the scale the severity of proteinurea could be determined as follows: 0 negativ, 1 <75 mg/dl, 2 <125 mg/dl, 3 ≥125 mg/dl.

2.3.4 Biochemical and Analytical Methods

2.3.4.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The integrity of expressed antibodies was verified by denaturating polyacrylamid gel electrophoresis. In brief, proteins were first heated in a reducing SDS and β-mercaptoethanol containing buffer (protein sample buffer) for 5 minutes at 95°C. High temperatures, β-mercaptoethanol and SDS assure the denaturating of protein samples. To generate the resolving gel, 15% acrylamide (stock 30% acryl-bisacrylamide) was added to the SDS resolving gel buffer (*2.2.4*). The addition of 0.1% TEMED (Invitrogen) and 0.1% ammonium persulphate (APS, Carl-Roth) induced the polymerization of the resolving gel. The still fluid resolving gel was then immediately poured into the vertical gel chamber (BioRad). The stacking gel contained just 5% acrylamide added to the SDS stacking gel buffer (*2.2.4*). After polymerization of the resolving gel, the stacking gel was poured into the gel chamber. Gel electrophoresis was performed in 1x SDS PAGE buffer with 25mA/gel. To estimate the size of the proteins, a marker (Kaleidoscope, BioRad) was loaded. Proteins were detected by staining of the gel with Coomassie Brilliant Blue.

2.3.4.2 Enzyme-linked immunosorbent Assay (ELISA)

ELISA is a biochemical technique used to detect the presence and specifity of antibodies. ELISAs were done as previously described (Wardemann, Yurasov et al. 2003; Ehlers, Fukuyama et al. 2006). In detail, 96-well plates were coated with 100µl of antigens or anti murine IgG Fc or IgM antibodies in coating buffer for two hours at room temperatur or overnight at 4°C. Subsequent to coating the antigens, the wells

were washed triply with PBS and then incubated with block/wash buffer for one hour at room temperature. Serum samples were diluted 1:100 (for detection of antigen specific antibodies) or 1:500 (for detection of total IgM antibodies) or 1:2000 (for detection of total IgG antibodies) in block/wash buffer and incubated for one hour at room temperature. Purified antibodies were incubated in dilution series. Plates were washed again 4 times with PBS. Ig subclasses and isotypes were detected with 100 µl horseradish peroxidase (HRP)–coupled polyclonal goat anti–mouse IgM, IgG, IgG2c or IgG2b specific secondary antibodies (Bethyl Laboratories). HRP- conjugated antibodies were used for detection in a 1:5000 dilution. After washing the microtiter plates again five times the colourimetric turnover was induced by application of 100 µl TMB and the reaction was stopped with 100 µl 1 N H_2SO_4. Absorption was measured at 450 nm using a photometric ELISA plate reader.
ELISA plates for the detection of anti-nucleosome, anti-RNP-70 and anti-nuclear (ANA-Detect) antibodies were purchased from Orgentec. The mouse IL-6 and IL-10 ELISA Kits were purchased from BD Biosciences Pharmingen. For anti-IgG RF ELISA, 5 µg/ml protein-G-sepharose-purified human IgG was coated on the plates. For anti-insulin, anti-dsDNA, anti-ssDNA and anti-LPS ELISA, 5µg/ml of the corresponding antigen was coated on the plates. Total IgM and total IgG antibody titers were measured using ELISA plates coated with 100 µl of 5 µg/ml polyclonal goat anti-mouse IgM or IgG Fc in Coating Buffer. Reference samples (Bethyl Laboratories) were used to calculate the IgM concentration of purified IgM antibodies.

2.3.5 Immunbiological Methods

2.3.5.1 Lymphocyte stimulation

Lymphocyte stimulation amplifies the secretion and enrichment of cytokines in the cell. In detail, prepared lymphocytes (see *2.3.3.2*) were resuspended in 200 µl RPMI supplemented with 10 ng/ml PMA and 1 µg/ml ionomycin and incubated for 1 hour at 37°C. PMA acts as a stimulant on the activation status of cells, especially T cells, independent of their antigen-specificity whereas Ionomycin, an Ionophor, is required for the transfer of PMA into the cells. After 1h 5 µg/ml Brefeldin A was added. Brefeldin A blocks the intracellular transport system, and therefore

promotes the accumulation of produced cytokines in the cells. After an incubation period of 3 hours at 37°C, the cells were washed and resuspended in PBS/BSA.

2.3.5.2 Flow cytometric Analyses

Flow cytometry (or fluorescence activated cell sorting, FACS) enables detection and analysis of cells on a single cell level. Certain parameters, as e.g. size, granularity and fluorescence can be quantified using FACS. In general, cells are stained with antibodies coupled to a fluorophore showing specificities to cell surface or intracellular proteins. Therefore, single cells can be characterized by cell surface and intracellular markers, size and granularity.

Staining of cell surface proteins

Cell surface protein stainings for FACS were performed in cold PBS/BSA in 96-well plates (V-bottom, non-treated). Cells were incubated with the appropriate, primary antibody (see table 2.10) in a total volume of 50 µl, for 15 minutes on ice. The proper dilution of the antibody was determined by preliminary test stainings. Subsequent to inbubation, cells were washed with 200 µl PBS/BSA and after that either prepared for a following intracellular staining or directly resuspended in 200 µl PBS/BSA for flow cytometry analysis.

Staining of intacellular proteins

For intracellular staining of proteins, cells were fixed with 100 µl Cytofix/Cytoperm for 20 minutes at 4°C. This step enables fixation and permeabilization of the cells which is necessary for the intracellular staining of proteins, e.g. cytokines. Afterwards cells were washed with 200 µl Perm/wash buffer and then stained with a primary antibody (see table 2.10) in a total volume of 50 µl Perm/wash buffer for 30 minutes at 4°C.

2.3.5.3 Immunhistological Stainings

Immunhistological stainings with the help of fluorophore-coupled antibodies enable the visualization of cells and molecules in tissue sections. In this work immunhistological stainings of the kidney were performed.

Histological processing

Isolated kidneys were directly transferred into histocassettes filled with OCT-medium and frozen on dry ice. Frozen organs were stored at -80°C and later used for preparing fine sections for immunhistostainings. The sections (5µm) were produced using the microtome (Microm- HM 500 OM) and mounted gently on a glass frosted slide. After fixation in acetone for 10min at -20°C the tissue sections were either stored at -80°C or further processed immediately.

Staining Procedure

After defrosting at room temperature, tissue sections on the glass slides were encircled with the Super Pap Pen creating a water repellent circle around the section. Subsequent, the slides were triply washed with PBS and blocked with PBS/3% BSA for 30 minutes. Tissue sections were stained for MacI (MacI-Cy5, in house preparation), IgG2c (IgG2c-FITC, BD Bioscience), F4/80 (F4/80-Cy5), CD4 (CD4-Alexa 546, in house preparation) and IL17 (IL17-FITC, BD Bioscience). Flourophore-coupled antibodies were all used in a dilution between 1:50 and 1:150. The following incubation for 1h was carried out in a dark and humid chamber, to protect samples from drying out. After another washing step with PBS, tissue sections on the glass slides were embedded in Flouromount, covered with a coverslip and sealed with nail polish. Stained tissue samples were analyzed using a confocal laser scanning microscope (LSM710, Carl Zeiss AG).

2.3.6 *in vitro* assays

2.3.6.1 *Ex vivo* cultivation of total splenocytes and peritoneal cells

Spontaneous secretion of IgM, IL-6 or IL-10 from $2x10^7$ splenocytes, $5x10^6$ total peritoneal cells was induced in 24 or 48 well plates with 800 or 500 µl RPMI (GIBCO) containing 10% FCS, 1% Penicillin/Streptomycin (PS) and 50 µM 2-mercaptoethanol, respectively. B-1 cells were sorted using a FACS-Aria (Becton Dickinson) cell sorter. The purity obtained for positive cell sorting by FACS was higher than 95%. Culture supernatants were collected after 48h and secreted ANA IgM, IL-10 or IL-6 levels were determined by ELISA.

2.3.6.2 B-1 cell stimulation with TLR agonists

FACS sorted peritoneal IgM^{high} MacI^{int} B-1 cells (1.0 x 10^5) from 8 week-old FcγRIIB$^{-/-}$ females were incubated in 96-well-plates with 200 µl RPMI (GIBCO) containing 10% FCS, 1% Penicillin/Streptomycin (PS), 50 µM 2-mercaptoethanol and different combinations of 5µg/ml CpG 1826 (Invivogen), 5 µg/ml Imiquimod (Invivogen) as indicated. B-1 cells were sorted using a FACS-Aria (Becton Dickinson) cell sorter. The purity obtained for positive cell sorting by FACS was higher than 95%. Culture supernatants were collected after 96h and secreted total IgM was determined by ELISA.

2.4 Statistical Analysis

Statistics were done using GraphPad Prism 4. Data were expressed as mean ± SEM. Data were analysed using unpaired two-tailed t-test for comparison between two groups or Log-rank test for survival curves. Indicated p-Values are based on the significance standard of 5% and can be distinguished as shown in table *2.13*.

Table 2.13: P-Values

Indication	P-Value
ns	$p > 0.05$
☆	$p \leq 0.05$
☆☆	$p < 0.01$
☆☆☆	$p < 0.001$

3 Previous work
Characterization of FcγRIIB$^{-/-}$ mice lacking TLR9

Previous work was done together with Maria Mertes, Alexander Stöhr and Susanne Eiglmeier from the Laboratory of Tolerance and Autoimmunity at the DRFZ.

3.1 Self-reactive IgG antibodies

One diagnostic marker of SLE diseases is the development of pathogenic IgG autoantibodies specific to nuclear antigens and their detectability in serum. Serum analysis of 5-6 month-old wild type C57BL/6, FcγRIIB$^{-/-}$ and FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice using enzyme linked immunosorbent assay (ELISA) revealed significant differences in autoantibody titers. Here, IgG autoantibodies specific to nuclear antigens in general (ANA), to Fc parts of serum IgG (rheumatoid factor, RF), to RNA containing antigens (RNP) as well as specific to nucleosomes (Nuc) were analyzed. Mice lacking the inhibitory FcγRIIB showed increased levels of autoreactive IgG antibodies specific to all tested antigens compared to C57BL/6 wildtype mice. The additional loss of TLR9 even raised levels of anti-nuclear (ANA), anti-IgG rheumatoid factor (RF) and anti-RNP-70 IgG2c (IgG2a, haplotype b) and IgG2b serum autoantibodies in 5-6 month-old FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice compared to age-matched FcγRIIB$^{-/-}$ controls (*Fig 3.1 A*). However, the lack of TLR9 in FcγRIIB$^{-/-}$ mice was associated with low levels of anti-nucleosome antibodies *(Fig 3.1 B).* Thus, our data further confirmed previous studies of lupus mouse models which claim that TLR9 signaling is essential for the development of anti-nucleosome IgG serum antibodies (Lartigue, Courville et al. 2006).

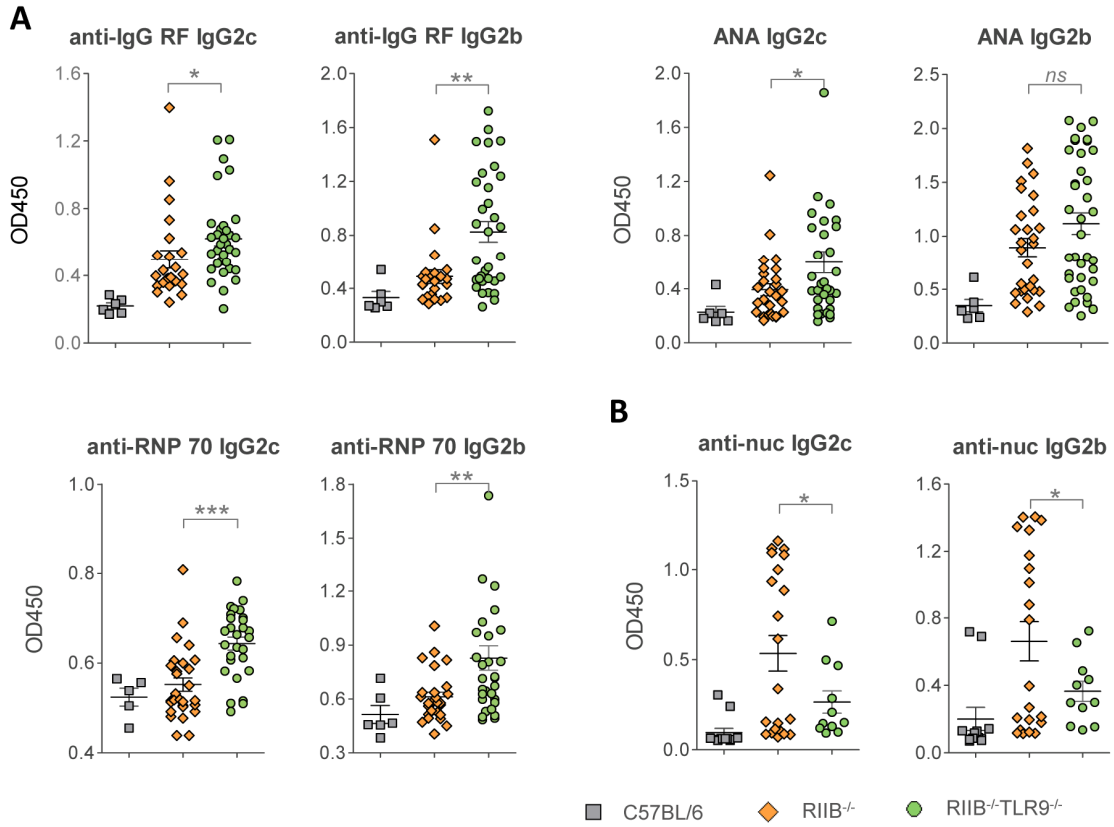

Figure 3.1 FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice exhibit enhanced levels of self-reactive IgG autoantibodies. **(A)** Anti-IgG RF IgG2c and IgG2b, ANA IgG2c and IgG2b and anti-RNP70 IgG2c and IgG2b serum autoantibody levels of 5-6 month-old wild-type C57BL/6, FcγRIIB$^{-/-}$ (RIIB$^{-/-}$) or FcγRIIB$^{-/-}$ TLR9$^{-/-}$ (RIIB$^{-/-}$TLR9$^{-/-}$) mice as analyzed by ELISA. **(B)** Anti-nucleosome (nuc) serum autoantibody levels of 5-6 month-old wild-type C57BL/6, RIIB$^{-/-}$ or RIIB$^{-/-}$TLR9$^{-/-}$ mice as analyzed by ELISA. Symbols represent data from individual mice. Horizontal lines show mean values with standard error of the mean (SEM). One representative ot of three independent ELISA is shown for each image.

3.2 Survival and Proteinuria

During the early course of lupus nephritis, immune complexes, consisting of self-reactive IgG antibodies and their antigens, start to deposit in the renal glomeruli. Consequent infiltration of inflammatory macrophages and T cells leads to severe damage of the kidney, resulting in the excretion of proteins in the urine, as their retention by glomeruli and tubuli becomes inefficient.

Analysis of proteins in the urine of 5-6 month-old FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice pointed out that around 55% suffered from proteinuria, whereas only 12% of the age-matched FcγRIIB$^{-/-}$ control group did *(Fig. 3.2 A)*. In the DRFZ mouse facility about 20% of

lupus prone FcγRIIB$^{-/-}$ mice developed nephritis and died from disease symptoms by the age of nine months *(Fig. 3.2 B)*. Notably, mortality was significantly increased to 80% in FcγRIIB$^{-/-}$ mice lacking TLR9 (FcγRIIB$^{-/-}$TLR9$^{-/-}$).

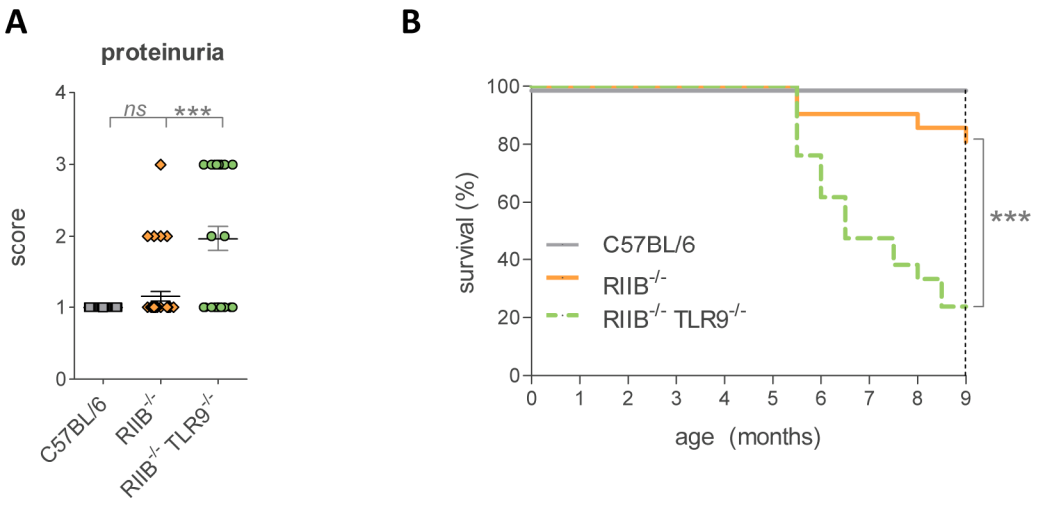

Figure 3.2 FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice exhibit exacerbated development of lupus disease (A) Proteinuria scores in 5-6 month-old C57BL/6 (n=15), FcγRIIB$^{-/-}$ (RIIB$^{-/-}$; n=39), and FcγRIIB$^{-/-}$ TLR9$^{-/-}$ (RIIB$^{-/-}$TLR9$^{-/-}$; n=29) mice. Symbols represent data from individual mice. Horizontal lines show mean values with standard error of the mean (SEM). **(B)** Kaplan-Meier survival curves for wild-type C57BL/6 (n=30), FcγRIIB$^{-/-}$ (RIIB$^{-/-}$; n=21) and FcγRIIB$^{-/-}$TLR9$^{-/-}$ (RIIB$^{-/-}$TLR9$^{-/-}$; n=21) mice.

3.3 Pro-inflammatory CD4^{+} T helper cell subsets

According to the literature, the Th1 cell subset is predominantly linked with the development of antibodies of the IgG2c and 2b subclasses, which are considered as the pathogenic isotypes in murine lupus (Snapper and Paul 1987; Kotzin 1996). To determine whether elevated titers of autoreactive IgG antibodies in TLR9 deficient FcγRIIB$^{-/-}$ mice are associated with increased Th1 cell frequencies, flow cytometric analysis of Th1 cells, which can be identified by the secretion of the cytokine interferone γ (IFNγ), was performed. An increase in pro-inflammatory Th1 cell frequencies was observed in blood, spleen and lymph nodes (LN) of FcγRIIB$^{-/-}$ mice lacking TLR9, thus correlating with exacerbated lupus disease (*Fig 3.3 A*, and data not shown).

Recent evidence indicates that also the Th17 cell subset plays a devastating role in the pathogenesis of autoimmunity and especially of SLE (Crispin and Tsokos 2010). Besides their effector function in promoting inflammation through the secretion of pro-inflammatory cytokines, particularly IL-17, they also seem to have stimulatory effects on B cells inducing germinal center formation and autoantibody production (Hsu, Yang et al. 2008; Mitsdoerffer, Lee et al. 2010). To see whether Th17 cells play a role in the augmented incidence of nephritis in TLR9 deficient FcγRIIB$^{-/-}$ mice, flow cytometric analysis of Th17 cells was performed as described above. Indeed, our results demonstrated a significant increase of Th17 cell frequencies in blood, spleen and lymph nodes of 5-6 month-old FcγRIIB$^{-/-}$ mice lacking TLR9 compared to the age-matched FcγRIIB$^{-/-}$ control mice (*Fig 3.3 B* and data not shown). To determine whether pro-inflammatory Th17 cells infiltrate into target organs such as renal tissues, immunohistological stainings of kidney sections with antibodies against MacI, CD4 and IL-17 were performed. Severe inflammation in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice was associated with increased frequent accumulation of Th17 cells as well as macrophages in the kidney, indicating the onset of lupus nephritis *(Fig 3.3 C).*

The activation status of CD4^{+} T cells in general can be characterized by the expression of the chemokine receptor CXCR3, which is essential for T cell migration, the cell surface glycoprotein CD44, which is important for cell-cell interactions and the L-selectin CD62L. Activated T cells express high levels of CXCR3 and CD44 on their surface whereas the expression of CD62L is downregulated. To analyze the frequencies of circulating activated CD4^{+} T cells we stained blood peripheral lymphocytes with anti-CXCR3, anti-CD44, anti-CD62L and anti-CD4 antibodies. Subsequent FACS analysis demonstrated, that high numbers of Th1 and Th17 cells in FcγRIIB$^{-/-}$ mice lacking TLR9, correlated with increased frequencies of activated peripheral blood CXCR3^{+}CD4^{+} and CD44^{+}CD62L^{-}CD4^{+} T helper cells *(Fig 3.3 D)*.

Besides the cytokine transforming growth factor beta (TGF-β), interleukin 6 (IL-6) contibutes to the formation of Th17 cells in mice (Korn, Bettelli et al. 2009). To evaluate whether high titers of Th17 cells in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice were generated due to the increased expression of IL-6, 2x10^{7} total splenocytes were cultured for 48h and supernatants were analyzed (ELISA) for IL-6 production. Severe inflammation and increased numbers of Th17 cells in TLR9 deficient FcγRIIB$^{-/-}$ mice were associated

with elevated production of the pro-inflammatory cytokine IL-6 in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice *(Fig 3.3 E).*

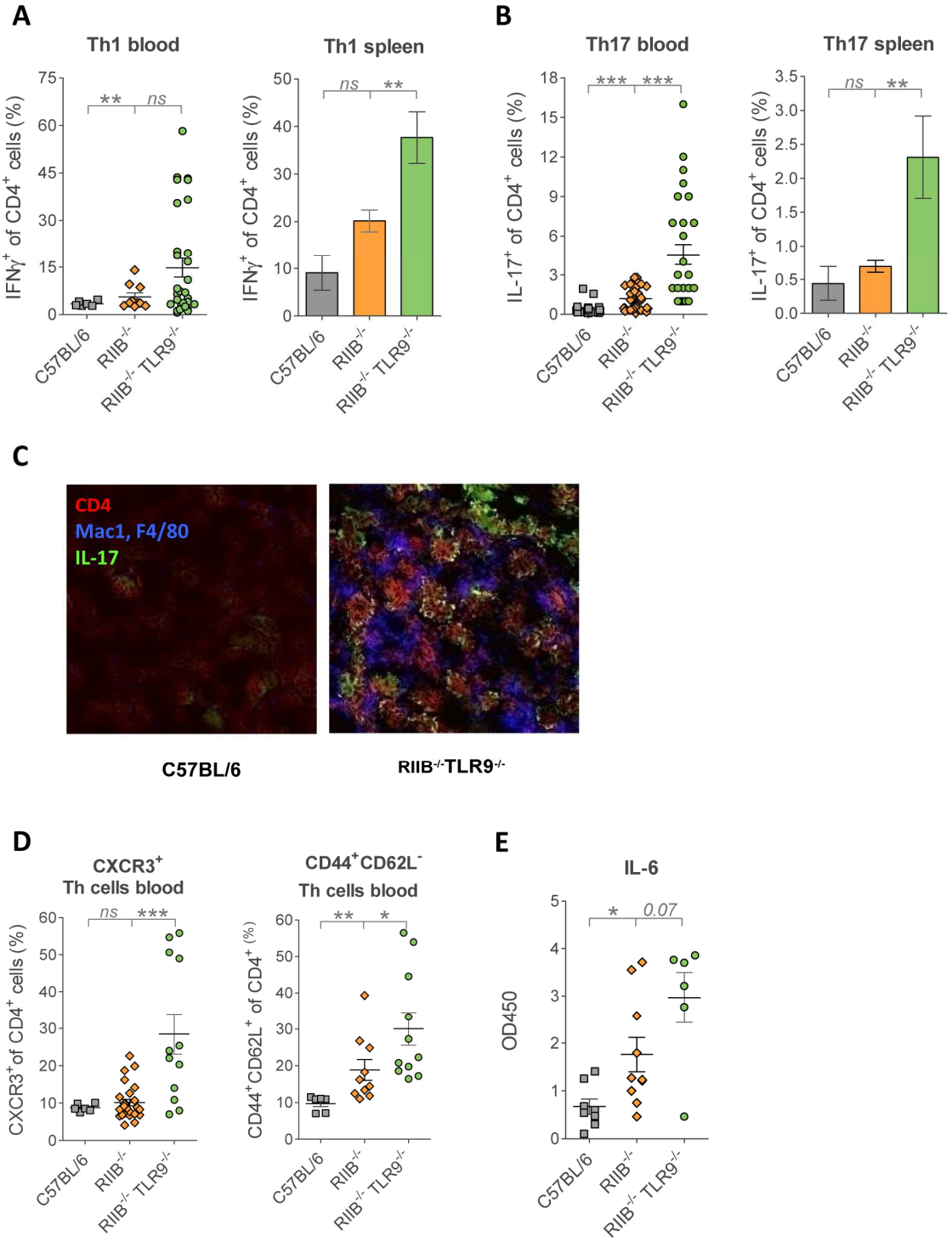

Figure 3.3 FcγRIIB$^{-/-}$TLR9$^{-/-}$ display increased levels of pro-inflammatory Th1, Th17 cells and activated T helper cells. Frequencies of CD4^{+}IFNγ^{+} Th1 cells **(A)** and CD4^{+}IL-17^{+} Th17 cells **(B)** as determined by FACS in blood or spleen of 5-6 month-old wild type C57BL/6, FcγRIIB$^{-/-}$ (RIIB$^{-/-}$) and FcγRIIB$^{-/-}$TLR9$^{-/-}$(RIIB$^{-/-}$TLR9$^{-/-}$) mice (Th1 and Th17 cells in spleen; C57BL/6 mice,

n=3; RIIB$^{-/-}$ mice, n=9; RIIB$^{-/-}$TLR9$^{-/-}$ mice, n=3-4).**(C)** Immunofluorescence staining of macrophages and CD4^{+}IL17^{+} Th17 cells in kidney sections of wild type C57BL/6 mice and RIIB$^{-/-}$TLR9$^{-/-}$ mice with proteinuria. Images are representative of 5 different mice per group. **(D)** Frequencies of CD4^{+}CXCR3^{+} and CD4^{+}CD44^{+}CD62L^{-} activated T helper in the blood of 5-6 month-old wild type C57BL/6, FcγRIIB$^{-/-}$ (RIIB$^{-/-}$) and FcγRIIB$^{-/-}$TLR9$^{-/-}$(RIIB$^{-/-}$TLR9$^{-/-}$) mice as determined by FACS. **(E)** IL-6 levels in 48h supernatant of cultured splenocytes of 5-6 month-old wild-type C57BL/6, RIIB$^{-/-}$ and RIIB$^{-/-}$TLR9$^{-/-}$ mice. Symbols represent data from individual mice. Horizontal lines show mean values with standard error of the mean (SEM). Bar graphs show the mean value with standard error of the mean (SEM) for each group of mice.One representative out of two independent experiments is shown in each image.

There has already been evidence that TLR9 stimulation mediates tolerance in various lupus prone mouse models (Lartigue, Courville et al. 2006; Christensen and Shlomchik 2007). So far, our data confirmed the tolerogenic effect of TLR9 in the FcγRIIB$^{-/-}$ lupus mouse model. Hence, the loss of TLR9 in lupus prone FcγRIIB$^{-/-}$ mice led to the accumulation of pro-inflammatory Th1, Th17 cells and IgG2c and IgG2b autoantibodies inducing exacerbated lupus disease.

4 Results

4.1 Loss of TLR9 in FγRIIB$^{-/-}$ mice results in low levels of self-reactive IgM antibodies

As shown in *3.1* loss of TLR9 in FcγRIIB$^{-/-}$ mice is associated with increased titers of self-reactive antibodies of the IgG2c and IgG2b subclasses. Interestingly, analysis of self-reactive serum IgM titers revealed converse results. 5-6 month-old FcγRIIB$^{-/-}$ mice exhibited elevated levels of self-reactive ANA and anti-IgG RF serum IgM antibody levels in comparison to age-matched C57BL/6 wild-type control mice *(Fig 4.1 A)*.

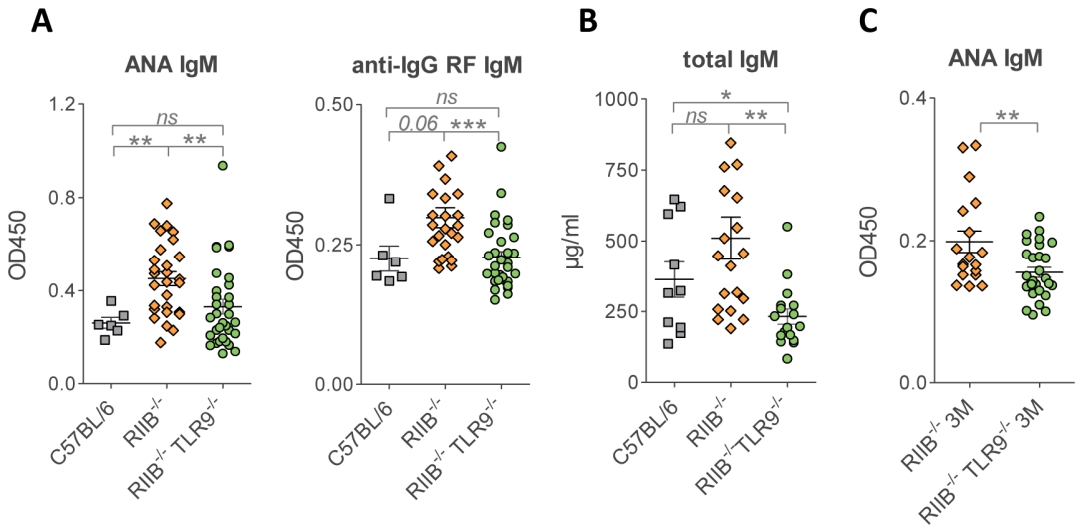

Figure 4.1 TLR9 stimulation in FcγRIIB$^{-/-}$ mice regulates self-reactive IgM levels.(A) ANA and anti-IgG RF IgM autoantibody levels in serum of 5-6 month-old wild-type C57BL/6, FcγRIIB$^{-/-}$ (RIIB$^{-/-}$) or FcγRIIB$^{-/-}$TLR9$^{-/-}$ (RIIB$^{-/-}$TLR9$^{-/-}$) mice as analyzed by ELISA. **(B)** Total IgM antibody levels in serum of 5-6 month-old wild-type C57BL/6, FcγRIIB$^{-/-}$ (RIIB$^{-/-}$) or FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice as analyzed by ELISA. **(C)** ANA autoantibody levels in serum of 3 month-old FcγRIIB$^{-/-}$ (RIIB$^{-/-}$) or FcγRIIB$^{-/-}$TLR9$^{-/-}$(RIIB$^{-/-}$TLR9$^{-/-}$) mice as analyzed by ELISA. Symbols represent data from individual mice. Horizontal lines show mean values with standard error of the mean (SEM). One representative ELISA out of at least two independent ELISAs is shown for each plot.

But contrary to IgG autoantibodies, self-reactive serum IgM antibody levels were significantly reduced in 5-6 month-old TLR9-deficient FcγRIIB$^{-/-}$ mice and were thus comparable to the levels measured in age-matched C57BL/6 wild-type controls. Same tendencies were observed considering total IgM antibody levels *(Fig 4.1 B).*

To evaluate whether the reduction of self-reactive IgM levels was dependent on the age of the mice, serum of 3 month-old, young animals were analyzed. Results were consistent with the results for 5-6 month old FcγRIIB$^{-/-}$ and FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice *(Fig 4.1 C)* and thus demonstrated that reduction of self-reactive IgM titers in FcγRIIB$^{-/-}$ TLR9$^{-/-}$ was independent of age.

Hence, we conclude that the development of self-reactive IgM serum antibodies in autoimmune FcγRIIB$^{-/-}$ mice is dependent on TLR9-mediated signaling.

4.2 Reconstitution of self-reactive IgM levels in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice with recombinant monoclonal self-reactive IgM antibodies

In contrast to self-reactive antibodies of the IgG isotype, self-reactive IgM autoantibodies are considered to play a role in the protection from the development of lupus disease symptoms in mice and humans (Boes, Schmidt et al. 2000; Forger, Matthias et al. 2004; Werwitzke, Trick et al. 2005). Boes *et al.* demonstrated the protective role of IgM in a murine model of SLE lacking soluble IgM antibodies. Mice showed aggravated lupus disease symptoms due to the lack of soluble IgM antibodies. Furthermore, Werwitzke *et al.* found that the transfer of monoclonal anti-DNA IgM antibodies delays the onset of nephritis in NZB/W mice.
Based on these findings we decided to reconstitute self-reactive IgM titers in FcγRIIB$^{-/-}$ mice lacking TLR9. Therefore a monoclonal polyreactive (ED38) and a monoclonal control (mgO53) IgM antibody were cloned and produced (Wardemann, Yurasov et al. 2003; Meffre, Schaefer et al. 2004). The human heavy and kappa constant regions in respective expression vectors of the antibody ED38 and the control antibody were exchanged for the murine IgM heavy chain and murine kappa light chain constant regions *(see 2.3.1.5).* The antibodies were produced in Hek293T cells and purified with the help of a constructed anti-murine IgM Purification Column *(see 2.3.2).* Polyreactivity and self-reactivity of the generated antibodies were verified by ELISA. Monoclonal antibodies were considered as

polyreactive if they were binding to at least three different self-antigens. Here, insulin, dsDNA, ssDNA and also LPS were used as representative antigens. ED38 IgM antibody was binding to all tested antigens whereas the control IgM antibody could be designated to as non-reactive *(Fig 4.2 A).*

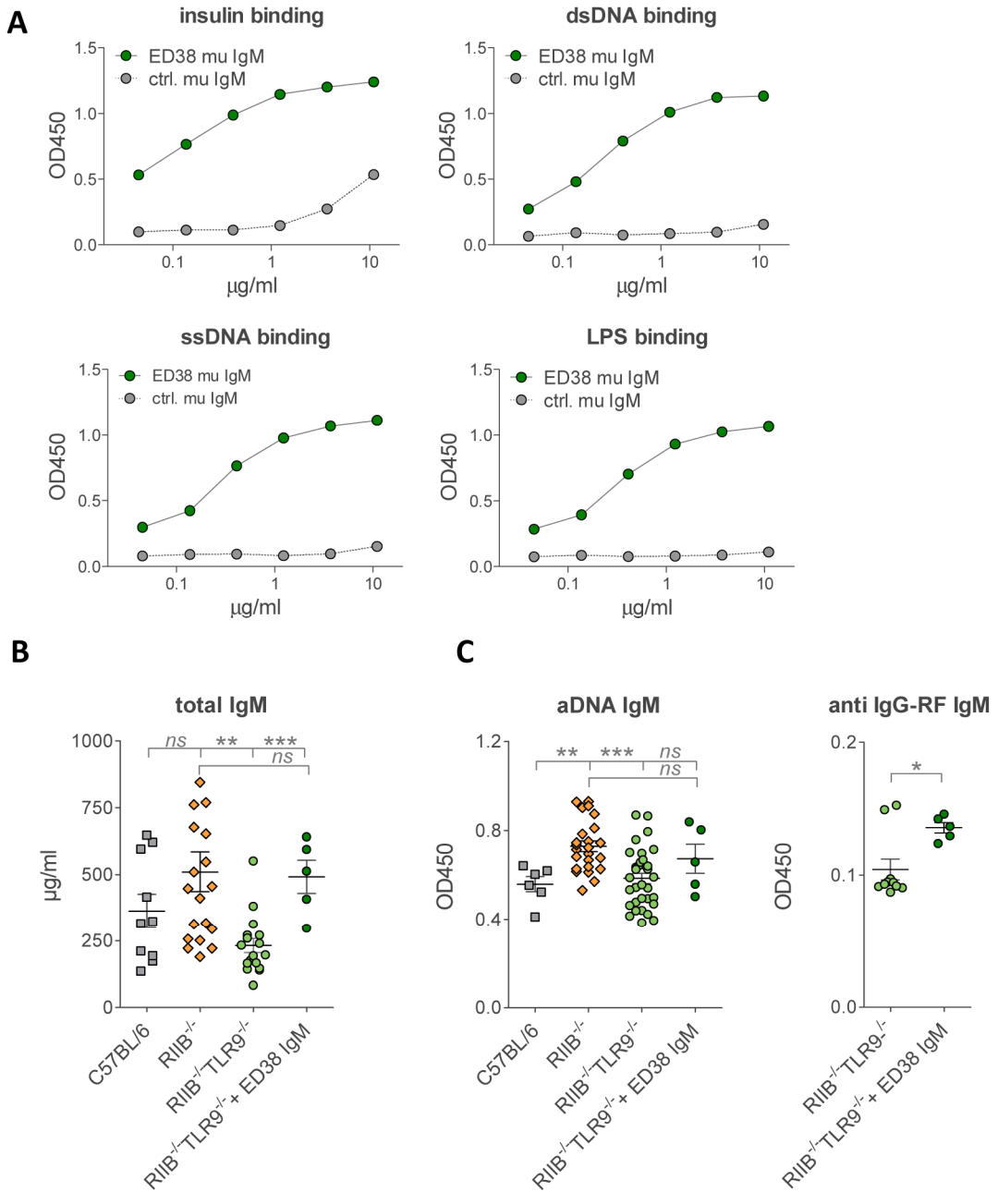

Figure 4.2 Administration of poly- and self-reactive IgM antibodies raises total and self-reactive IgM levels in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice. (**A**) Affinity analysis of the monoclonal self- and polyreactive ED38 antibodies to insulin, dsDNA, ssDNA and LPS in comparison to a control (ctrl.) IgM antibody (mgo53) as determined by ELISA. One representative out of two independent ELISA is shown for each plot. **(B)** Total IgM antibody levels in serum of 6

month-old wild-type C57BL/6, FcγRIIB$^{-/-}$ (RIIB$^{-/-}$), FcγRIIB$^{-/-}$TLR9$^{-/-}$ or ED38 IgM treated FcγRIIB$^{-/-}$ TLR9$^{-/-}$ mice as analyzed by ELISA. **(C)** Anti-DNA or anti-IgG RF IgM antibody levels in serum of 6 month-old wild-type C57BL/6, FcγRIIB$^{-/-}$ (RIIB$^{-/-}$), FcγRIIB$^{-/-}$TLR9$^{-/-}$ or ED38 IgM treated FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice as analyzed by ELISA. Symbols represent data from individual mice. Horizontal lines show mean values with standard error of the mean (SEM). One representative out of two independent ELISA is shown for each plot.

To investigate whether reconstitution of self-reactive IgM levels can substantially control lupus disease in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice, 200µg of the recombinant monoclonal ED38 and control IgM antibody were applicated intraperitoneally (i.p.) twice a week starting at the age of 3.5 months. Long-term administration of ED38 IgM was consequently followed by the rise of total serum IgM antibodies *(Fig 4.2 B)* and increased self-reactive serum IgM antibody titers. At the age of 6 months, FcγRIIB$^{-/-}$ TLR9$^{-/-}$ mice treated with ED38 IgM antibodies and FcγRIIB$^{-/-}$ mice exhibited nearly similar levels of DNA reactive IgM. Administration of ED38 IgM also caused augmented titers of anti-IgG rheumatoid factor (RF) IgM antibodies *(Fig 4.2 C).*

4.2.1 Monoclonal self-reactive IgM antibodies reduce nephritis-induced mortality of FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice

The treatment of FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice with ED38 IgM and the non-reactive control IgM antibodies was performed over a period of 5.5 months until the age of nine months. At 6 months of age, the excretion of proteins in the urine, due to the damage of renal glomeruli, was analyzed and compared to untreated control groups. 12% of the FcγRIIB$^{-/-}$ mice developed proteinuria whereas 55% of the untreated control group of FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice did. Strikingly, the incidence of proteinuria in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice could be significantly reduced to 20% by the treatment with ED38 IgM antibodies whereas the application of control IgM antibodies did not affect the development of proteinuria in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice *(Fig 4.3 A).*

The occurence of proteinuria is one of the first disease symptoms in lethal lupus nephritis. Observations revealed that at the latest three to four weeks after the development of proteinuria diseased mice died from lupus nephritis. Hence, the proteinuria data were reflected in the respective survival curves. In the DRFZ mouse facility about 80% of lupus prone FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice developed nephritis and died from disease symptoms by the age of nine months. No improvement of survival was

observed in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice, treated with the monoclonal unspecific control IgM antibody. But remarkably, mortality was significantly decreased to 25% in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice treated with monoclonal polyreactive ED38 IgM antibodies *(Fig 4.3 B).* Thus, the here self-constructed and produced polyreactive IgM antibody ED38 indeed was able to control lupus disease in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice.

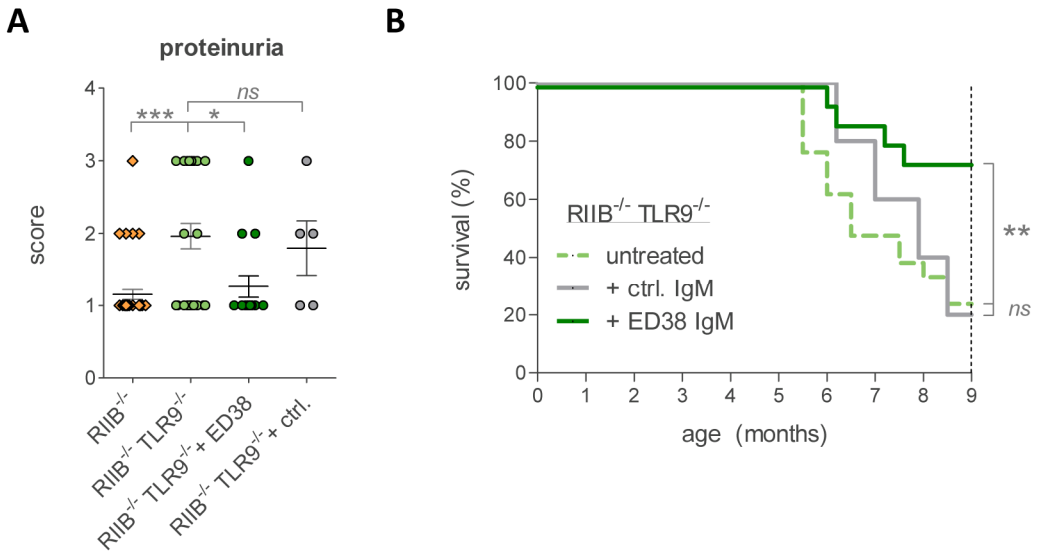

Figure 4.3 Reconstitution of self-reactive IgM levels in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice prevents lupus disease. (A) Proteinuria scores in 6 month-old FcγRIIB$^{-/-}$ (RIIB$^{-/-}$; n=39), FcγRIIB$^{-/-}$TLR9$^{-/-}$ (n=29) and ED38 IgM (n=15) or control IgM (n=5) treated mice. Symbols represent data from individual mice. Horizontal lines show mean values with standard error of the mean (SEM). **(B)** Kaplan-Meier survival curves for untreated FcγRIIB$^{-/-}$TLR9$^{-/-}$ (RIIB$^{-/-}$TLR9$^{-/-}$) mice (n=21) and ED38 IgM (n=15) and control IgM (ctrl. mgo53; n=5) treated RIIB$^{-/-}$TLR9$^{-/-}$ mice (200 µg twice a week starting at the age of 3.5 months).

4.2.2 Monoclonal self-reactive IgM antibodies prevent the accumulation of pro-inflammatory CD4$^+$ T cells in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice

Increased frequencies of activated pro-inflammatory CD4$^+$ T helper cells, precisely Th1 and Th17 cells, were associated with exacerbated lupus disease in FcγRIIB$^{-/-}$ mice lacking TLR9 *(see 3.3).* To ascertain whether long-term application of self-reactive IgM antibodies affected T helper cell differentiation, expansion and activation, flow cytometric analysis of peripheral blood lymphocytes and peripheral lymphoid organs was performed. At 6 month of age, FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice treated with ED38 IgM antibodies and the age-matched untreated control group displayed no

differences in blood Th1 cell frequencies, thus indicating that ED38 IgM antibodies did not affect Th1 cell frequencies in peripheral blood cells *(Fig 4.4 A).* In contrast, long-term treatment of FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice with ED38 IgM reduced Th17 cell frequencies in the peripheral blood cell pool compared to the age-matched 5-6 month-old FcγRIIB$^{-/-}$TLR9$^{-/-}$ control mice *(Fig 4.4 B).*

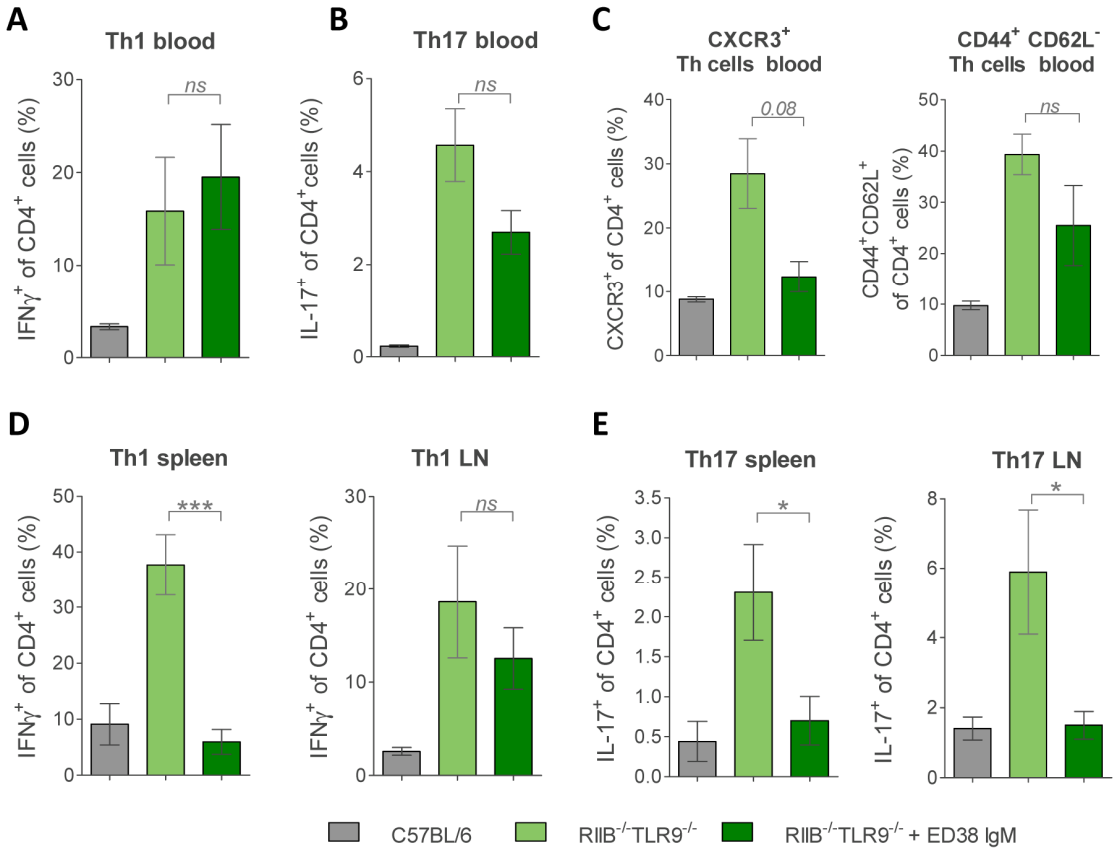

Figure 4.4 Reconstitution of self-reactive IgM levels in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice inhibits accumulation of pro-inflammatory Th1, Th17 and activated T helper cells. (A) Frequencies of CD4$^+$IFNγ$^+$ Th1 cells in blood of 5-6 month-old wild-type C57BL/6 (n=10) and 5-6 month-old FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice (n=5) or 6 month-old FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice treated with ED38 IgM (n=5). **(B)** Frequencies of CD4$^+$IL-17$^+$ Th17 cells in blood of 5-6 month-old wild-type C57BL/6 (n=15) and 5-6 month-old FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice (n=28) or 6 month-old FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice treated with ED38 IgM (n=5). **(C)** Frequencies of CD4$^+$CXCR3$^+$ and CD4$^+$CD44$^+$CD62L$^-$ activated T helper as analyzed by FACS in blood of 5-6 month-old wild-type C57BL/6 (n=6) and 5-6 month-old FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice (n=12) or 6 month-old FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice treated with ED38 IgM (n=5). **(D)** Frequencies of CD4$^+$IFNγ$^+$ Th1 cells in spleen and pooled lymph nodes of 5-6 month-old wild-type C57BL/6 (n=3) and 5-6 month-old FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice (n=4) or 9 month-old FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice treated with ED38 IgM (n=5). **(E)** Frequencies of CD4$^+$IL-17$^+$ Th17 cells in spleen and pooled lymph nodes of 5-6 month-old wild-type C57BL/6 (n=3) and 5-6 month-old FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice (n=4) or 9 month-old FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice treated with ED38 IgM (n=5) Bar graphs show the mean value with standard error of the mean (SEM) for each group of mice.

Lowered frequencies of Th17 cells thereby correlated with decreased frequencies of activated peripheral blood $CXCR3^+CD4^+$ and $CD44^+CD62L^-CD4^+$ T helper cells *(Fig 4.4 C).* At 9 months of age, mice treated with ED38 IgM antibodies were sacrificed and Th1 and TH17 cell frequencies in peripheral lymphoid organs were compared to a 5-6 month-old control group. Significantly reduced Th1 cell frequencies were observed in the spleens of $Fc\gamma RIIB^{-/-}TLR9^{-/-}$ mice treated with ED38 IgM antibodies and slight reductions of Th1 cell frequencies were noticed in pooled lymph nodes (mesenteric, inguinal, lumbal) *(Fig 4.4 D).* Adressing pro-inflammatory Th17 cells in peripheral lymphoid organs of $Fc\gamma RIIB^{-/-}TLR9^{-/-}$ mice treated with monoclonal ED38 IgM antibodies, significantly decreased frequencies were assessed in spleens and pooled lymph nodes *(Fig 4.4 E).*

Thus, long-term treatment of $Fc\gamma RIIB^{-/-}TLR9^{-/-}$ mice with ED38 IgM antibodies prevented the accumulation of pro-inflammatory $CD4^+$ T cells.

4.2.3 Monoclonal self-reactive IgM antibodies lower splenic plasma cell numbers and IgG autoantibody titers in $Fc\gamma RIIB^{-/-}TLR9^{-/-}$ mice

To test whether the application of monoclonal polyreactive IgM antibodies influenced plasma cell numbers, flow cytometric analysis of $CD138^+$ splenic plasma cells was performed.

As $Fc\gamma RIIB^{-/-}TLR9^{-/-}$ mice treated with ED38 IgM antibodies exhibited decreased frequencies of pro-inflammatory Th1 and Th17 cells, B cells obviously did not obtain sufficient T cell help. Hence, strong reductions of $CD138^+$ splenic plasma cells compared to untreated control mice were observed *(Fig 4.5 A).* Reduced numbers of splenic $CD138^+$ plasma cells in $Fc\gamma RIIB^{-/-}TLR9^{-/-}$ mice treated with ED38 IgM antibodies, resulted in lowered levels of total IgG2c and IgG2b serum antibodies, which thus were comparable to the levels of the proteinuria negative (PU-) healthy fraction of the age-matched control mice *(Fig 4.5 B).*

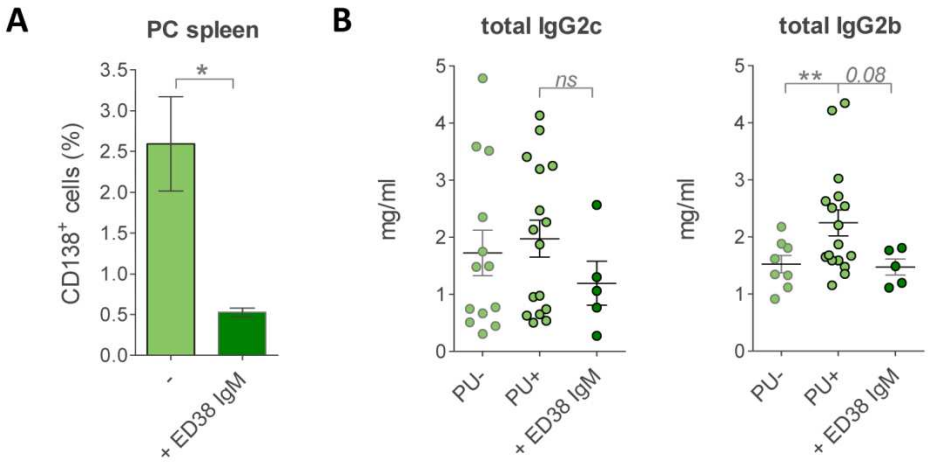

Figure 4.5 Reconstitution of self-reactive IgM levels in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice reduces splenic plasma cell numbers and total IgG antibody titers. (A) Frequencies of splenic CD138^{+} plasma cells (PC) in 5-6 month-old untreated FcγRIIB$^{-/-}$TLR9$^{-/-}$ (RIIB$^{-/-}$TLR9$^{-/-}$) mice (n=7) and 9 month-old RIIB$^{-/-}$TLR9$^{-/-}$ mice treated with ED38 IgM (n=5) as determined by FACS. **(B)** Total IgG2c and IgG2b serum levels as determined by ELISA in 5-6 month-old untreated RIIB$^{-/-}$TLR9$^{-/-}$ mice without or with proteinuria (PU- or PU+) or 6 month-old ED38 IgM treated RIIB$^{-/-}$TLR9$^{-/-}$ mice Symbols represent data from individual mice. Horizontal lines show mean values with standard error of the mean (SEM). One representative out of two independent ELISA is shown for each plot.

.

Subdivision of FcγRIIB$^{-/-}$TLR9$^{-/-}$ control mice into a proteinuria positive (PU+) and proteinuria negative (PU-) control group furthermore revealed a correlation of self-reactive IgG antibodies with the development of lupus nephritis. Heavily increased levels of anti-nuclear (ANA), anti-IgG rheumatoid factor (RF) and anti-RNP-70 IgG2c and IgG2b serum antibodies were observed only in the proteinuria positiv control group *(Fig 4.6 A-C).* These data inversely indicated that high titers of IgG serum autoantibodies were directly associated with the development of proteinuria. Nevertheless, also proteinuria negativ mice partially showed elevated levels of autoreactive serum antibodies.

Besides the reduction of total IgG antibody levels, treatment with ED38 IgM affected titers of autoreactive IgG serum antibodies. Hence, FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice treated with ED38 IgM antibodies and proteinuria negative control FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice displayed comparable levels of total, anti-nuclear (ANA), anti-IgG rheumatoid factor (RF) and anti-RNP-70 IgG2c and IgG2b serum antibodies *(Fig 4.6 A-C).* By contrast, treatment of FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice with control IgM antibodies did not affect autoreactive IgG antibody titers as serum analysis revealed similar ANA, anti-IgG RF and anti-RNP-70 IgG2c and IgG2b levels like the proteinuria positiv control group *(Fig 4.6 A-C).* These

results were in line with high mortality rates and incidences of proteinuria in mice treated with control IgM antibodies.

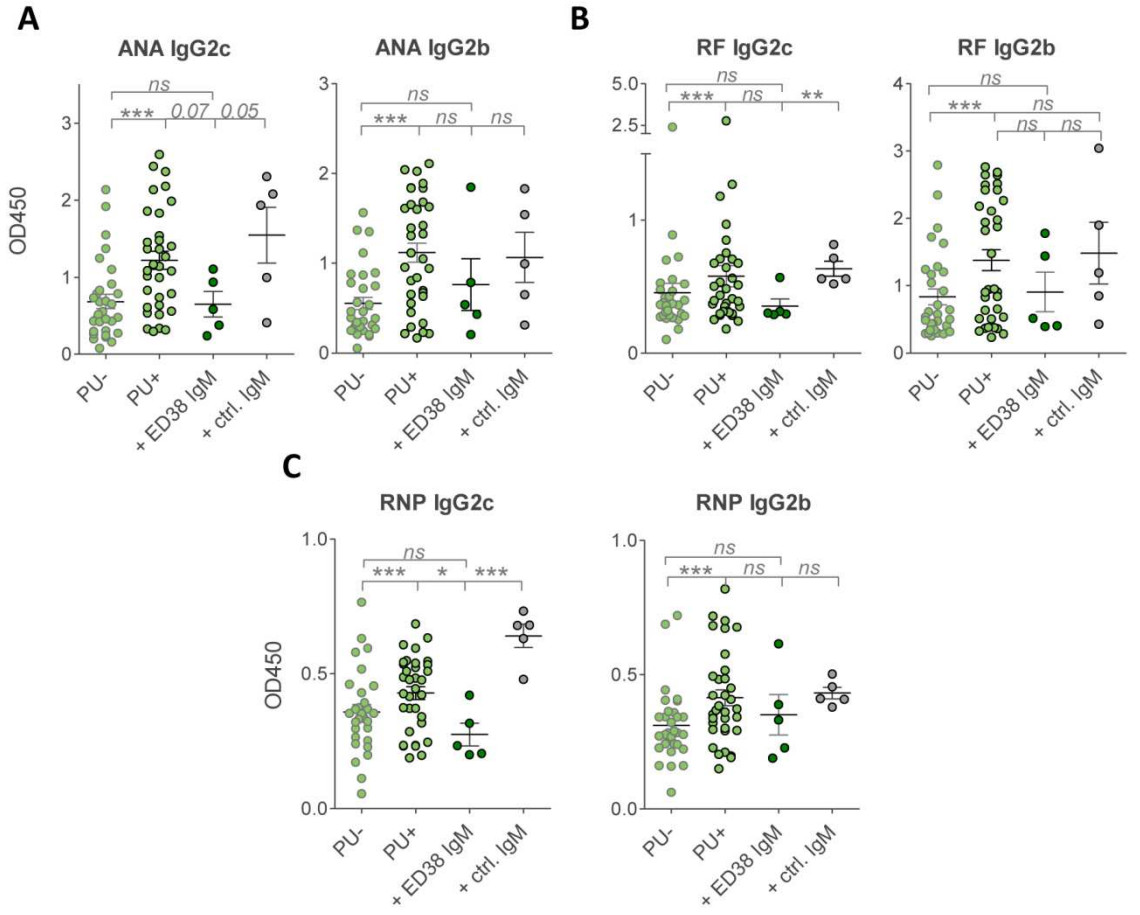

Figure 4.6 Reconstitution of self-reactive IgM levels in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice reduces self-reactive IgG antibody titers. (A) ANA, **(B)** anti-IgG RF and **(C)** anti-RNP-70 IgG2c, IgG2b serum autoantibody levels of 5-6 month-old untreated RIIB$^{-/-}$TLR9$^{-/-}$ mice without or with proteinuria (PU- or PU+), ED38 IgM or control IgM treated RIIB$^{-/-}$TLR9$^{-/-}$ mice as analyzed by ELISA. Symbols represent data from individual mice. Horizontal lines show mean values with standard error of the mean (SEM). One representative out of three independent ELISA is shown.

4.2.4 Monoclonal self-reactive IgM antibodies prevent macrophage infiltration into renal tissues of FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice

In systemic lupus erythematosus, autoantibodies and expansion of pro-inflammatory T cells indicate systemic autoimmunity, but organ damage involves additional mechanisms of inflammation. Cellular immune responses mediated by infiltrating mononuclear cells have an important role in the amplification and the progression of renal injury. To test whether monoclonal self-reactive IgM antibodies

influence the infiltration of activated macrophages into renal tissues, cryo-sections were prepared and stained for IgG2c immune complexes (anti-murine IgG2c), and macrophages (anti-murine MacI, anti-murine F4/80) Immunhistological stainings indicated the deposition of immune complexes in renal glomeruli of both, treated and also non-treated FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice. But interestingly infiltration of macrophages and their accumulation around the glomeruli could only be observed in the non-treated FcγRIIB$^{-/-}$TLR9$^{-/-}$ control group *(Fig 4.7).*

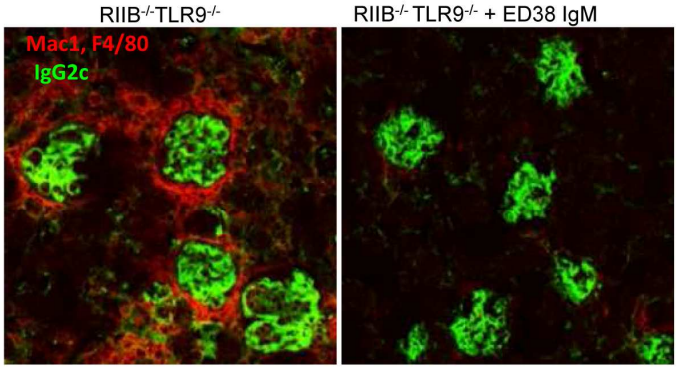

Figure 4.7 Reconstitution of self-reactive IgM levels suppresses infiltration of macrophages in renal tissues of FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice. Immunofluorescence analysis of IgG2c immune complex deposition and macrophage (Mac1 and F4/80) infiltration in kidney sections of an untreated FcγRIIB$^{-/-}$TLR9$^{-/-}$ mouse with proteinuria and a 9 months old FcγRIIB$^{-/-}$TLR9$^{-/-}$ mouse treated with polyreactive IgM (ED38 IgM) showing no signs of proteinuria. Stainings are representative for at least 5 different mice per group.

4.2.5 Single treatment with monoclonal self-reactive IgM antibodies diminishes T helper 17 cell frequencies in aged lupus prone FcγRIIB$^{-/-}$ TLR9$^{-/-}$ mice

Flow cytometric analysis of pro-inflammatory CD4^{+} T cells in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice displayed that during the conventional course of SLE the frequencies of Th17 cells are consistently increasing dependent on age and disease progression *(Fig 4.8 A).* As already mentioned, recent studies have shown that Th17 cell-mediated inflammation is central to the development and pathogenesis of SLE (Nalbandian, Crispin et al. 2009). By breeding FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice to IL-17R$^{-/-}$ mice, the fatal role of Th17 cells in the FcγRIIB$^{-/-}$ TLR9$^{-/-}$ lupus model was demonstrated as FcγRIIB$^{-/-}$TLR9$^{-/-}$IL-17R$^{-/-}$ mice exhibited significantly reduced mortality rates (Susanne Eiglmeier,

personel communication). Accordingly, Th17 cells and the cytokine IL-17 might be potential therapeutic targets in ameliorating lupus disease. On that account, we tested whether monoclonal self-reactive IgM antibodies are able to reduce Th17 cell frequencies in aged FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice and thus could be used therapeutically. Aged mice were screened for high levels of Th17 cells before treatment. Here, a second generated monoclonal self-reactive murine IgM antibody, 1RIIgc7 IgM, was used. Besides its polyreactivity (data not shown), 1RIIgc7 antibodies exhibited high affinities to dsDNA (Tiller et al., 2010 JEM in press).

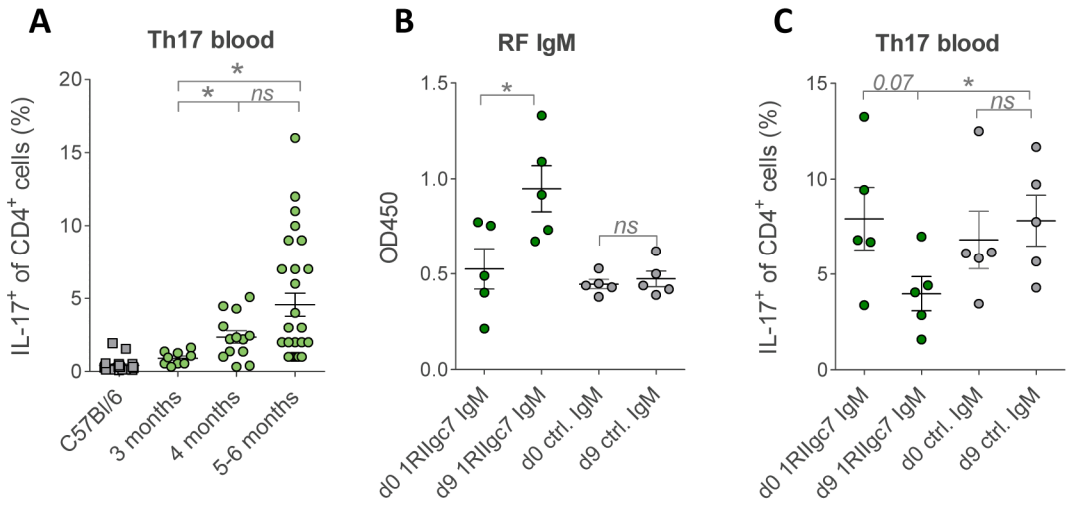

Figure 4.8 Single treatment of aged FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice with self-reactive IgM antibodies reduces Th17 cell frequencies within 9 days. (A) Frequencies of CD4^{+}IL-17^{+} Th17 cells as analyzed by FACS in blood of 3, 4 and 5-6 month-old FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice and 6 month old wild type C57BL/6 mice. Bar graphs show the mean value with standard error of the mean (SEM) for each group of mice. **(B)** Anti-IgG RF IgM antibody levels in serum of 6 month-old FcγRIIB$^{-/-}$TLR9$^{-/-}$ on day 0 and d 9 after treatment with 200µg 1RIIgc7 IgM or 200µg ctrl. IgM as analyzed by ELISA. Symbols represent data from individual mice. Horizontal lines show mean values with standard error of the mean (SEM). One representative out of two independent ELISA is shown for each image. **(C)** Frequencies of CD4^{+}IL-17^{+} Th17 cells as analyzed by FACS in blood of 6 month-old FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice on day 0 and d 9 after treatment with 200µg 1RIIgc7 or ctrl. IgM antibody. Symbols represent data from individual mice. Horizontal lines show mean values with standard error of the mean (SEM). One representative out of three independent experiments is shown.

Due to their high affinities to DNA, we expected even more considerable effects by using 1RIIgc7 IgM antibodies. One intraperitoneal (i.p.) transfer of 200µg monoclonal self-and polyreactive 1RIIgc7 IgM or unspecific control IgM antibody into 5-6 month-old FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice was performed. Nine days after the single shot of IgM antibodies, self-reactive IgM serum antibody titers were analysed by ELISA. 1RIIgc7 IgM antibody treated FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice showed significantly

elevated levels of anti-IgG rheumatoid factor (RF) serum IgM antibodies, whereas no increase in self-reactive IgM antibodies was detectable in control IgM antibody treated mice *(Fig 4.8 B).* Flow cytometric analysis of Th17 cell frequencies in the peripheral blood cell pool revealed a reduction nine days after administration of self-reactive 1RIIgc7 but not after treatment with unspecific control IgM antibodies *(Fig 4.8 C).* Strikingly, one single dose of self-reactive IgM antibodies was sufficient to reduce high frequencies of pro-inflammatory blood Th17 cells after 9 days. Thus, injection of monoclonal self-reactive IgM antibodies is indeed able to diminish high levels of Th17 cells in aged FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice and could therefore be applied in a therapeutical manner.

In summary, we were able to ameliorate lupus disease by long-term reconstitution of self-reactive IgM titers in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice using self-constructed, recombinant produced, monoclonal self-reactive IgM antibodies. Thereby reconstituted mice exhibited decreased frequencies of activated pro-inflammatory CD4^{+} cells, decreased plasma cell numbers and pathogenic IgG autoantibody titers as well as no renal infiltration of activated macrophages and reduced nephritis and mortality.

4.3 Low levels of self-reactive IgM antibodies in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice correlate with reduced frequencies of peritoneal B-1 cells

Low levels of self-reactive serum IgM antibodies in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice compared to FcγRIIB$^{-/-}$ mice noticeably correlated with augmented disease progression. Hence, as already demonstrated in the Mrl/*lpr* lupus mouse model (Boes, Schmidt et al. 2000) high titers of self-reactive IgM antibodies seem to protect FcγRIIB$^{-/-}$ mice from fatal disease progression. Cross-breeding of FcγRIIB$^{-/-}$ mice with TCRβ$^{-/-}$ mice, which produce neither CD4^{+} nor CD8^{+} T cells, indicated that the development of pathogenic self-reactive antibodies of the IgG isotype was dependent on T cell help *(Fig 4.9 A).* By contrast, the generation of protective self-reactive IgM antibodies in FcγRIIB$^{-/-}$ mice did not require any T cell help. Serum analysis of 6 month-old FcγRIIB$^{-/-}$ and age-matched FcγRIIB$^{-/-}$TCRβ$^{-/-}$ mice clearly demonstrated that IgM antibodies specific for nuclear antigens and IgG RF were generated T cell independently (TI) *(Fig 4.9 B).*

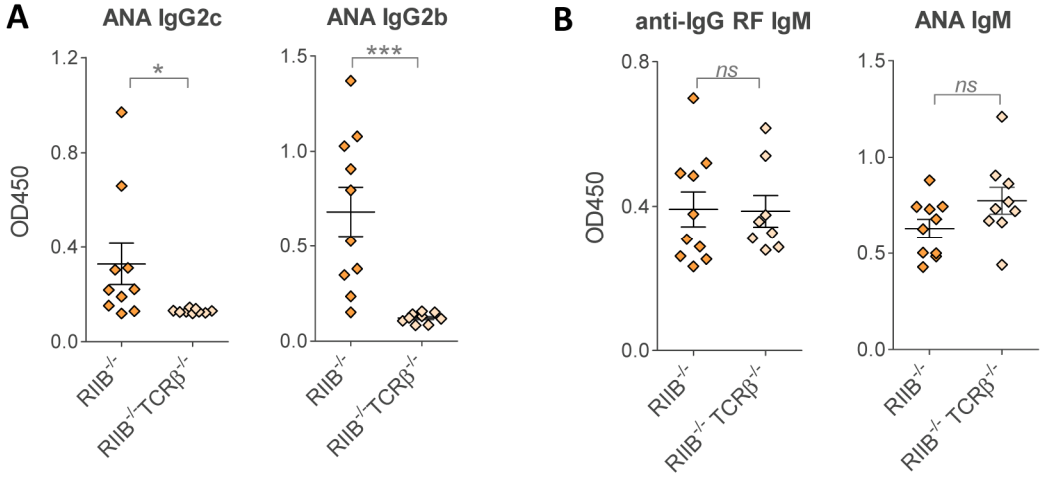

Figure 4.9 The development of self-reactive IgM antibodies in FcγRIIB$^{-/-}$ mice is T helper cells independent (TI). (A) ANA IgG2c and IgG2b serum autoantibody levels of 6 month-old FcγRIIB$^{-/-}$ (RIIB$^{-/-}$) and FcγRIIB$^{-/-}$TCRβ$^{-/-}$ (RIIB$^{-/-}$TCRβ$^{-/-}$) mice as analyzed by ELISA. **(B)** ANA IgM and anti IgG RF IgM serum autoantibody levels of 6 month-old FcγRIIB$^{-/-}$ (RIIB$^{-/-}$) and FcγRIIB$^{-/-}$ TCRβ$^{-/-}$ (RIIB$^{-/-}$TCRβ$^{-/-}$) mice as analyzed by ELISA. Horizontal lines show mean values with standard error of the mean (SEM). One representative out of two independent ELISA is shown for each image.

During the last years, two distinct B-lymphocyte populations, with innate-like functions have been associated with TI IgM antibody responses: Splenic marginal zone (MZ) B cells and B-1 cells, that reside within the peritoneal and pleural cavities (Martin, Oliver et al. 2001). TI generated IgM antibodies were thereby denotated as "natural IgM antibodies". The major proportion of natural IgM antibodies is said to be poly- and also self-reactive to among others phylogenetically conserved structures, such as nucleic acids (Martin, Oliver et al. 2001; Viau and Zouali 2005; Ferry, Potter et al. 2007). Thus we hypothesized that either MZ B cells or peritoneal B-1 cells might be the source of self-reactive IgM. To illustrate whether differences in self-reactive serum IgM levels in 5-6 month-old FcγRIIB$^{-/-}$ and FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice were associated with abnormalities in either MZ or B-1 B cell compartments the frequency of splenic and peritoneal B cell populations were measured by flow cytometry. Peritoneal cells were isolated by peritoneal lavage. No significant differences in the frequencies of total splenic B220^{+}IgM^{+} B cells *(Fig 4.10 A),* CD21^{+}CD23^{-}B220^{+}IgM^{+} MZ B cells *(Fig 4.10 B)* as well as peritoneal IgM^{+}MacI^{-} B-2 cells *(Fig 4.10 C)* were observed comparing FcγRIIB$^{-/-}$ and FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice**.**

By contrast, analysis of the peritoneal B-1 cell compartment revealed that FcγRIIB$^{-/-}$ mice exhibit decreased peritoneal B-1 cell populations compared to C57BL/6 wild

type mice. Interestingly, the most prominent reduction of peritoneal IgM^{high} MacI^{int} B-1 cell frequencies was observed in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice. More precisely, the peritoneal cavity of FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice contained only two-thirds of the B-1 cells measured in FcγRIIB$^{-/-}$ mice and compared to wild type mice B-1 cell frequencies were reduced to less than 50% *(Fig 4.10 D).*

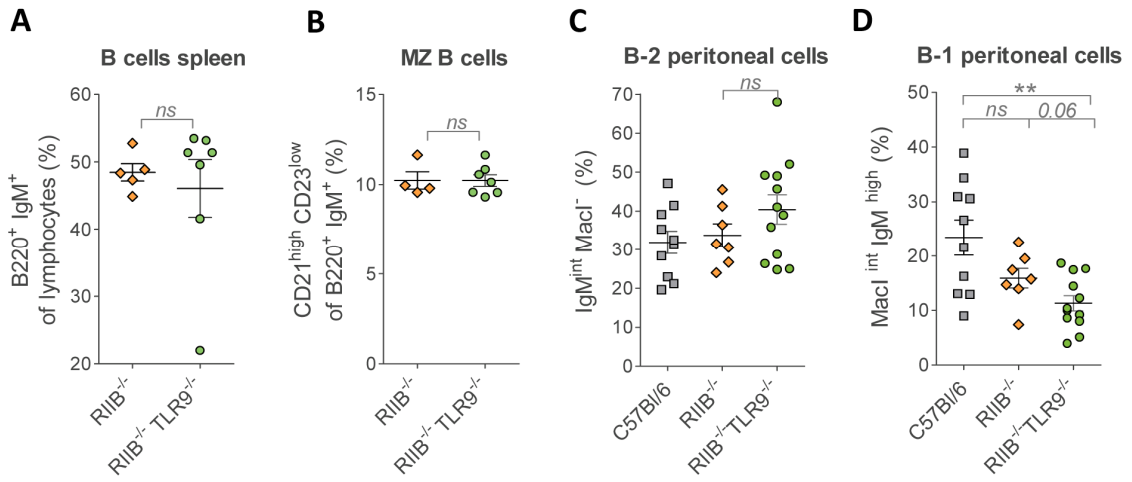

Figure 4.10 TLR9 stimulation in FcγRIIB$^{-/-}$ mice regulates peritoneal B-1 cell frequencies. Frequencies of **(A)** total B220^{+}IgM^{+} splenic B cells, **(B)** CD21^{high}CD23^{low} marginal zone (MZ) B cells out of B220^{+}IgM^{+} spleen cells, **(D)** IgM^{int} Mac^{-}peritoneal B-2 cells, **(E)** IgM^{high} MacI^{+} peritoneal B-1 cells of 5-6 month-old C57BL/6 wild type, FcγRIIB$^{-/-}$ (RIIB$^{-/-}$) or RIIB$^{-/-}$TLR9$^{-/-}$ mice. Symbols represent data from individual mice. Horizontal lines show mean values with standard error of the mean (SEM).

As FcγRIIB$^{-/-}$ TLR9$^{-/-}$ mice showed no differences in MZ B cell frequencies compared to FcγRIIB$^{-/-}$ mice, we assumed that MZ B cells are not associated with the formation of self-reactive IgM antibodies.

This was further confirmed by the depletion of marginal zone B cells in FcγRIIB$^{-/-}$ mice via the i.p. injection of an anti-CD21 antibody (Whipple, Shanahan et al. 2004). For the depletion experiments 4µg of anti-CD21 antibody were injected on day 0 and day 8. Flow cytometric analysis demonstrated that two days after the administration of anti-CD21 antibody, the marginal zone B cell compartment was depleted for the most part *(Fig 4.11 A).* Serum samples of anti-CD21 antibody treated mice were taken at day 0, 5 and 13 and analyzed for total IgM antibody titers and self-reactive IgM antibody levels by ELISA. On day 13 total IgM antibody titers decreased from 272µg/ml to 220µg/ml but notably no reduction of anti-nuclear (ANA) IgM antibody levels were observed *(Fig 4.11 B and C).* Thus, depletion of MZ

B cells in FcγRIIB$^{-/-}$ mice did not result in diminished levels of self-reactive IgM antibodies; therefore we conclude that MZ B cells do not contribute to their generation.

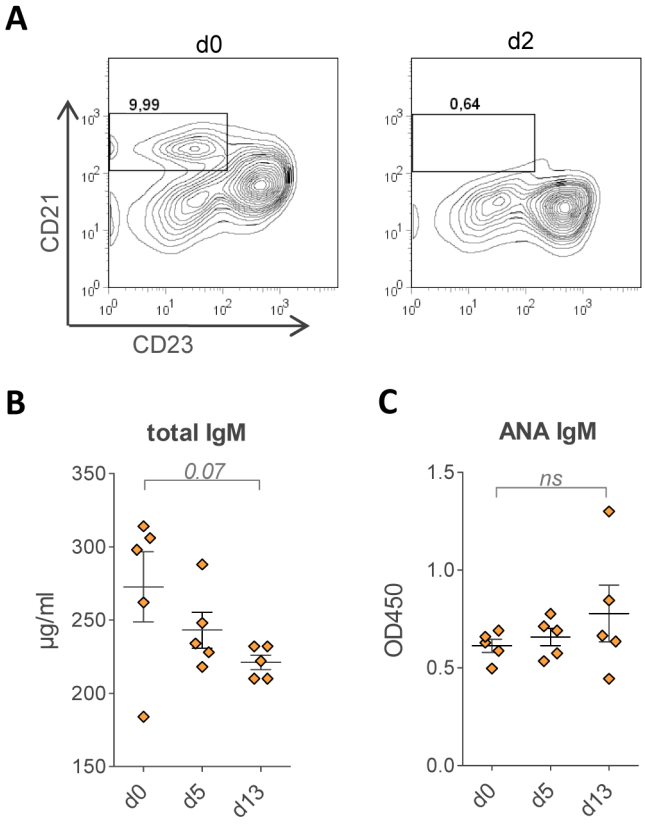

Figure 4.11 Marginal zone B cells do not contribute to the generation of self-reactive IgM in FcγRIIB$^{-/-}$ mice. FcγRIIB$^{-/-}$ (RIIB$^{-/-}$) mice were treated i.p. with PBS or 4µg aCD21 (7G6) antibody at day 0 and 8. **(A)** Efficiency of treatment was monitored in parallel approaches by FACS at day (d) 2. **(B)** Total serum IgM and **(C)** ANA serum IgM levels at d 0, 5 and 13 as analyzed by ELISA. Symbols represent data from individual mice. Horizontal lines show mean values with standard error of the mean (SEM). One representative out of two independent experiments is shown.

To evaluate whether differences in self-reactive IgM serum antibody levels comparing the two mouse strains were due to the low frequencies of peritoneal B-1 cells in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice, 2x10^7 total splenocytes and 5x10^6 total peritoneal cells were cultured *ex vivo* for 48h. Supernatants were analysed for the spontaneous production of self-reactive IgM antibodies by ELISA. As mentioned before, the here analyzed 6 month-old FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice again displayed decreased titers of ANA serum IgM antibodies compared to age-matched FcγRIIB$^{-/-}$ mice *(Fig 4.12 A, first panel).* Splenocytes from FcγRIIB$^{-/-}$ and FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice produced equal amounts of ANA IgM antibodies *(Fig 4.12 A, second panel).* However, the production of ANA

IgM by peritoneal cells was significantly lower in the absence of TLR9 *(Fig 4.12 A, third panel).* Thus, low levels of self-reactive IgM serum antibodies in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice correlated with low frequencies of peritoneal B-1 cells.

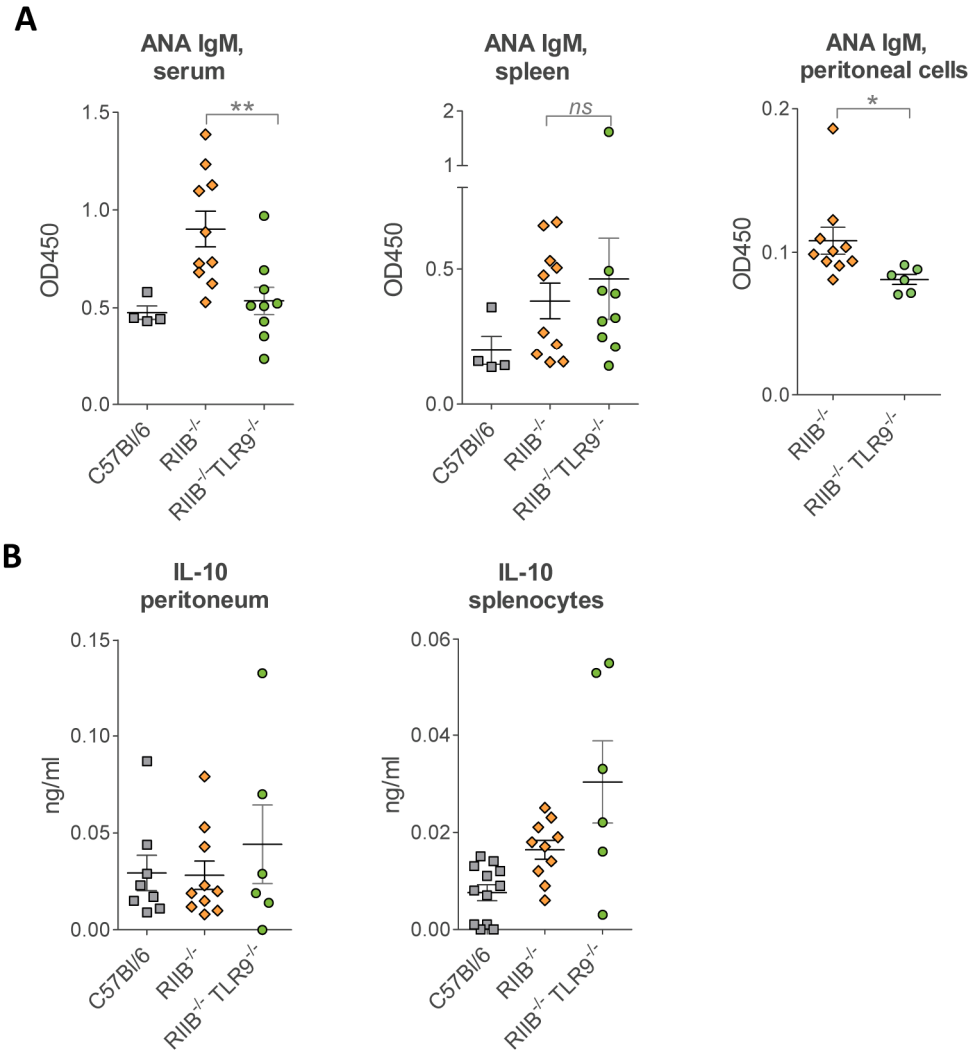

Figure 4.12 Reduced frequencies of peritoneal B-1 cells in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice are associated with low titers of self-reactive IgM but do not affect IL-10 levels. (A) ANA IgM antibody levels in serum, supernatants of 2x10^7 cultured (48h) total splenocytes or supernatants of 5x10^6 *ex vivo* cultured (48h) total peritoneal cells of 6 months old wild type C57BL/6, FcγRIIB$^{-/-}$ or FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice as analyzed by ELISA. **(B)** IL-10 levels in supernatants of 2x10^7 cultured (48h) total splenocytes or supernatants of 5x10^6 cultured (48h) total peritoneal cells of 6 months old wild type C57BL/6, FcγRIIB$^{-/-}$ or FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice as analyzed by ELISA. Symbols represent data from individual mice. Horizontal lines show mean values with standard error of the mean (SEM). One representative out of three independent experiments is shown.

Recent publications have identified B cell regulatory activities and negative regulation through IL-10 production. Particularly peritoneal B-1 B cells are mentioned in this context (Sindhava, Woodman et al. 2010). However, there are

controversial data regarding the role of IL-10 in lupus-like animal models and in human SLE (Yin, et al. 2002; Ishida H et al, 1994). Nevertheless, we tested the IL-10 production by peritoneal cells and splenocytes of FcγRIIB$^{-/-}$ and FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice to see if there are detectable differences due to the reduction of B-1 cell frequencies in FcγRIIB$^{-/-}$ mice lacking TLR9. Peritoneal cells from FcγRIIB$^{-/-}$ and FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice produced equal amounts of IL-10 *(Fig 4.12 B, first panel)* and FcγRIIB$^{-/-}$ splenocytes lacking TLR9 secreted even slightly more IL-10 than FcγRIIB$^{-/-}$ splenocytes *(Fig 4.12 B, second panel)*. Thus, exacerbated disease progression in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice is apparently not based on reduced IL-10 levels but on reduced self-reactive IgM serum levels due to lowered peritoneal B-1 cell numbers.

4.4 Missing TLR9 stimulation in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice affects particularly B-1b cell frequencies

Based on the expression of surface markers, two populations of B-1 B cells, B-1a and B-1b cells, can be discriminated in the peritoneal cavity, which are both IgM^{+}MacI^{int}. Whereas B-1a cells are characterized as CD5^{+} and B220^{dull}, B-1b cells are B220^{+} and do not express CD5 on their surface (Stall et al, 1992).

In chapter *4.3* we demonstrated that TLR9 deficiency in FcγRIIB$^{-/-}$ mice results in reduced frequencies of total peritoneal B-1 cells. To illustrate whether the low B-1 B cell frequencies were due to either the reduction of B-1a cells or B-1b cells, the B-1 cell populations of C57BL/6 wild type, FcγRIIB$^{-/-}$ and FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice were analyzed for the surface expression of CD5 and B220 by flow cytometry *(Fig 4.13 A)*. Whereas lowered frequencies of B-1 cells in FcγRIIB$^{-/-}$ mice compared to wild type mice were caused by a slight reduction of both, B-1a and B-1b cell frequencies, reduced frequencies of B-1 cells in FcγRIIB$^{-/-}$ mice lacking TLR9 compared to FcγRIIB$^{-/-}$ mice were clearly associated with a significant reduction in peritoneal CD5^{-} B-1b cells. The absence of TLR9 had no influences on B-1a cell frequencies in FcγRIIB$^{-/-}$ mice *(Fig 4.13 B)*.

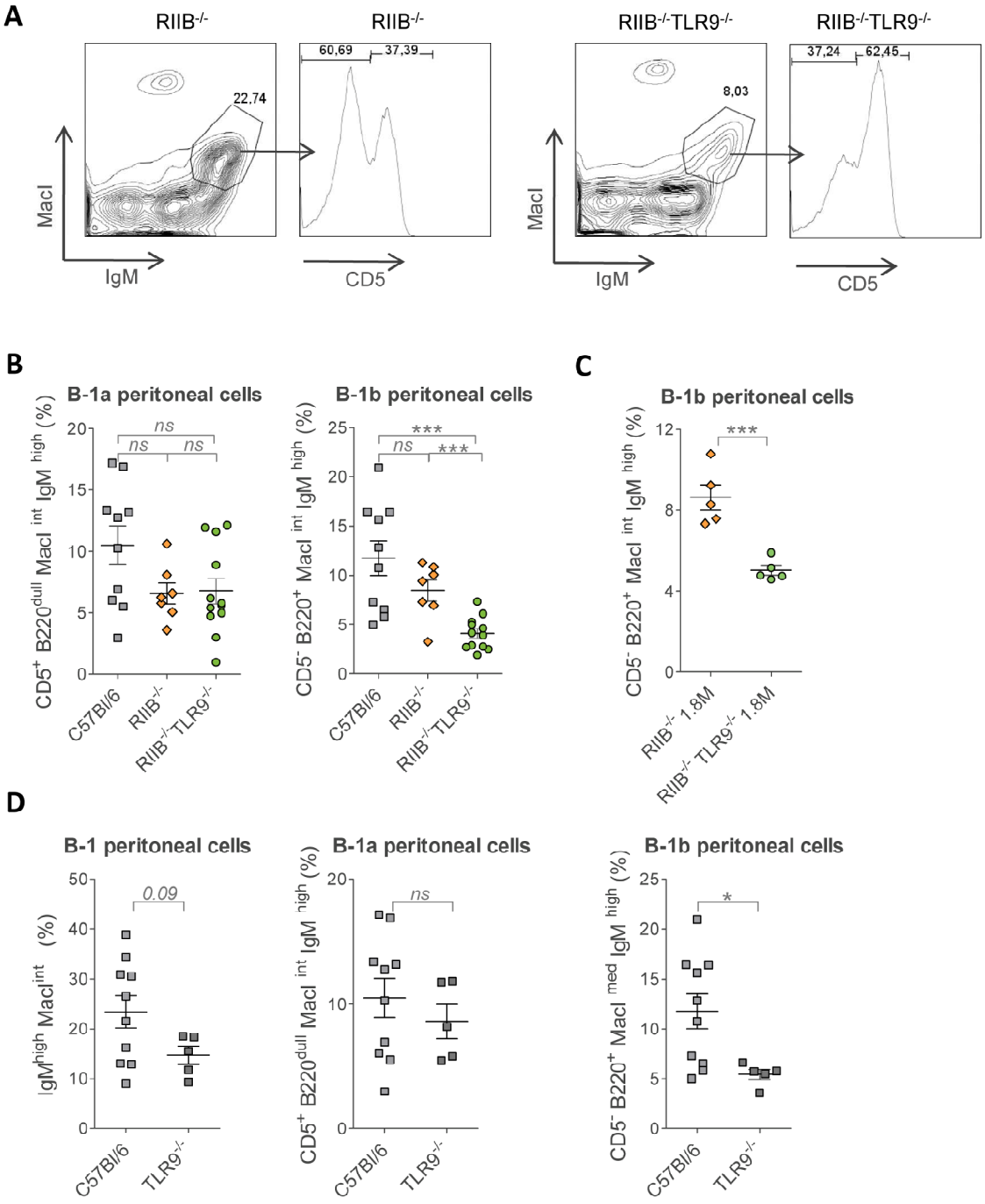

Figure 4.13 FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice exhibit reduced frequencies of peritoneal B-1b cells. (A) Representative FACS analyses of peritoneal lymphocytes of FcγRIIB$^{-/-}$ and FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice. Cells were primarily stained for IgM and MacI. The MacI^{int} IgM^{high} B-1 cell population was further analyzed for CD5 and B220 expression. **(B+C)** Frequencies of CD5^{+} B220^{dull} MacI^{int} IgM^{high} B-1a cells or CD5^{-} B220^{+} MacI^{int} IgM^{high} B-1b cells from peritoneal lymphocytes of **(B)** 5-6 month-old C57BL/6, FcγRIIB$^{-/-}$, or FcγRIIB$^{-/-}$TLR9$^{-/-}$mice and **(C)** of 1.8 month-old FcγRIIB$^{-/-}$, or FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice. **(D)** Frequencies of MacI^{int} IgM^{high} B-1 cells, CD5^{+} B220^{dull} MacI^{int} IgM^{high} B-1a cells or CD5^{-} B220^{+} MacI^{int} IgM^{high} B-1b cells from peritoneal lymphocytes of 6 month old C57BL/6 and TLR9$^{-/-}$ mice. Symbols represent data from individual mice. Horizontal lines show mean values with standard error of the mean (SEM). One representative out of three independent experiments is shown.

Adressing the question whether the reduction of B-1b cell frequencies in FcγRIIB$^{-/-}$ TLR9$^{-/-}$ mice was dependent on age, the distribution of B-1 cells into CD5^{+} B220^{dull} B-1a and CD5^{-} B220^{+} B-1b cells of young, 1.8 months old FcγRIIB$^{-/-}$ and FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice was compared. Results indicated that already in young TLR9 deficient FcγRIIB$^{-/-}$ mice frequencies of B-1b cells were diminished *(Fig 4.13 C).* To confirm that reduced frequencies of B-1b cells in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice were explicitely dependent on the lack of TLR9, we additionally analyzed 6 month old TLR9$^{-/-}$ mice. TLR9 deficiency led to a decrease of total peritoneal B-1 cells and to a clear recuction of the B-1b cell population whereas peritoneal B-1a cells were not affected *(Fig 4.13 D).* Thus, TLR9 stimulation plays a key role in regulating peritoneal B-1b cell frequencies.

4.5 TLR9 stimulation of B-1 cells promotes the proliferation of B-1b but hardly B-1a cells and induces the generation of self-reactive IgM antibodies *in vitro* and *in vivo*

Absence of TLR9 in FcγRIIB$^{-/-}$ mice led to decreased numbers of peritoneal B-1b cells and was associated with low levels of self-reactive IgM antibodies. Recent publications have indicated an association of TLR9 signaling in B-1 cells with the generation of self-reactive IgM (Genestier, Taillardet et al. 2007; Kubo, Uchida et al. 2009).

To evaluate the role of TLR9 signaling in IgM production by B-1 cells, we used FACS sorted FcγRIIB$^{-/-}$ peritoneal IgM^{+}MacI^{int} B-1 cells. The purity obtained for positive cell sorting by FACS was higher than 95% *(Fig 4.14 A).* Subsequently, sorted B-1 cells were *in vitro* stimulated with 5µg/ml CpG ODN 1826. Moreover, TLR7 stimulation by TLR7 agonist Imiquimod (5µg/ml) was performed to additionally evaluate, whether TLR7 signaling is able to induce self-reactive IgM antibodies. Both, TLR9 and TLR7 are located in the endosomes and stimulation of TLR9 and TLR7 by pathogenic nucleotide structures triggers signal transduction via the adaptor protein MyD88. Thus, the two TLRs might exhibit similar capacities (Takeda and Akira 2005). After 96h stimulated B-1 cells and supernatants were analyzed. B-1 cell stimulation with CpG ODN 1826 alone induced the highest proliferation rates *(Fig 4.14 B)* and therefore the highest amounts of total IgM antibodies in the supernatants *(Fig 4.14 C).* This TLR9-dependent effect was inhibited by the addition of the TLR7 ligand Imiquimod *(Fig 4.14 B and C).* An antagonism between TLR7 and TLR9 in B cells *in vitro* has been

described before (Booth, Buza et al. 2010). TLR7 stimulation alone was not able to induce comparable proliferation rates and IgM antibody production was negligible *(Fig 4.14 B and C).*

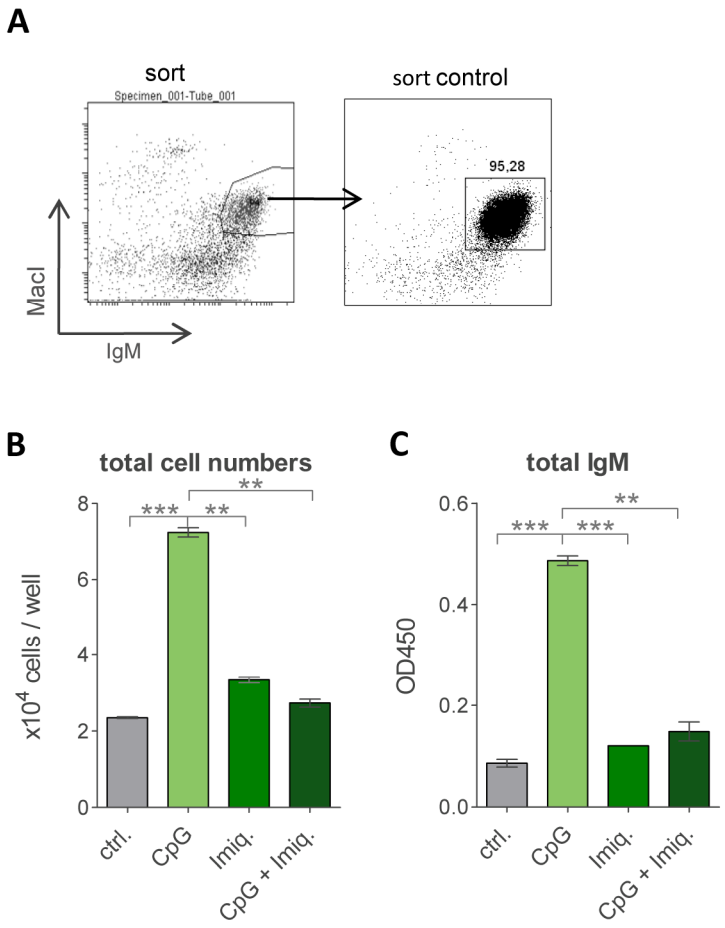

Figure 4.14 TLR9 stimulation of FcγRIIB$^{-/-}$ B-1 cells *in vitro* induces cell proliferation and the accumulation of IgM antibodies. Sorted MacI^{int} IgM^{high} FcγRIIB$^{-/-}$ B-1 cells were *in vitro* stimulated for 96h with either 5µg/ml CpG ODN 1826 or 5µg/ml Imiquimod or a combination of both. **(A)** Representative plot of MacI^{int} IgM^{high} sorted FcγRIIB$^{-/-}$ B-1 cells by FACS Aria cell sorter and respective sort control. **(B)** Total cell numbers of sorted FcγRIIB$^{-/-}$ B-1 cells 96h after stimulation as estimated using a Neubauer chamber. **(C)** Total IgM levels in supernatants of 96h stimulated MacI^{int} IgM^{high} FcγRIIB$^{-/-}$ B-1 cells as analyzed by ELISA. Bar graphs show the mean value with standard error of the mean (SEM) for each group. One representative out of three independent experiments is shown.

After 96h, sorted peritoneal IgM^{+}MacI^{int} B-1 cells were analyzed for CD5 and B220 expression by flow cytometry. Results indicated that CpG stimulation primarily affected the B220^{+}CD5^{-} B-1b cell population (*Fig 4.15 A*). Minor effects on B-1a cells upon CpG stimulation were observed. TLR7 stimulation showed virtually no effect on B-1a or B-1b cells compared to non-stimulated B-1 cells *(Fig 4.15 A).* Referring to

total cell numbers, TLR9 stimulation induced a more than 10-fold increase in B-1b cell numbers compared to non-stimulated and Imiquimod stimulated B-1 cells, whereas marginal increases in B-1a cells were observed *(Fig 4.15 B).* Concurrent stimulation of TLR9 and TLR7 by the addition of CpG and Imiquimod did not increase B-1b cell numbers, thus again demonstrating the inhibitory capacity of TLR7 *(Fig 4.15 A and B).*

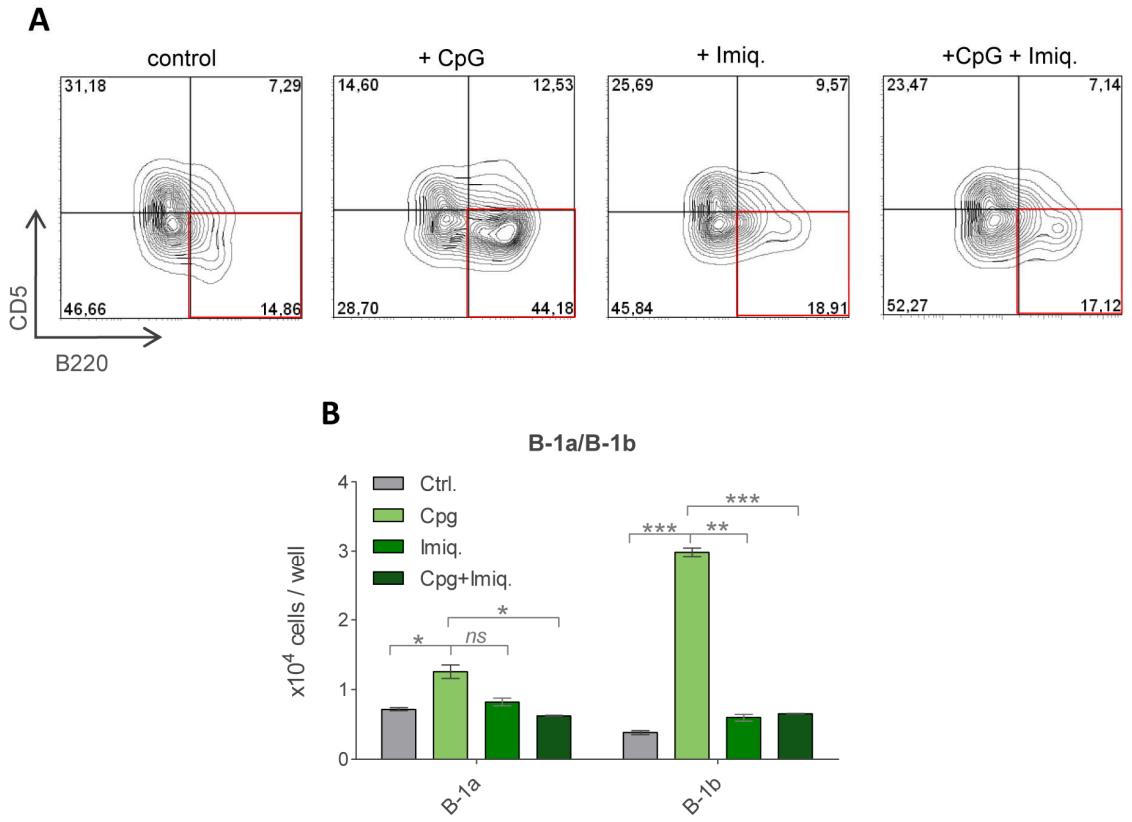

Figure 4.15 TLR9 stimulation of FcγRIIB$^{-/-}$ B-1 cells *in vitro* promotes the proliferation of particularly B-1b cells. Sorted MacI^{int} IgM^{high} FcγRIIB$^{-/-}$ B-1 cells were *in vitro* stimulated for 96h with either 5µg/ml CpG ODN 1826 or 5µg/ml Imiquimod or a combination of both. **(A)** Representative plots of FACS analysis of CD5^{+} B220^{dull} B-1a or CD5^{-} B220^{+} B-1b cells (marked red) after 96h of stimulation. One representative plot is shown for each image. **(B)** Total numbers of FcγRIIB$^{-/-}$ CD5^{+} B220^{dull} B-1a or CD5^{-} B220^{+} B-1b cells after 96h of stimulation. Bar graphs show the mean value with standard error of the mean (SEM) for each group. One representative out of three independent experiments is shown.

We conclude therefore that high amounts of produced IgM antibodies upon TLR9 stimulation derived from activated proliferating B-1b cells. Furthermore, TLR7 signaling does not compensate the loss of TLR9 signaling in B-1 cells and is therefore not able to induce the generation of self-reactive IgM antibodies in our *in vitro* assay.

To verify these findings *in vivo*, FcγRIIB$^{-/-}$ mice were i.p. immunized with either 50µg CpG ODN 1826, 25µg Imiquimod or a combination of both. 7 days after administration of TLR stimuli, FcγRIIB$^{-/-}$ mice were sacrificed and peritoneal cells were isolated by peritoneal lavage. Consistent with the *in vitro* data, CpG stimulation of FcγRIIB$^{-/-}$ mice induced the highest proliferation rate in total peritoneal cells. As already observed *in vitro*, additional injection of TLR7 agonist Imiquimod inhibited CpG induced cell proliferation. TLR7 agonist alone did not induce the proliferation of peritoneal cells *(Fig 4.16 A).* Considering the level of total serum IgM antibodies, a significant increase was observed on day 7 in serum of CpG stimulated mice, whereas Imiquimod as well as CpG and Imiquimod stimulated mice showed no rise of serum IgM antibodies *(Fig 4.16 B).*

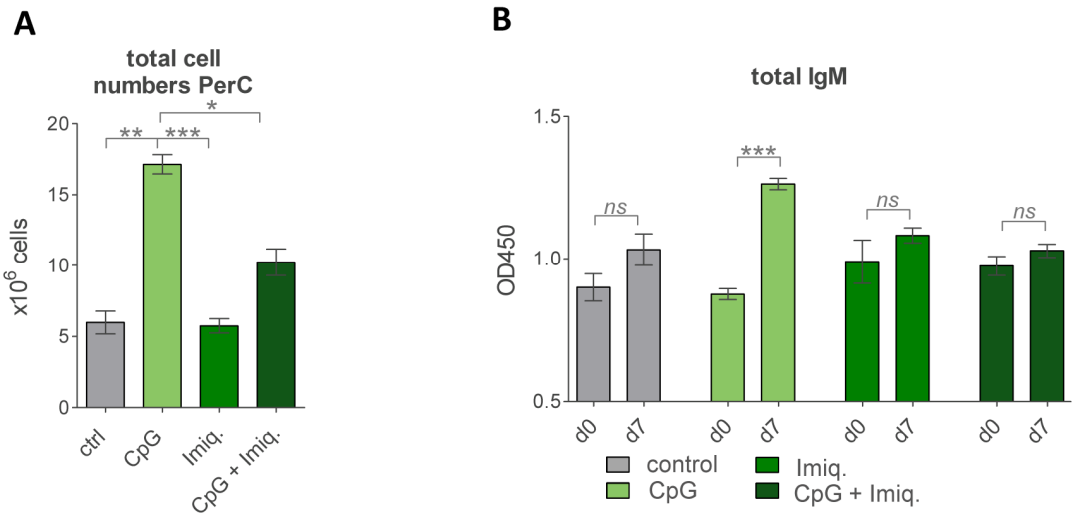

Figure 4.16 TLR9 stimulation *in vivo* induces the proliferation of peritoneal cells and the accumulation of IgM antibodies. FcγRIIB$^{-/-}$ mice were *in vivo* stimulated with either 50µg/ml CpG ODN 1826 (n=5) or 25µg/ml Imiquimod (n=6) or a combination of both (n=6) and analyzed at day 7 (d 7). **(A)** Total cell numbers of peritoneal cells in FcγRIIB$^{-/-}$ mice 7 days after stimulation as estimated using a Neubauer chamber. **(B)** Total IgM levels in serum of FcγRIIB$^{-/-}$ mice before (d 0) and 7 days after (d 7) stimulation as analyzed by ELISA. Bar graphs show the mean value with standard error of the mean (SEM) for each group. One representative out of two independent experiments is shown.

Flow cytometric analysis and subsequent subdivision of peritoneal IgM^{high} MacI^{int} B-1 cells in CD5^{+} B-1a and CD5^{-} B-1b cells 7 days after TLR stimulation indicated again, that CpG hardly induced the proliferation of CD5^{+} B-1a cells *(Fig 4.17 A).* Instead, CpG administration particularily stimulated peritoneal CD5^{-} B-1b cells, which led to a 6-fold increase in B-1b cell numbers *(Fig 4.17 B)* and thereby elevated total B-1 cell

numbers *(Fig 4.17 C).* Also *in vivo* TLR7 stimulation was not able to induce the proliferation of B-1b cells *(Fig 4.17 A and B).* Thus, results shown here were consistent with the *in vitro* data. Notably, TLR9 stimulation *in vitro* and *in vivo* resulted in proliferation of FcγRIIB$^{-/-}$ peritoneal B-1b cells and led to the generation of significantly increased amounts of IgM antibodies.

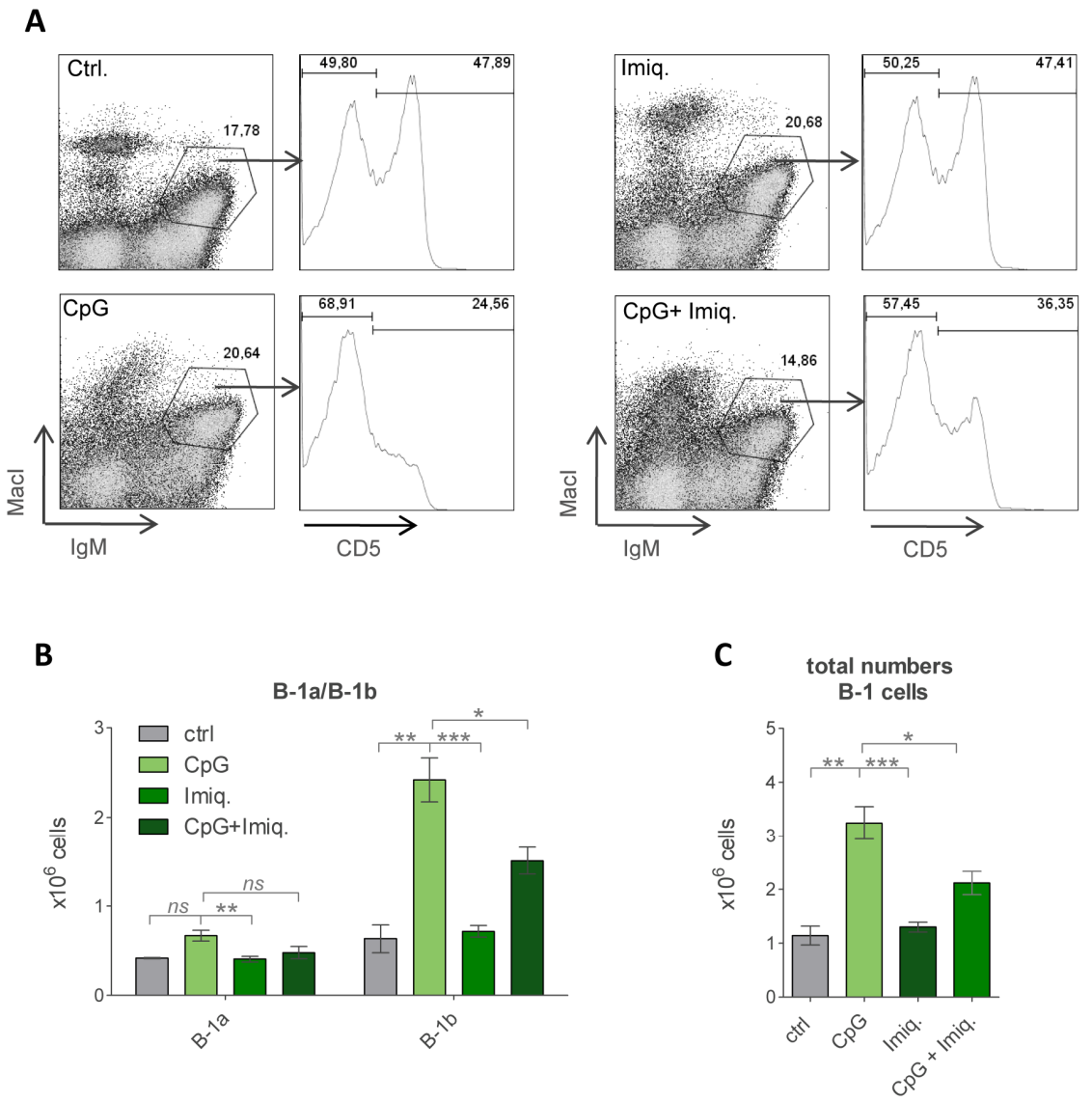

Figure 4.17 TLR9 stimulation *in vivo* promotes the proliferation of particularly peritoneal B-1b cells. FcγRIIB$^{-/-}$ mice were *in vivo* stimulated with either 50µg/ml CpG ODN 1826 (n=5) or 25µg/ml Imiquimod (n=6) or a combination of both (n=6) and analyzed at day 7 (d 7). **(A)** Representative FACS plot of IgM^{high} MacI^{int} B-1 cells, further subdivided into CD5^{+} B-1a or CD5^{-} B-1b cells at d 7 after treatment with either CpG, Imiquimod or a combination of both. One representative out of at least 6 plots is shown for each image. **(B)** Total numbers of FcγRIIB$^{-/-}$ CD5+ B-1a or CD5- B-1b cells at d7 after treatment with either Cpg Imiquimod or a combination of both. **(C)** Total numbers of peritoneal B-1 cells in FcγRIIB$^{-/-}$ mice at day 7 after stimulation. Bar graphs show the mean value with standard error of the mean (SEM) for each group. One representative out of two independent experiments is shown.

4.6 Transfer of TLR9 sufficient FcγRIIB$^{-/-}$ peritoneal B-1b cells suppresses Th17 cell development and rescues FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice from severe autoimmunity

So far, we were able to show that FcγRIIB$^{-/-}$ mice lacking TLR9 exhibit reduced self-reactive IgM levels and B-1b cell frequencies. Furthermore, reconstitution of self-reactive IgM levels by the administration of monoclonal self-reactive IgM antibodies prevented the accumulation of pro-inflammatory Th17 cells and the induction of lupus nephritis in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice. Finally TLR9 stimulation *in vivo* and *in vitro* led to the proliferation of particularly B-1b cells and most notably to an increase of IgM antibody levels indicating an essential role of TLR9 in B-1b cells.
To link these results and to verify the tolerogenic effect of TLR9 in peritoneal B-1b cells via the generation of self-reactive IgM, cell transfer experiments were performed. Transfer experiments were partially done together with the PhD student Alexander Stoehr in our laboratory.

4.6.1 Transfer of total FcγRIIB$^{-/-}$ peritoneal cells

Primarily, total FcγRIIB$^{-/-}$ peritoneal cells were transplanted into FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice *(Fig 4.18 A).* 0.5-1.5 x 10^7 FcγRIIB$^{-/-}$ donor cells per FcγRIIB$^{-/-}$TLR9$^{-/-}$ recipient mouse were injected i.p every other week over a period of 5.5 months until the age of 9 months starting at the age of 3.5 months. As expected, transfer of total peritoneal cells from FcγRIIB$^{-/-}$ mice induced significantly increased levels of ANA and anti-IgG RF IgM autoantibodies after recieving five transfers on day 74 compared to a 6 month-old control group *(Fig 4.18 B)*.
Remarkably, FcγRIIB$^{-/-}$ peritoneal cells suppressed the uncontrolled accumulation and expansion of pro-inflammatory Th17 cells. After recieving five transfers (d74) of FcγRIIB$^{-/-}$ total peritoneal cells, FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice showed reduced frequencies of blood Th17 cells compared to the age matched control group *(Fig 4.18 C).*

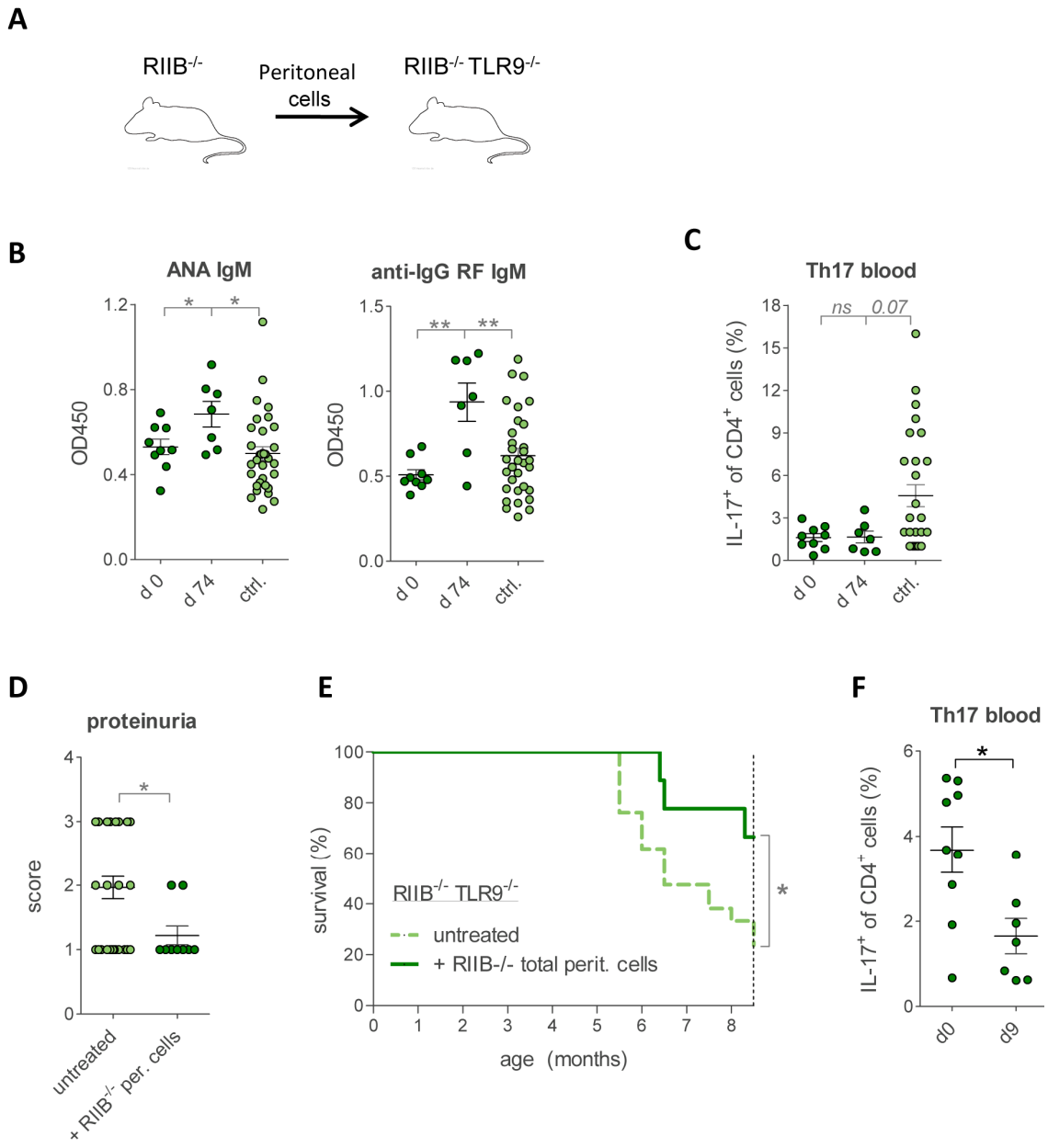

Figure 4.18 Transfer of FcγRIIB$^{-/-}$ total peritoneal cells into FcγRIIB$^{-/-}$ TLR9$^{-/-}$ mice prevents Th17 cell accumulation and lupus nephritis. (A) Scheme; 3.5-4 month old FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice were transplanted with 0.5-1.5 x 10^7 RIIB$^{-/-}$ peritoneal donor cells on day (d) 0, 14, 28, 49 and 64. **(B)** ANA and anti-IgG RF serum IgM autoantibody levels in FcγRIIB$^{-/-}$TLR9$^{-/-}$ (RIIB$^{-/-}$TLR9$^{-/-}$) mice before (d 0) and after 5 i.p. transfers (d74) of total peritoneal cells from donor RIIB$^{-/-}$ mice in comparison to untreated 5-6 month-old RIIB$^{-/-}$TLR9$^{-/-}$ mice as analyzed by ELISA. Horizontal lines show mean values with standard error of the mean (SEM). One representative out of three independent ELISA is shown**. (C)** Frequencies of blood CD4$^+$IL-17$^+$ Th17 cells in RIIB$^{-/-}$ TLR9$^{-/-}$ mice before (d 0) and after 5 i.p. transfers (d74) of peritoneal cells from donor RIIB$^{-/-}$ mice in comparison to untreated 5-6 month-old RIIB$^{-/-}$TLR9$^{-/-}$ mice as analyzed by FACS. Symbols represent data from individual mice. Horizontal lines show mean values with standard error of the mean (SEM). **(D)** Proteinuria scores in 6 month-old FcγRIIB$^{-/-}$ (RIIB$^{-/-}$; n=39), FcγRIIB$^{-/-}$TLR9$^{-/-}$ (n=29) and FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice transplanted with FcγRIIB$^{-/-}$ peritoneal cells (n=9). Symbols represent data from individual mice. Horizontal lines show mean values with standard error of the mean (SEM). **(E)** Kaplan-Meier survival curves for untreated

FcγRIIB$^{-/-}$TLR9$^{-/-}$ (RIIB$^{-/-}$TLR9$^{-/-}$) mice (n=21) and peritoneal cell treated RIIB$^{-/-}$TLR9$^{-/-}$ mice (n=9). **(F)** Frequencies of blood CD4^{+}IL-17^{+} Th17 cells in 6 month old FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice before (d0) and 9 days after i.p transfer of 1x10^{7} FcγRIIB$^{-/-}$ peritoneal cells (d 9) as analyzed by FACS. Symbols represent data from individual mice. Horizontal lines show mean values with standard error of the mean (SEM).

Additionally, the incidence of proteinuria was significantly reduced in FcγRIIB$^{-/-}$TLR9$^{-/-}$ recipient mice *(Fig 4.18 D).* At 6 months of age only 22% of the recipient mice suffered from proteinuria compared to 55% of the age-matched control group. These results were in line with the nephritis-induced mortality rate. Transfer of TLR9-sufficient peritoneal cells clearly improved survival rates of FcγRIIB$^{-/-}$TLR9$^{-/-}$ recipient mice *(Fig 4.18 E).*

To test whether FcγRIIB$^{-/-}$ peritoneal cells were able to reduce high frequencies of T helper 17 cells in aged, lupus-prone mice, one single transfer of 1x10^{7} FcγRIIB$^{-/-}$ total peritoneal cells in 6 month old FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice was performed. Recipient mice were screened for high levels of Th17 cells before the transfer. Impressively, one single transfer of FcγRIIB$^{-/-}$ peritoneal cells dropped the Th17 cell frequencies in peripheral blood to less than 50% after nine days *(Fig 4.18 F).*

To confirm the central role of TLR9 in this approach, also FcγRIIB$^{-/-}$TLR9$^{-/-}$ total peritoneal cells (1x10^{7} cells per recipient mouse) were transferred into aged FcγRIIB$^{-/-}$TLR9$^{-/-}$ recipient mice *(Fig 4.19 A).* Recipient mice were again screened for high levels of Th17 cells in the blood before the transfer. Serum analysis indicated that transferred FcγRIIB$^{-/-}$TLR9$^{-/-}$ peritoneal cells did not induce elevated levels of self-reactive IgM antibodies nine days after the transfer *(Fig 4.19 B).* In contrast to FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice transplanted with TLR9 sufficient FcγRIIB$^{-/-}$ peritoneal cells, Th17 cell frequencies in the blood of mice receiving FcγRIIB$^{-/-}$TLR9$^{-/-}$ peritoneal cells even showed a slightly increase *(Fig 4.19 C).*
Hence, only TLR9 sufficient FcγRIIB$^{-/-}$ total peritoneal cells were able to induce high amounts of self-reactive IgM and suppress pro-inflammatory Th17 response.

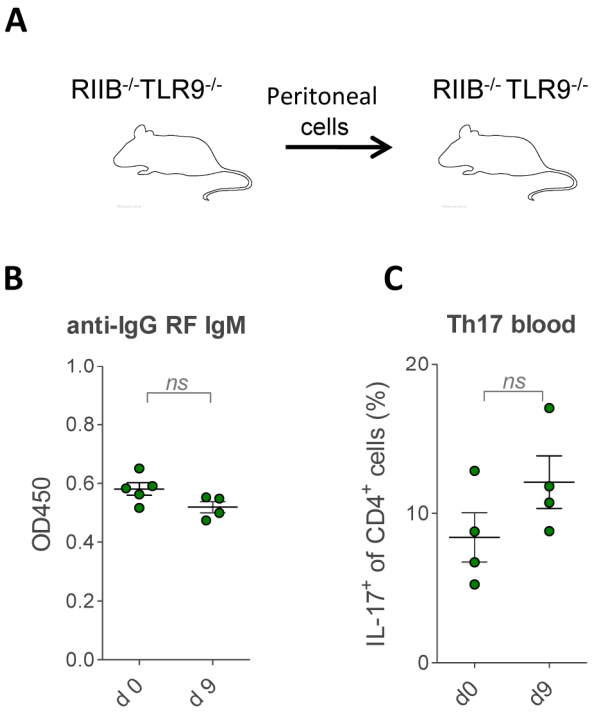

Figure 4.19 Transfer of FcγRIIB$^{-/-}$TLR9$^{-/-}$ total peritoneal cells into FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice does not reduce Th17 cell accumulation within 9 days. (A) Scheme; 1 x 10^7 RIIB$^{-/-}$TLR9$^{-/-}$ peritoneal donor cells were transferred into FcγRIIB$^{-/-}$TLR9$^{-/-}$ recipient mice on day 0. **(B)** Anti-IgG RF serum IgM autoantibody levels in FcγRIIB$^{-/-}$TLR9$^{-/-}$ (RIIB$^{-/-}$TLR9$^{-/-}$) mice before (d 0, 6 month-old) and 9 days after i.p. transfer of total peritoneal cells from donor RIIB$^{-/-}$TLR9$^{-/-}$ mice (d9) as analyzed by ELISA. Symbols represent data from individual mice. Horizontal lines show mean values with standard error of the mean (SEM). One representative out of three independent ELISA is shown. **(C)** Frequencies of blood CD4$^+$IL-17$^+$ Th17 cells in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice before (d 0, 6 month-old) and 9 days after i.p. transfer of peritoneal cells from donor RIIB$^{-/-}$ TLR9$^{-/-}$ mice as analyzed by FACS. Symbols represent data from individual mice. Horizontal lines show mean values with standard error of the mean (SEM).

4.6.2 Transfer of total FcγRIIB$^{-/-}$µS peritoneal cells

Flow cytometric analysis indicated that FcγRIIB$^{-/-}$ total peritoneal cells were amongst others composed of around 20% IgM$^+$ MacI^{int} B-1 cells, 30% IgM$^+$ MacI$^-$ B-2 cells, 15% MacI^{high} macrophages, 3% CD8$^+$ T cells and 6% CD4$^+$CD8$^+$ T cells *(Fig 4.20).* Consistent with recent findings, we virtually did not find any CD4 single positiv T cells in the peritoneal cavity (Glik and Douvdevani 2006).

Thus, besides the enrichment of TLR9 sufficient B-1 cells the transfer of FcγRIIB$^{-/-}$ total peritoneal cells resulted in the accumulation of various other peritoneal cells and also might have implicated augmented production of anti-inflammatory cytokines, such as IL-10.

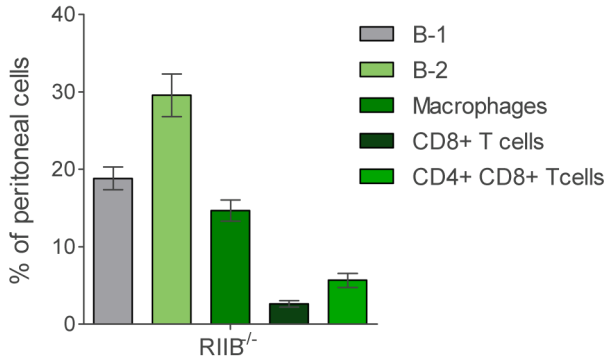

Figure 4.20 Composition of FcγRIIB$^{-/-}$ total peritoneal cells. Frequencies of MacI^{int} IgM^{high} B-1 cells, IgM^{+} MacI^{-} B-2 cells, MacI^{high} macrophages, CD8^{+} T cells and CD4^{+} CD8^{+} T cells in the peritoneum of 6 months old FcγRIIB$^{-/-}$ mice (n=4) as analyzed by FACS. Bar graphs show the mean value with standard error of the mean (SEM) for each group.

In order to prove that indeed self-reactive IgM produced by peritoneal B cells acted as mediator of tolerance, we used mice deficient in secreted IgM and cross-bred them with FcγRIIB$^{-/-}$ mice (FcγRIIB$^{-/-}$ μS). Subsequently, FcγRIIB$^{-/-}$μS total peritoneal cells were transferred into FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice *(Fig 4.21 A).* More precisely, 0.5-1.5 x 10^{7} FcγRIIB$^{-/-}$μS donor cells per FcγRIIB$^{-/-}$TLR9$^{-/-}$ recipient mouse were injected i.p every other week over a period of 5.5 months until the age of 9 months starting at the age of 3.5 months. In contrast to FcγRIIB$^{-/-}$ total peritoneal cells, the transfer of peritoneal cells from FcγRIIB$^{-/-}$μs mice into FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice did not prevent the accumulation of pro-inflammatory Th17 cells *(Fig 4.21 B).* Furthermore, the incidence of proteinuria, as a sign of renal damage, was not reduced at an age of 6 months in FcγRIIB$^{-/-}$TLR9$^{-/-}$ recipient mice compared to an age-matched conrol group *(Fig 4.21 C).* These results were reflected in the respective survival rates; the transfer of total peritoneal cells from FcγRIIB$^{-/-}$μS mice in FcγRIIB$^{-/-}$TLR9$^{-/-}$ recipient mice did not improve the survival *(Fig 4.21 D).* Notably, the attempt of reducing high frequencies of pro-inflammatory blood Th17 cells in aged FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice by only one transfer of 1x10^{7} FcγRIIB$^{-/-}$ μS total peritoneal cells failed, confirming the central role of IgM in the control of Th17 cells *(Fig 4.21 E).*

Accordingly, self-reactive IgM antibodies produced by peritoneal B cells play an essential role in the suppression of lupus nephritis.

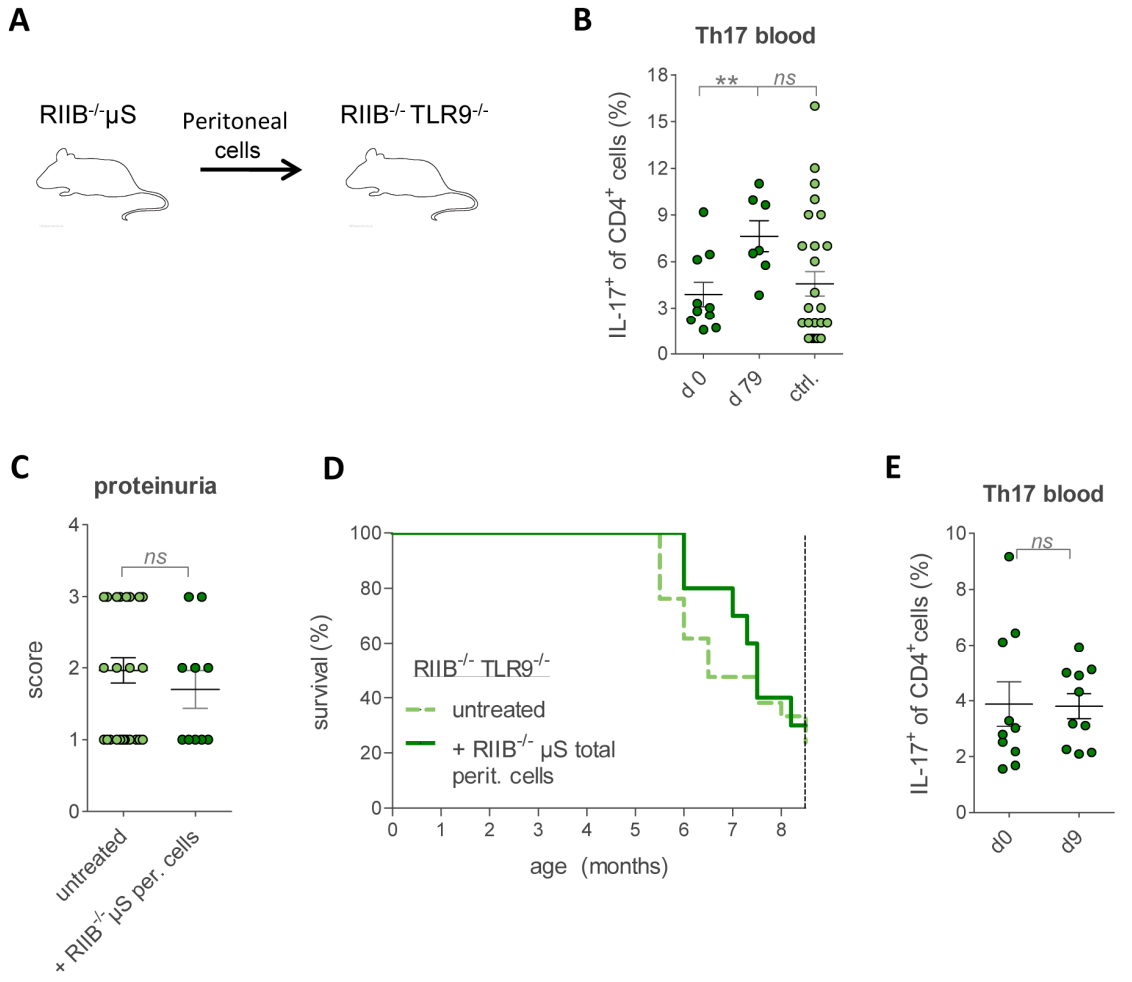

Figure 4.21 Transfer of FcγRIIB$^{-/-}$µS total peritoneal cells in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice does not inhibit Th17 cell accumulation and lupus nephritis.(A) Scheme; 3.5-4 month old FcγRIIB$^{-/-}$ TLR9$^{-/-}$ mice were transplanted with 0.5-1.5 x 10^7 RIIB$^{-/-}$µS peritoneal donor cells on day (d) 0, 14, 28, 49 and 64. **(B)** Frequencies of blood CD4$^+$IL-17$^+$ Th17 cells in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice before (d 0) and after 5 i.p. transfers (d79) of peritoneal cells from donor RIIB$^{-/-}$ µS mice in comparison to untreated 5-6 month-old RIIB$^{-/-}$TLR9$^{-/-}$ mice as analyzed by FACS. Symbols represent data from individual mice. Horizontal lines show mean values with standard error of the mean (SEM). **(C)** Proteinuria scores in 6 month-old FcγRIIB$^{-/-}$ (RIIB$^{-/-}$; n=39), FcγRIIB$^{-/-}$ TLR9$^{-/-}$ (n=29) and FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice treated with RIIB$^{-/-}$µS peritoneal cells (n=10). Symbols represent data from individual mice. Horizontal lines show mean values with standard error of the mean (SEM). **(D)** Kaplan-Meier survival curves for untreated FcγRIIB$^{-/-}$TLR9$^{-/-}$ (RIIB$^{-/-}$TLR9$^{-/-}$) mice (n=21) and RIIB$^{-/-}$µS peritoneal cell treated RIIB$^{-/-}$TLR9$^{-/-}$ mice (n=10). **(E)** Frequencies of blood CD4$^+$IL-17$^+$ Th17 cells in 6 month old FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice before (d0) and 9 days after i.p transfer of 1x10^7 FcγRIIB$^{-/-}$ µS peritoneal cells (d9) as analyzed by FACS. Symbols represent data from individual mice. Horizontal lines show mean values with standard error of the mean (SEM).

4.6.3 Transfer of FcγRIIB$^{-/-}$ peritoneal B-1b cells

As we have demonstrated, the reduction of peritoneal B-1 cells in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice compared to FcγRIIB$^{-/-}$ mice was exclusively based on decreased frequencies of peritoneal B-1b cells. Hence, transfer of sorted FcγRIIB$^{-/-}$ peritoneal IgM^{high}MacI^{int} B220^{+}CD5^{-} B-1b cells into FcγRIIB$^{-/-}$ TLR9$^{-/-}$ mice was performed *(Fig 4.22 A).* 0.5-1x 10^6 FcγRIIB$^{-/-}$ B-1b donor cells per FcγRIIB$^{-/-}$TLR9$^{-/-}$ recipient mouse were injected i.p every other week over a period of 5.5 months until the age of 9 months starting at the age of 3.5 months. Consistent with the transfer of FcγRIIB$^{-/-}$ total peritoneal cells, the transfer of sorted B-1b cells suppressed the expansion of pro-inflammatory Th17 cells. After recieving five transfers of sorted FcγRIIB$^{-/-}$ B-1b cells at an age of 6 month, FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice exhibited decreased frequencies of blood Th17 cells compared to the age-matched control group and to the initially measured value *(Fig 4.22 B).* As expected, proteinuria scores were highly decreased in 6 month old FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice receiving FcγRIIB$^{-/-}$ B-1b cells. Only 14% of FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice transplanted with FcγRIIB$^{-/-}$ B-1b cells displayed signs of renal damage whereas 55% of the age-matched control group did *(Fig 4.22 C).*

By the age of 8.5 months almost 80% of the FcγRIIB$^{-/-}$TLR9$^{-/-}$ control group died due to glomerulonephritis whereas FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice receiving FcγRIIB$^{-/-}$ B-1b cells showed clearly decreased mortality rates with the death of only 24% of the transplanted mice *(Fig 4.22 D).* The transfer of FcγRIIB deficient B-1b cells in FcγRIIB$^{-/-}$ TLR9$^{-/-}$ also resulted in the decrease of autoreactive antibodies of the IgG isotype *(Fig 4.22 E).* Single transfer of 1x10^6 FcγRIIB$^{-/-}$ peritoneal B-1b cells in aged FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice screened for high titers of blood Th17 cells, resulted in a slight increase of self-reactive IgM serum antibodies on day 9 and consistent with the data obtained from the total peritoneal cell transfers, the Th17 cell frequencies were significantly lowered from around 6% of CD4^{+} T cells to 3.3% *(Fig 4.22 F).*

Thus, transfer of TLR9 expressing peritoneal B-1b cells from FcγRIIB-deficient mice into FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice inhibits the accumulation of pro-inflammatory Th17 cells and thereby rescues FcγRIIB$^{-/-}$TLR9$^{-/-}$ from severe autoimmunity.

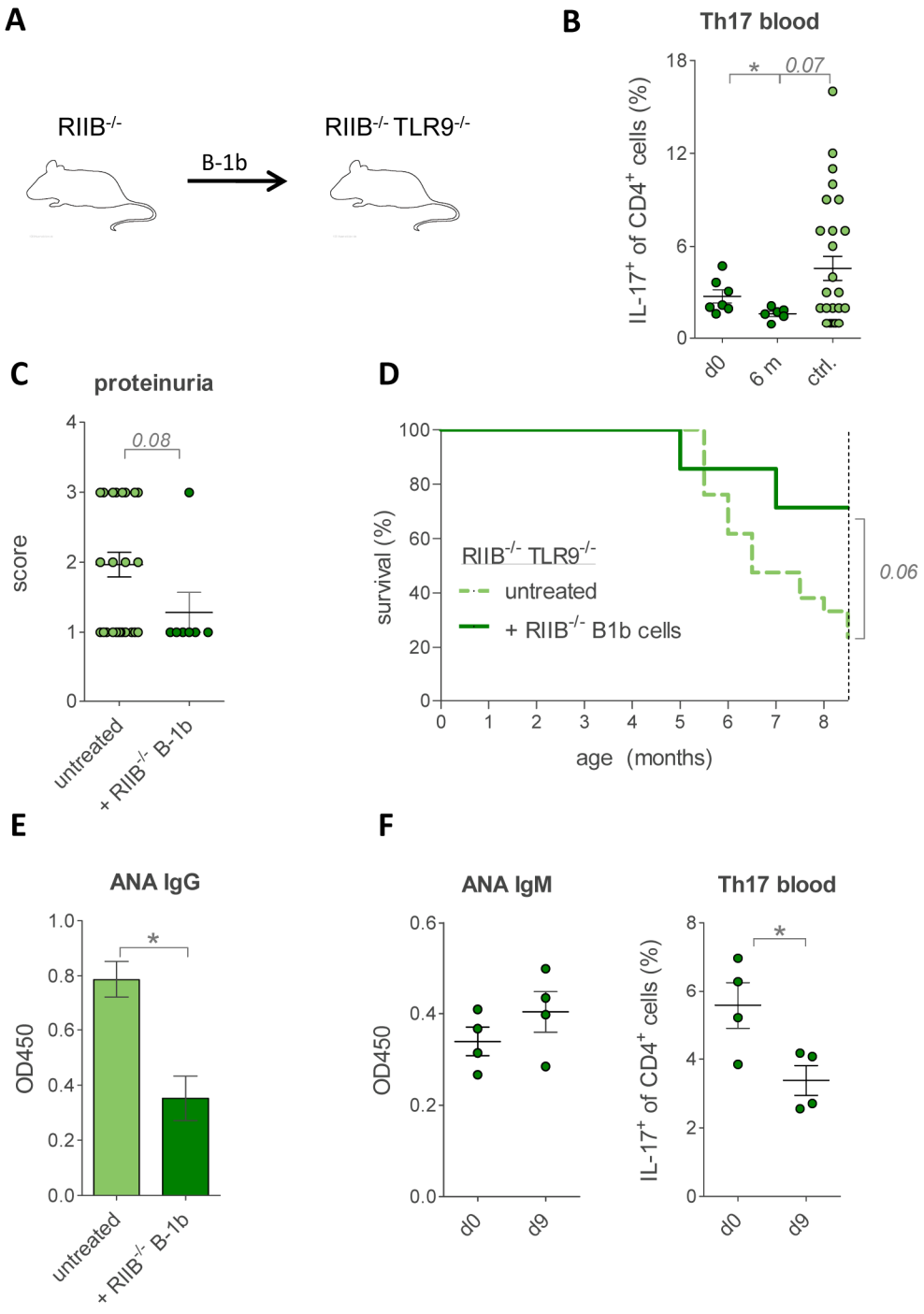

Figure 4.22 Transfer of sorted FcγRIIB$^{-/-}$B-1b cells into FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice prevents Th17 cell accumulation and lupus nephritis. (A) Scheme; 3.5-4 month old FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice were transplanted with 1x10^{6}RIIB$^{-/-}$ B-1b donor cells on day (d) 0, 14, 28, 49 and 64. **(B)** Frequencies of blood CD4^{+}IL-17^{+} Th17 cells in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice before (d 0) and after 5 i.p. transfers (6M) of 1x10^{6} B-1b cells from donor RIIB$^{-/-}$ mice in comparison to untreated 5-6 month-old RIIB$^{-/-}$TLR9$^{-/-}$ mice as analyzed by FACS. Symbols represent data from individual mice. Horizontal lines show mean values with standard error of the mean (SEM). **(C)** Proteinuria scores in 6 month-old FcγRIIB$^{-/-}$ (RIIB$^{-/-}$; n=39), FcγRIIB$^{-/-}$TLR9$^{-/-}$ (n=29) and FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice treated with B-1b cells (n=7). Symbols represent data from individual mice. Horizontal lines

show mean values with standard error of the mean (SEM). **(D)** Kaplan-Meier survival curves for untreated FcγRIIB$^{-/-}$TLR9$^{-/-}$ (RIIB$^{-/-}$TLR9$^{-/-}$) mice (n=21) and B-1b cell transplanted RIIB$^{-/-}$TLR9$^{-/-}$ mice (n=7). **(E)** ANA serum IgG autoantibody levels in 6 month old FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice after 5 i.p. transfers of 1x10^{6} B-1b cells from donor RIIB$^{-/-}$ mice in comparison to untreated 5-6 month-old RIIB$^{-/-}$TLR9$^{-/-}$ mice as analyzed by ELISA. Bar graphs show the mean value with standard error of the mean (SEM) for each group. One representative out of two independent ELISA is shown**. (F)** ANA IgM levels and frequencies of blood CD4^{+}IL-17^{+} Th17 cells in 6 month old FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice before (d0) and 9 days after i.p transfer of 1x10^{7} FcγRIIB$^{-/-}$ peritoneal cells (d9) as analyzed by ELISA and FACS, respectively. Symbols represent data from individual mice. Horizontal lines show mean values with standard error of the mean (SEM).

5 Discussion

Systemic Lupus Erythematosus (SLE) is a fairly heterogeneous autoimmune disease associated with the formation of autoreactive antibodes specific to nuclear antigens (Rahman and Isenberg 2008). During the course of SLE disease, high titers of pathogenic serum IgG autoantibodies form immune complexes and accumulate in the kidney, resulting in the recruitment of inflammatory immune cells promoting renal damage and thus induce glomerulonephritis. As reason for the humoral response against self-antigens, an unefficient clearance of apoptotic material and the therewith related uncontrolled exposure to self-antigens has been suggested (Casciola-Rosen, Anhalt et al. 1994). In the 1990`s B cells were identified to play the central role in SLE pathogenesis (Chan and Shlomchik 1998). The generation of BCR transgenic B cells with specificities to self-antigens demonstrated that in the context of an autoimmune-prone genetic background those B cells escape self-tolerance and differentiate into antibody-secreting cells (Roark, Kuntz et al. 1995; Mandik-Nayak, Seo et al. 1999; Chen, Li et al. 2006). By using a RF-transgenic system (AM14) with transgenic B cells specific to self-IgGs, it has been shown that autoreactive B cells are activated by dual engagement of BCR and innate Toll-like receptors, most notably TLR9 and TLR7 (Leadbetter, Rifkin et al. 2002; Viglianti, Lau et al. 2003; Lau, Broughton et al. 2005). Activation of autoreactive B cells via TLR7 thereby induces antibodies specific to RNA containing antigens (Pisitkun, Deane et al. 2006), whereas TLR9 stimulation induces the generation of anti-dsDNA antibodies (Leadbetter, Rifkin et al. 2002). On the basis of these findings, numerous TLR deficient lupus mouse models have been generated to investigate the role of TLRs in autoantibody production and lupus disease progression (Christensen, Kashgarian et al. 2005; Lartigue, Courville et al. 2006; Wu and Peng 2006). But whereas the *knock out* of TLR7 results in the amelioration of lupus disease (Christensen, Shupe et al. 2006; Christensen and Shlomchik 2007; Santiago-Raber, Dunand-Sauthier et al. 2010), loss of TLR9 is associated with enhanced disease progression in lupus prone mouse models indicating a tolerogenic function of TLR9 in SLE (Christensen, Kashgarian et al. 2005; Lartigue, Courville et al. 2006; Wu and Peng 2006; Christensen and

Shlomchik 2007; Ehlers and Ravetch 2007). In this thesis, the FcγRIIB$^{-/-}$ lupus mouse model was used to study the role of TLR9 in SLE.

5.1 The Phenotype of FcγRIIB$^{-/-}$ mice lacking TLR9

Consistent with other works, TLR9 stimulation in the FcγRIIB$^{-/-}$ lupus mouse model is essential for the generation of IgG serum antibodies specific to nucleosomes (Christensen, Kashgarian et al. 2005; Lartigue, Courville et al. 2006; Christensen and Shlomchik 2007). By contrast, missing TLR9 stimulation extensively increases pathogenic IgG2c and IgG2b antibody responses to various other nuclear antigens including RNA containing antigens and self-IgG.

As T cell help is required for the generation of highly affine autoreactive IgG antibodies, pro-inflammatory T cells are known to be associated with the development of SLE (Kotzin 1996). Analysis of CD4^{+} T cells in various peripheric lymphoid organs of FcγRIIB$^{-/-}$ mice lacking TLR9 shows a massiv increase in activated Th1 and Th17 cells compared to TLR9 sufficient FcγRIIB$^{-/-}$ mice. Hence, TLR9 stimulation and signaling seems to be involved in the suppression of pathogenic T cell responses (Fukata, Breglio et al. 2008). Th1 cells are known to play a major role in the generation of IgG antibodies, thus explaining elevated titers of autoreactive IgG antibodies in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice. The typical Th1-type cytokine IFNγ thereby provokes class-switch to antibodies of the IgG2c subclass (Snapper and Paul 1987; Kotzin 1996). The stimuli promoting the development of antibodies of the IgG2b subclass are not clearly identified but B cell class-switching to IgG2b seems to be dependent on TGF-β (Stavnezer 1996; Stavnezer 1996) . The Fc parts of IgG2c and IgG2b antibodies are known to exhibit extremely high affinities to activating Fcγ receptors (Nimmerjahn and Ravetch 2005). Thus IgG2c and IgG2b are described as the pathogenic isotypes in SLE causing immune complex deposition in the kidney (Nimmerjahn and Ravetch 2005; Giorgini, Brown et al. 2008). IL-17 producing T cells (Th17) have been studied extensively in both infection and autoimmunity, but in contrast to Th1 cells their exact role within the mechanisms that lead to SLE is still not identified. Primarily, their effector function includes the promotion of inflammation via the secretion of pro-inflammatory cytokines like IL-17A, IL-17F, IL-21 and IL-22. Particularily IL-17 induces the production of chemokines such as CXCL-

8, MIP-1 and MCP-1 to recruit macrophages to the site of inflammation (Koenders and van den Berg 2010). Additionally, Th17 cells seem to be able to stimulate B cells (Mitsdoerffer, Lee et al. 2010). In this context, Hsu *et al.* could show that mice lacking IL-17 receptor exhibit reduced germinal center B cell development and humoral responses (Hsu, Yang et al. 2008). We could detect pro-inflammatory Th17 cells in renal tissues of FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice, thus indicating their main role in promoting inflammation and tissue destruction of target organs. Recent unpublished investigations demonstrate that the deletion of IL-17A receptor in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice delays lupus disease manifestation, indicating a devastating role of IL17-producing T cells in the FcγRIIB$^{-/-}$TLR9$^{-/-}$ mouse model (Susanne Eiglmeier, personal communication). This is in line with previous observations in various murine autoimmune mouse models (Hsu, Yang et al. 2008; Kyttaris, Zhang et al. 2010). Autoimmune BXD2 mice exhibit high levels of IL-17 in their serum and IL-17 overexpression in these mice results in enhanced disease progression whereas the blocking of IL-17 receptor signaling reduced its intensity (Hsu, Yang et al. 2008). Consistent with observations in lupus mouse models, SLE patients show higher serum levels of IL-17 and increased frequencies of IL-17 producing T cells in peripheral blood during active flares (Crispin, Oukka et al. 2008; Garrett-Sinha, John et al. 2008; Wong, Lit et al. 2008). Hence, Th17 cells and the cytokine IL-17 might be potential therapeutic targets in the treatment of lupus disease. We found, that the excessive expansion of pro-inflammatory Th17 cells in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice correlates with augmented production of IL-6 in splenocytes. IL-6 is known as a pro-inflammatory cytokine, which besides its effect on the induction of B lymphocyte maturation into plasma cells and augmentation of immunoglobulin secretion, together with TGF-β promotes the differentiation of naïve T cells into Th17 cells (Bettelli, Carrier et al. 2006; Rabe, Chalaris et al. 2008). Although IL-6 is predominantly synthesized by monocytes, fibroblasts, and endothelial cells, activated Th17 cells themselves are also capable of producing IL-6, thus encouraging additional pro-inflammatory responses (Yap and Lai 2010). The pivotal role of IL-6 in the pathogenesis of SLE has been supported by murine experiments (Finck, Chan et al. 1994; Mihara, Takagi et al. 1998). The deletion of IL-6 in MRL/*lpr* mice for instance reduced renal macrophage infiltration, autoantibody production and T cell activation and delayed lupus nephritis (Cash, Relle et al. 2010)

In this work, we demonstrate that TLR9 deficiency in FcγRIIB$^{-/-}$ mice results in i) increased generation of pathogenic IgG autoantibodies, ii) augmented production of IL-6 and iii) excessive expansion of pro-inflammatory Th1 and Th17 cells, thus leading to increased incidences of proteinuria and mortality rates. Hence, these findings confirm a tolerogenic role of TLR9 in the FcγRIIB$^{-/-}$ lupus mouse model. The term "tolerance" has already been mentioned together with TLR9 stimulation in previous investigations. Lampropoulou *et al.* ascribed TLR9 tolerance in B cells to the production of IL-10 in a murine model of EAE (Lampropoulou, Hoehlig et al. 2008), whereas the group around E. Raz demonstrated a tolerogenic effect of TLR9 in intestinal epithelial cells (IEC). The stimulation of TLR9 in IECs thereby is essential for the maintenance of tolerance to commensal bacteria (Lee, Gonzales-Navajas et al. 2008). However, the definite mechanism of TLR9 mediated tolerance in murine lupus remained to be investigated.

5.2 Loss of TLR9 in FcγRIIB$^{-/-}$ mice is associated with reduced titers of self-reactive IgM serum antibodies

Here we demonstrate that TLR9 deficiency in FcγRIIB$^{-/-}$ mice is accompanied by highly reduced titers of self-reactive IgM antibodies. Even young FcγRIIB$^{-/-}$ mice lacking TLR9 exhibit lowered levels of self-reactive antibodies of the IgM isotype. Hence, TLR9 stimulation and signaling is essential for the generation of self-reactive IgM antibodies. It has been described that in contrast to autoreactive IgG antibodies, self-reactive IgM antibodies are not associated with disease activity or specific symptoms in human SLE. Clinical studies even reveal a protective role of autoreactive IgM antibodies as SLE patients with a ratio of autoreactive IgG/IgM beneath 0.8 are unlikely to be affected by glomerulonephritis whereas patients displaying higher ratios exhibit active flares (Forger, Matthias et al. 2004). Conferring these findings to the FcγRIIB$^{-/-}$ mouse model, missing TLR9 stimulation promotes the imbalance of the ratio of autoreactive IgG/IgM as on the one hand it results in extensively increased titers of autoreactive IgG antibodies and on the other hand diminishes self-reactive IgM levels.

Recently, the importance of IgM serum antibodies in the maintenance of self-tolerance in murine models of SLE has been demonstrated. By breeding mice that are unable to secret IgM but secret other classes of immunoglobulins with *lpr*

(lupus prone) mice, Boes *et al.* examined the effect of the absence of secreted IgM in murine SLE. Thereby they could demonstrate that lupus prone mice lacking soluble IgM suffered from more severe glomerulonephritis and subsequently mortality was increased. The investigators unsuccessfully tried to restore the normal development of glomerulonephritis with the use of monoclonal IgM antibodies specific to dsDNA. As discussed in the report, the lack of success was probably due to an IgG response against the injected IgM antibody (Boes, Schmidt et al. 2000). Even mice, which have been generated on a non-autoimmune, normal background exhibit the development of IgG autoantibodies and renal immune complex depositions on exposure to lipopolysaccharides, while lacking soluble IgM antibodies, suggesting that the absence of secreted IgM may predispose to autoimmunity (Ehrenstein, Cook et al. 2000). Apart from SLE, a protective role of self-reactive IgM in the manifestation of several other autoimmune diseases, such as artherosclerosis and multiple sclerosis has been described recently (Warrington, Bieber et al. 2007; Lewis, Malik et al. 2009).

We show that reduced levels of self-reactive IgM antibodies are associated with intense accumulation of pro-inflammatory Th1 and Th17 cells and excacerbated lupus disease in the FcγRIIB$^{-/-}$TLR9$^{-/-}$ lupus mouse model, thus confirming a protective role of self-reactive IgM in murine lupus.

5.3 Monoclonal self-reactive IgM antibodies prevent the accumulation of Th17 cells and lupus nephritis

Whereas injection of IgG antibodies reactive to dsDNA in murine models of lupus induces glomerulonephritis (Ehrenstein, Katz et al. 1995; Koren, Koscec et al. 1995), recent studies reveal, that the treatment of lupus-prone NZB/W mice with a murine monoclonal IgM antibody specific for dsDNA prevented renal damage (Werwitzke, Trick et al. 2005).

In this work, we used two monoclonal polyreactive IgM antibodies, ED38 IgM and 1RIIgc7 IgM, to reconstitute IgM levels in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice. We could clearly demonstrate that reconstitution of self-reactive IgM titers ameliorates lupus disease, confirming that self-reactive IgM antibodies indeed mediate protection in FcγRIIB$^{-/-}$ TLR9$^{-/-}$ mice. Long-term application of newly constructed polyreactive and self-reactive IgM antibodies in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice thereby significantly delays the onset

of proteinuria and prolongs survival whereas unspecific control IgM antibodies do not influence disease manifestation. Reduced mortality rates in self-reactive IgM treated mice correlate with reduced numbers of pro-inflammatory T helper cells, particularly Th17 cells in peripheral lymphoid organs. In this context, we could furthermore show that only one transfer of monoclonal self-reactive IgM antibodies is sufficient to reduce Th17 cell numbers in aged FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice already after 9 days. Consistent with other publications, these results suggest that Th17 cells might be rather short-lived (Pepper, Linehan et al. 2010). Whereas high numbers of pre-existing Th17 cells rapidly die, administration of monoclonal self-reactive IgM antibodies might prevent anew maturation of activated Th17 cells.

As a consequence of low numbers of T helper cells, long-term treatment of FcγRIIB$^{-/-}$ TLR9$^{-/-}$ mice with monoclonal self-reactive IgM antibodies resulted in reduced plasma cell numbers and IgG autoantibody titers. This observation supports previous investigations, which reveal that lupus mice lacking soluble IgM antibodies show accelerated development of autoreactive IgG antibodies (Boes, Schmidt et al. 2000). Nevertheless, we have found renal immune complex deposition in FcγRIIB$^{-/-}$ TLR9$^{-/-}$ mice treated with self-reactive IgM. But in contrast to the kidneys of control mice, kidneys of IgM treated mice do not exhibit infiltrations of activated macrophages. Studies done in our laboratory demonstrate that immune complex depositions in kidneys of lupus prone mice are not sufficient to induce glomerulonephritis (M. Mertes, unpublished data). As additional stimulus for inflammation, M.Mertes suggested the presence of elevated numbers of pro-inflammatory Th17 cells. As already mentioned, we could detect pro-inflammatory Th17 cells in renal tissues of FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice. Hence, most likely, high frequencies of Th17 cells in the kidneys of FcγRIIB$^{-/-}$TLR9$^{-/-}$ control mice cause augmented renal infiltration of macrophages, as secreted IL-17 promotes the recruitment of macrophages (Koenders and van den Berg 2010). Low numbers of Th17 cells in IgM treated mice might therefore be not sufficient to trigger macrophage infiltration. The definite role of macrophages in the development of SLE is still discussed as it has traditionally been assumed that impaired phagocytosis by monocytes and macrophages in SLE allows the accumulation of apoptotic cell waste leading to activation of autoreactive B and T cells (Gaipl, Munoz et al. 2007). However, recent studies reveal the pathogenic role of macrophages in SLE. Thus, deposition of immune complexes in renal tissues results in tissue pathology most

likely through the engagement of FcγRIV expressed on infiltrating myeloid effector cells (Nimmerjahn, Bruhns et al. 2005) and subsequent induction of aberrant cytokine expression (Katsiari, Liossis et al. 2010). These studies indicate the direct participation of macrophages in late lethal glomerulonephritis.

So far, our data show that loss of TLR9 in FcγRIIB$^{-/-}$ mice exacerbates lupus disease and correlates with the reduction of self-reactive IgM antibodies. Subsequent reconstitution of self-reactive IgM levels rescues FcγRIIB$^{-/-}$ TLR9$^{-/-}$ mice from severe disease progression by most notably reducing pro-inflammatory Th17 cell frequencies. Considering these results, we suggest protective self-reactive IgM antibodies as mediators of tolerance initiated by TLR9 stimulation.

5.4 TLR9 signaling in peritoneal B-1b cells induces the generation of protective self-reactive IgM

Toll-like receptor 9 is widely expressed in the innate and adaptive immune system, being located in the endosomes of macrophages, dendritic cells as well as in T cells and most notably B cells (Kabelitz 2007). Parallel investigations of the PhD student Alexander Stoehr in our laboratory reveal that loss of TLR9 in bone marrow-derived B cells promotes excacerbated lupus disease in FcγRIIB$^{-/-}$ mice. Mixed bone marrow chimeric FcγRIIB$^{-/-}$ mice with FcγRIIB/TLR9-double-deficient B cells showed increased frequencies of pro-inflammatory Th17 cells correlating with reduced levels of autoreactive IgM and thereby resemble the phenotype of FcγRIIB$^{-/-}$ TLR9$^{-/-}$ mice (Stoehr and Schoen *et al.* submitted; in revision).
Thus, in line with other investigations, we support the idea that B cells are involved in TLR9 mediated tolerance (Lampropoulou, Hoehlig et al. 2008). By cross-breeding FcγRIIB$^{-/-}$ mice with TCRβ$^{-/-}$ mice we demonstrated that protective self-reactive IgM antibodies, in contrast to autoantibodies of the IgG isotype, in FcγRIIB$^{-/-}$ mice are generated independently of T cell help. Splenic marginal zone B cells (MZ), and peritoneal B-1 B cells are known to be the major source of IgM generated independently of T cell help and to maintain and regulate IgM antibody homeostasis under steady state conditions (Martin, Oliver et al. 2001; Ehrenstein and Notley 2010). In fact, B-1 cells have been shown to contribute 80-95% of serum IgM in non-infected mice (Baumgarth, Herman et al. 1999). IgM antibodies produced by

MZ B cells and most notably B-1 cells are referred to as natural IgM and due to germline encoded V gene segments, these antibodies show low affinities but broad specificites to both foreign and self structures (Boes 2000). MZ B cells as well as B-1 cells are described as innate like B cells and both peripheral B cell subsets are reported to develop partly from progenitors in adult bone marrow (Hardy 2006). Hence, both, MZ and B-1 cells seemed to be promising candidates for the generation of protective self-reactive IgM antibodies. However, determination of splenic marginal zone B cell frequencies in FcγRIIB$^{-/-}$ and FcγRIIB$^{-/-}$ TLR9$^{-/-}$ mice did not reveal any differences, indicating that either MZ B cells do not participate in TLR9 mediated tolerance or that MZ B cells are not activated in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice and thus they do not produce self-reactive IgM. Depletion experiments in FcγRIIB$^{-/-}$ mice confirmed that MZ B cells are not involved in protective self-reactive IgM generation. By contrast, we could demonstrate that the lack of TLR9 in FcγRIIB$^{-/-}$ mice highly affects peritoneal B-1 cell frequencies. TLR9 signaling in B-1 cells has been associated with the generation of self-reactive IgM antibodies by other groups (Genestier, Taillardet et al. 2007; Kubo, Uchida et al. 2009). Thus, consistent with earlier publications, spontaneous self-reactive IgM production by FcγRIIB$^{-/-}$ peritoneal cells and FcγRIIB$^{-/-}$ peritoneal cells lacking TLR9 *ex vivo* revealed that absence of TLR9 in peritoneal B cells results in reduced production of autoreactive IgM antibodies. These findings indicate that reduced numbers of B-1 cells in FcγRIIB/TLR9 double-deficient mice entail low levels of self-reactive IgM.

After Hayakawa *et al.* discovered that B-1 cells are present in high numbers in the peritoneal cavitiy in 1985 (Hayakawa, Hardy et al. 1985), detailed flow cytometry analysis demonstrated, that the IgM^{high}MacI^{int} population of peritoneal B-1 cells can be distinguished in two subpopulations (Hayakawa, Hardy et al. 1985; Arnold, Pennell et al. 1994; Wells, Kantor et al. 1994). Both populations express equal amounts of IgM, IgD, CD43, CD23 and MacI and are, thus, distinct from mature B-2 cells. However, CD5, originally the distinguishing feature of B-1 cells, is only expressed on one of these B-1 cell subsets. Whereas CD5^{pos} B-1 cells are referred to as B-1a cells, the CD5^{neg} population is described as B-1b cells (Stall, Adams et al. 1992). Transfer experiments revealed that besides their differential expression of CD5, B-1a and B-1b cells are developmentally distinct. Whereas B-1a cells only develop from fetal liver, B-1b cells can be generated from both, fetal liver and adult

bone marrow (Clarke and Arnold 1998; Fagarasan, Watanabe et al. 2000; Haas, Poe et al. 2005; Hardy 2006; Hardy 2006; Dorshkind and Montecino-Rodriguez 2007).

Analysis of CD5 expression of peritoneal B-1 cells revealed that both, TLR9 sufficient and TLR9 deficient FcγRIIB$^{-/-}$ mice exhibited diminished levels of CD5^{+} B-1a cells compared to wild-type mice. Hence, the reduction of peritoneal B-1a cell numbers in FcγRIIB$^{-/-}$ mice was independent of TLR9. Various investigations have associated the peritoneal B-1a sub-population with autoimmunity in humans and mice (Dauphinee, Tovar et al. 1988; Smith and Olson 1990; Duan and Morel 2006). In steady state conditions B-1a cells secrete natural IgM antibodies independently of antigenic stimulation (Duan and Morel 2006). In the context of our model, the loss of FcγRIIB might enhance peritoneal B-1a cell activation (Ono, Bolland et al. 1996). By transfering labelled B-1a cells of aged lupus prone NZB/W F1 mice in either aged or young NZB/W F1 mice, Ishikawa *et al.* were able to show that B-1a cell homing to the peritoneal cavity is disturbed in aged diseased mice. Instead of homing to the peritoneal cavity, B-1a cells are recruited to the target organs such as the lung, kidney, and thymus (Ishikawa, Sato et al. 2001; Ito, Ishikawa et al. 2004; Ishikawa and Matsushima 2007). In line with these investigations, it has recently been supposed that upon activation, B-1a cells undergo class switch and migrate to target organs participating in the generation of autoreactive IgG antibodies (Duan and Morel 2006; Enghard, Humrich et al. 2010). Thus, migration of activated FcγRIIB$^{-/-}$ B-1a cells to peripheral lymphoid organs and tissues might result in lowered frequencies of B-1a cells in the peritoneal cavity. However, this process seems to be TLR9 independent. By contrast, we could clearly demonstrate that the absence of TLR9 in FcγRIIB$^{-/-}$ mice leads to a strong reduction of peritoneal CD5^{neg} B-1b cell frequencies even at young age, whereas B-1a cell frequencies are virtually not affected, suggesting that TLR9 plays a key role in regulating peritoneal B-1b cell frequencies. This observation was confirmed by the fact that already TLR9$^{-/-}$ mice showed reduced B-1b cell numbers. Stimulation of TLR9 either *in vivo* by i.p. injection of CpG in FcγRIIB$^{-/-}$ mice or *in vitro* by CpG stimulation of sorted FcγRIIB$^{-/-}$ B-1 cells resulted in extensive proliferation of B-1b cells. In contrast, B-1a cells only hardly responded to CpG. Thus, TLR9 seems to be essential for the maintenance of peritoneal B-1b cell numbers. Furthermore, we could demonstrate that high proliferation rates of B-1b cells upon TLR9 stimulation *in vitro* and *in vivo* were associated with augmented production of IgM antibodies, identifying B-1b cells as an important source of self-reactive IgM antibodies.

Although TLR7 resembles TLR9 in various properties, as it is located in the endosomes, recognizes pathogenic nucleotide structures and triggers signal transduction via the adaptor protein MyD88 (Takeda and Akira 2005), TLR7 stimulation *in vitro* and *in vivo* is not able to induce either B-1b cell proliferation or increased production of IgM antibodies. Hence, TLR7 signaling does not compensate the loss of TLR9 in peritoneal B-1 cells.

In contrast to B-1a cells, secretion of IgM antibodies by B-1b cells requires antigen-mediated activation (Alugupalli, Leong et al. 2004; Alugupalli and Gerstein 2005; Haas, Poe et al. 2005; Montecino-Rodriguez and Dorshkind 2006). Loss of FcγRIIB-dependent negative feedback mechanisms in self-reactive peritoneal B-1b cells may promote their activation by TLR9-ligand containing antigens via the B cell receptor (BCR) and TLR9 co-stimulation (Ono, Bolland et al. 1996; Leadbetter, Rifkin et al. 2002; Ferry, Potter et al. 2007; Avalos, Busconi et al. 2010) and thus induce the secretion of self-reactive IgM serum antibodies (Kawahara, Ohdan et al. 2003). Hence, two mechanisms might be responsible for the reduced levels of self-reactive IgM serum autoantibodies in TLR9-deficient FcγRIIB$^{-/-}$ mice: (i) less B-1b cells in general and (ii) reduced B-1b cell activation.

5.5 TLR9 sufficient FcγRIIB$^{-/-}$ peritoneal B-1b cells prevent the accumulation of Th17 cells and lupus nephritis in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice

Recent investigations have already linked B-1b cells to the induction of tolerance, e.g. oral tolerance (De Lorenzo, Brito et al. 2007; Shimizu, Kawahara et al. 2007). Here, we now demonstrate a tolerogenic role of B-1b cells in murine lupus via the stimulation of TLR9. Transfer of TLR9 sufficient FcγRIIB$^{-/-}$ total peritoneal cells as well as sorted B-1b cells in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice results in increased levels of self-reactive IgM antibodies and prolonged survival. Increased IgM levels thereby correlate with decreased levels of pro-inflammatory Th17 cells and lowered levels of pathogenic autoreactive IgG antibodies, thus supporting the idea that the tolerogenic function of B-1b cells may rely on the generation of self-reactive serum IgM antibodies. In contrast, transfer of TLR9 deficient total peritoneal cells does not increase self-reactive serum IgM levels in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice and therefore does not lead to diminished levels of Th17 cells, confirming TLR9 stimulation in peritoneal cells as initial act of tolerance induction.

In the last years, the anti-inflammatory function of IL-10 in autoimmunity has attracted huge attention (Bettelli, Nicholson et al. 2003; Roncarolo, Battaglia et al. 2003). Several peripheral B cell subsets have been demonstrated to produce IL-10 upon TLR stimulation and particularily B-1 cells have been mentioned in this context (Mizoguchi and Bhan 2006; Serra and Santamaria 2006; Fillatreau, Gray et al. 2008; Yanaba, Bouaziz et al. 2008; Yanaba, Bouaziz et al. 2009; DiLillo, Matsushita et al. 2010). In their study, Lampropoulou *et al.* ascribed the tolerogenic effect of TLR9 stimulation to the production of IL-10 by B-cells (Fillatreau, Sweenie et al. 2002; Lampropoulou, Hoehlig et al. 2008; Lampropoulou, Calderon-Gomez et al. 2010). We were able to show, that decreased frequencies of B-1 cells and more precisely B-1b cells in TLR9 deficient FcγRIIB$^{-/-}$ mice do not result in a reduction of IL-10 production in neither peritoneum nor spleen, thus indicating that IL-10 plays a minor role in the induction of TLR9 dependent tolerance in FcγRIIB$^{-/-}$ mice. These results were further confirmed by the transfer of FcγRIIB$^{-/-}$µS total peritoneal cells, which are able to produce IL-10 but concurrently are not able to generate soluble IgM antibodies. Thereby transferred FcγRIIB$^{-/-}$µS total peritoneal cells could not protect FcγRIIB$^{-/-}$ TLR9$^{-/-}$ mice from exaggerated Th17 responses and subsequently from getting severe glomerulonephritis.
Thus, results obtained from transfer experiments confirm that (i) TLR9 stimulation in peritoneal B-1b cells induces protection and that (ii) not IL-10 but self-reactive IgM functions as mediator of tolerance induction upon TLR9 stimulation.

5.6 Possible mechanisms of protection by self-reactive IgM antibodies

The mechanisms by which self-reactive IgM antibodies produced by B-1b cells might prevent the accumulation of Th17 cells and autoimmunity could not been defined in this work. However, there might be a number of different possibilities *(Fig 5.1).*
Most likely, self-reactive IgM antibodies contribute to the clearance of apoptotic cell waste as the major source of autoantigens (Casciola-Rosen, Anhalt et al. 1994; Peng, Kowalewski et al. 2005; Quartier, Potter et al. 2005). Due to its polymeric structure, IgM is known to be the most potent activator of the classical complement cascade amongst the various immunoglobulin isotypes (Bjornson and Detmers 1995). More precisely, IgM antibody polymers show a 1000-fold greater binding affinity to the

complement component C1q than IgG antibodies (Ehrenstein and Notley 2010). In humans as well as murine models, deficiencies in early components of the complement cascade such as C1q, C4 or complement receptors CD21/CD35 are associated with a high incidence of SLE due to high titers of ANA IgG antibodies and glomerulonephritis (Botto, Dell'Agnola et al. 1998; Pozdnykova, Prodeus et al. 1998; Prodeus, Goerg et al. 1998). Mice deficient in components of the complement pathway therefore show defects similar to those observed in mice which do not produce soluble IgM antibodies (Boes, Schmidt et al. 2000). Furthermore, the effects of complement deficiency suspiciously resemble the phenotype of FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice, which exhibit low levels of self-reactive IgM, indicating the involvement of IgM-dependent complement activation in the protection from severe glomerulonephritis. And indeed, by measuring serum C5a levels, which is a cleavage product, detectable during complement activation, we found decreased complement activation in FcγRIIB$^{-/-}$ mice lacking TLR9 (data not shown). Recent investigations demonstrate an inverse correlation between C5a and the development of pro-inflammatory Th17 cells. In serveral commonly used mouse strains and related C5a knock out mice, a linear inverse relationship between absolute amount of serum C5a and the frequency of Th17 cells was observed (Lajoie, Lewkowich et al. 2010). Thus, protective self-reactive IgM antibodies might contribute to self-antigen clearance via complement activation and subsequently avoid activation of autoreactive B cells and Th17 cell priming by antigen-presenting cells. This hypothesis is in line with a report by Chen et al. in the year 2009 (Chen, Khanna et al. 2009). The investigators demonstrate that the process of clearance of apoptotic waste by a natural IgM antibody produced by B-1 cells (T15) is dependent on the recruitment of C1q and mannose-binding lectin (MBL). By recruiting the deposition of both C1q and MBL, IgM antibodies highly enhance the phagocytosis of apoptotic cells. This study additionally reveals that natural IgM antibodies together with C1q or MBL can effectively suppress maturation and activation of conventional DCs and therefore inhibit the secretion of proinflammatory cytokines like IL-6, which is known to enhance Th17 cell differentiation. Moreover, i*n vivo* experiments demonstrated the inhibition of autoimmune inflammatory arthritis by adminstration of high doses of self-reactive IgM (Chen, Khanna et al. 2009).

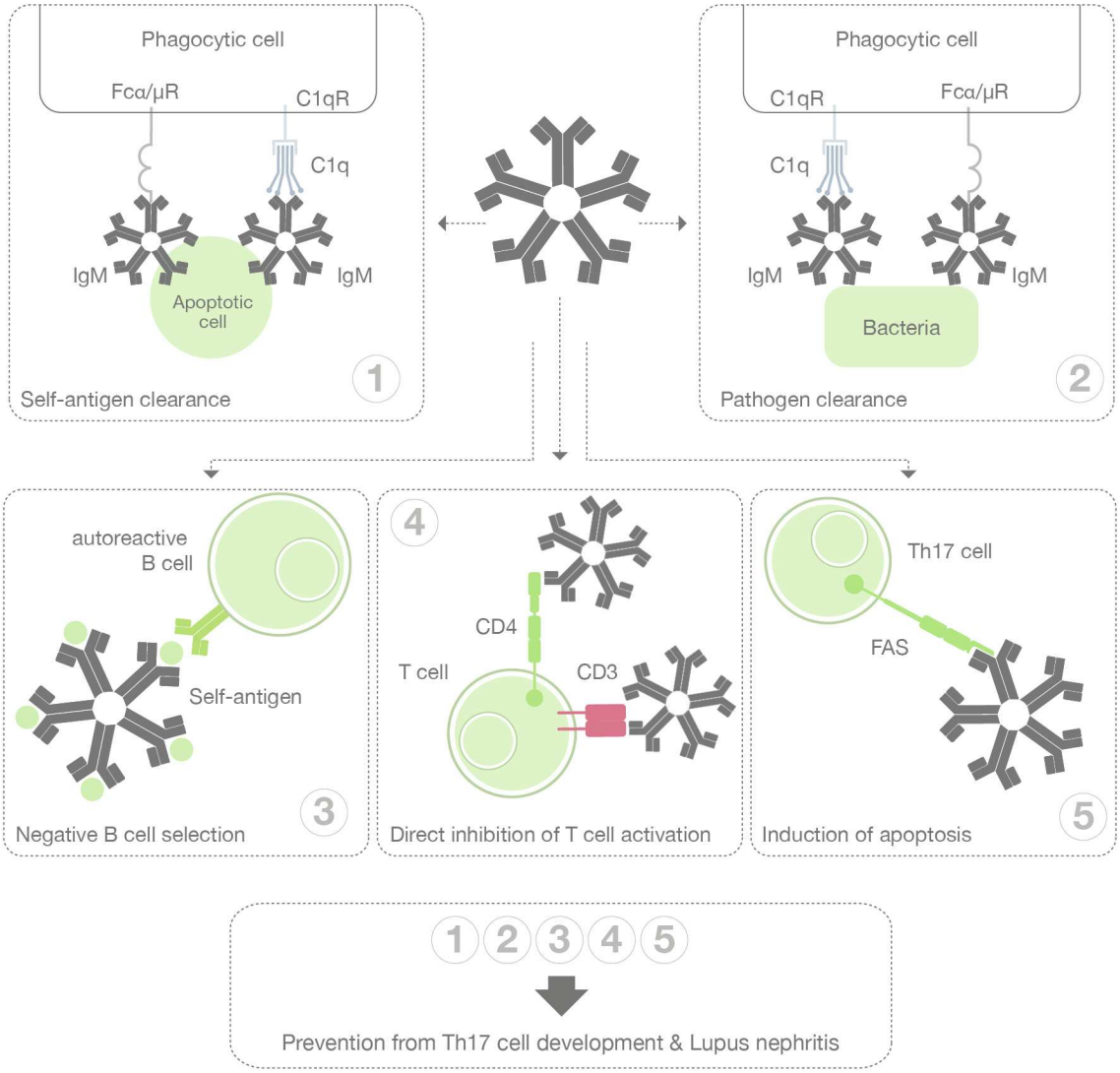

Figure 5.1 Self-reactive IgM antibodies might mediate protection from autoimmunity by various mechanisms. Self reactive IgM antibodies 1) most likely promote self-antigen clearance by binding to and eliminating apoptotic cell waste by either activating the complement system or interacting with Fcα/μ receptors on phagocytic cells; 2) might mediate the clearance of pathogenic bacteria by either activating the complement system or interacting with Fcα/μ receptors on phagocytic cells; 3) might induce negative selection of autoreactive B cells by promoting the exposure of self-antigens to immature, bone marrow resident B cells ; 4) might directly inhibit T cell activation by binding to CD4 or CD3 and 5) might be capable of inducing Fas mediated apoptosis in autoreactive lymphocytes. All together, these mechanisms prevent the accumulation of pro-inflammatory Th17 cells and therefore protect from lupus nephritis.

As it has been known for several years, the main function of naturally occuring self-reactive and polyreactive IgM is to bind to invading pathogens as a first line of defense (Boes 2000; Manson, Mauri et al. 2005). Hence, not only self-antigen

clearance but also the elimination of invading pathogens via complement activation has to be implicated as possible mode of action. Binding of IgM immune complexes to the recently identified Fcα/μ receptor on most notably macrophages and B cells might additionally encourage the removal of pathogenic invaders, as Fcα/μ receptor mediates endocytosis of IgM-coated microbial pathogens (Shibuya, Sakamoto et al. 2000; Shibuya and Honda 2006). Besides their role in autoimmunity, Th17 responses are essential for mucosal and epithelial host defense against extracellular bacteria and fungi (van de Veerdonk, Gresnigt et al. 2009). Thus, by binding to and eliminating extracellular pathogens, natural occuring self-reactive and polyreactive IgM antibodies might abolish inducable factors leading to Th17 cell maturation and therefore suppress the development of autoimmunity. Recent studies indicate that the treatment of FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice with antibiotics reduces Th17 cell frequencies and therefore ameliorates lupus disease, supporting the idea of the involvement of pathogens in the induction of SLE (M. Mertes; unpublished data).

Alternatively, self-reactive IgM antibodies might play a role in maintaining self-tolerance in earlier B cell development, by promoting the exposure of self-antigens to immature, bone marrow resident B cells, inducing negative selection processes (Ehrenstein and Notley 2010). A similar role in negative selection of autoreactive B cells has been demonstrated for the complement receptors CD21/CD35 (Prodeus, Goerg et al. 1998). Hence, less autoreactive B cells might result in less activated Th17 cells.

Moreover, it has been described, that naturally occuring IgM antibodies are capable of precipitating with CD3, CD4 and also CXCR4, thereby inhibiting T cell activation and proliferation, cytokine production and chemotaxis (Vassilev, Mihaylova et al. 2006; Lobo, Schlegel et al. 2008). Thus, natural self-reactive IgM antibodies might directly influence Th17 cell activation and differentiation, attenuating the inflammatory response especially to self-antigens.

Finally, various studies describe a role of natural polyreactive IgM in inducing apoptosis. IgM-induced cell death of lymphocytes was thereby shown to be mediated by Fas receptor cross-linking (Ollert, David et al. 1996; David, Heiligtag et al. 2001; Varambally, Bar-Dayan et al. 2004). As insufficient apoptosis leads to the persistence of autoreactive cells, defective apoptosis is known to be associated with the pathogenesis of several autoimmune diseases (George, Adler et al. 1995). Hence, the protective role of IgM in SLE might involve the induction of apoptosis of

autoreactive lymphocytes and therefore minimize their expansion. However, the exact mechanism of self-reactive IgM preventing the accumulation of Th17 cells in FcγRIIB$^{-/-}$TLR9$^{-/-}$ mice remains to be investigated.

5.7 Conclusion

Taken together, our data suggest that the tolerogenic function of TLR9-signaling is mediated by the production of self-reactive IgM antibodies by peritoneal B-1b cells. B-1b cells mainly reside in the peritoneal cavity, where they are activated by TLR9-ligand containing antigens via the B cell receptor (BCR) and TLR9 co-stimulation. Upon stimulation and activation, FcγRIIB$^{-/-}$ peritoneal B-1b cells undergo proliferation, thus promoting the production of self-reactive IgM antibodies (*Fig 5.2*).

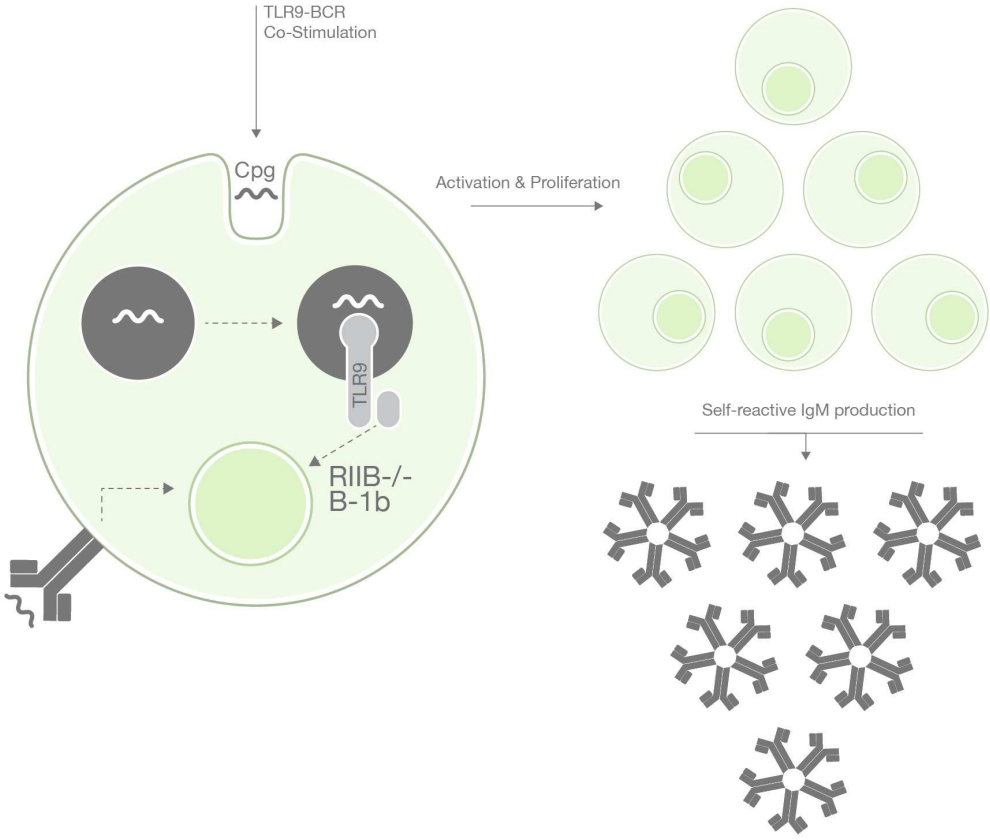

Figure 5.2 Co-Stimulation of TLR9 and BCR in FcγRIIB$^{-/-}$ B-1b cells results in proliferation and self-reactive IgM production. Schematic illustration of FcγRIIB$^{-/-}$ B-1b cells, stimulated by CpG. Co-stimulation of TLR9 and the B cell receptor activates FcγRIIB$^{-/-}$ B-1b cells, leading to B-1b cell expansion and increased production of protective self-reactive IgM.

Self-reactive IgM antibodies subsequently protect most notably from the excacerbated accumulation of Th17 cells and consequently from severe autoimmunity in the FcγRIIB$^{-/-}$ murine lupus model. The actual mechanism how self-reactive IgM antibodies prevent severe autoimmunity have not been identified in this work. However, the most probable function might be the clearance of apoptotic cells as major source of autoantigens as well as pathogens.

These findings provide new insight into the tolerogenic function of TLR9, B-1b cells and self-reactive IgM to control autoimmunity and should have implications in the development of therapies for SLE. Thus, B-cell targeted therapies as well as TLR9 silencing could be counterproductive as these therapies might induce reductions in serum IgM. A better understanding of therapeutic mechanisms of self-reactive IgM antibodies may provide novel approaches for the treatment of autoimmune disorders that are more specific and show less co-morbidities than symptomatically treatments with anti-inflammatory and immuno-suppressive substances. Immunoglobulin infusions, consisting of IgG (IvIg) or IgM antibodies (IvIgM) have already been used to treat autoimmune diseases with partly limited success. Our results should encourage the generation of a human monoclonal self-reactive IgM antibody that may be beneficial for the treatment of SLE and other autoimmune disorders.

Literature

Abbas, A. K., K. M. Murphy, et al. (1996). "Functional diversity of helper T lymphocytes." Nature **383**(6603): 787-793.

Aderem, A. and D. M. Underhill (1999). "Mechanisms of phagocytosis in macrophages." Annu Rev Immunol **17**: 593-623.

Allen, C. D., T. Okada, et al. (2007). "Germinal-center organization and cellular dynamics." Immunity **27**(2): 190-202.

Allman, D. and S. Pillai (2008). "Peripheral B cell subsets." Curr Opin Immunol **20**(2): 149-157.

Alugupalli, K. R. and R. M. Gerstein (2005). "Divide and conquer: division of labor by B-1 B cells." Immunity **23**(1): 1-2.

Alugupalli, K. R., J. M. Leong, et al. (2004). "B1b lymphocytes confer T cell-independent long-lasting immunity." Immunity **21**(3): 379-390.

Arnold, L. W., C. A. Pennell, et al. (1994). "Development of B-1 cells: segregation of phosphatidyl choline-specific B cells to the B-1 population occurs after immunoglobulin gene expression." J Exp Med **179**(5): 1585-1595.

Avalos, A. M., L. Busconi, et al. (2010). "Regulation of autoreactive B cell responses to endogenous TLR ligands." Autoimmunity **43**(1): 76-83.

Banchereau, J., F. Briere, et al. (2000). "Immunobiology of dendritic cells." Annu Rev Immunol **18**: 767-811.

Barry, M. and R. C. Bleackley (2002). "Cytotoxic T lymphocytes: all roads lead to death." Nat Rev Immunol **2**(6): 401-409.

Barton, G. M. and R. Medzhitov (2002). "Toll-like receptors and their ligands." Curr Top Microbiol Immunol **270**: 81-92.

Baumgarth, N., J. Chen, et al. (2000). "The role of B-1 and B-2 cells in immune protection from influenza virus infection." Curr Top Microbiol Immunol **252**: 163-169.

Baumgarth, N., O. C. Herman, et al. (1999). "Innate and acquired humoral immunities to influenza virus are mediated by distinct arms of the immune system." Proc Natl Acad Sci U S A **96**(5): 2250-2255.

Baumgarth, N., O. C. Herman, et al. (2000). "B-1 and B-2 cell-derived immunoglobulin M antibodies are nonredundant components of the protective response to influenza virus infection." J Exp Med **192**(2): 271-280.

Berek, C. (1993). "Somatic mutation and memory." Curr Opin Immunol **5**(2): 218-222.

Bertsias, G. K., J. E. Salmon, et al. (2010). "Therapeutic opportunities in systemic lupus erythematosus: state of the art and prospects for the new decade." Ann Rheum Dis **69**(9): 1603-1611.

Bettelli, E., Y. Carrier, et al. (2006). "Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells." Nature **441**(7090): 235-238.

Bettelli, E., L. B. Nicholson, et al. (2003). "IL-10, a key effector regulatory cytokine in experimental autoimmune encephalomyelitis." J Autoimmun **20**(4): 265-267.

Biron, C. A., K. B. Nguyen, et al. (1999). "Natural killer cells in antiviral defense: function and regulation by innate cytokines." Annu Rev Immunol **17**: 189-220.

Bjornson, A. B. and P. A. Detmers (1995). "The Pentameric Structure of Igm Is Necessary to Enhance Opsonization of Bacteroides-Thetaiotaomicron and Bacteroides-Fragilis Via the Alternative Complement Pathway." Microbial Pathogenesis **19**(2): 117-128.

Bjornson, A. B. and P. A. Detmers (1995). "The pentameric structure of IgM is necessary to enhance opsonization of Bacteroides thetaiotaomicron and Bacteroides fragilis via the alternative complement pathway." Microb Pathog **19**(2): 117-128.

Boes, M. (2000). "Role of natural and immune IgM antibodies in immune responses." Mol Immunol **37**(18): 1141-1149.

Boes, M., T. Schmidt, et al. (2000). "Accelerated development of IgG autoantibodies and autoimmune disease in the absence of secreted IgM." Proc Natl Acad Sci U S A **97**(3): 1184-1189.

Bolland, S. and J. V. Ravetch (2000). "Spontaneous autoimmune disease in Fc(gamma)RIIB-deficient mice results from strain-specific epistasis." Immunity **13**(2): 277-285.

Booth, J. S., J. J. Buza, et al. (2010). "Co-stimulation with TLR7/8 and TLR9 agonists induce down-regulation of innate immune responses in sheep blood mononuclear and B cells." Dev Comp Immunol **34**(5): 572-578.

Botto, M., C. Dell'Agnola, et al. (1998). "Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies." Nature Genetics **19**(1): 56-59.

Boyington, J. C. and P. D. Sun (2002). "A structural perspective on MHC class I recognition by killer cell immunoglobulin-like receptors." Mol Immunol **38**(14): 1007-1021.

Brown, J. S., T. Hussell, et al. (2002). "The classical pathway is the dominant complement pathway required for innate immunity to Streptococcus pneumoniae infection in mice." Proc Natl Acad Sci U S A **99**(26): 16969-16974.

Brownlie, R. J., K. E. Lawlor, et al. (2008). "Distinct cell-specific control of autoimmunity and infection by FcgammaRIIb." J Exp Med **205**(4): 883-895.

Burstein, H. J. and A. K. Abbas (1991). "T-cell-mediated activation of B cells." Curr Opin Immunol **3**(3): 345-349.

Buzas, E. I., B. Gyorgy, et al. (2006). "Carbohydrate recognition systems in autoimmunity." Autoimmunity **39**(8): 691-704.

Carlyle, J. R. and J. C. Zuniga-Pflucker (1998). "Requirement for the thymus in alphabeta T lymphocyte lineage commitment." Immunity **9**(2): 187-197.

Carpenter, A. C. and R. Bosselut (2010). "Decision checkpoints in the thymus." Nat Immunol **11**(8): 666-673.

Casali, P. and E. W. Schettino (1996). "Structure and function of natural antibodies." Curr Top Microbiol Immunol **210**: 167-179.

Casciola-Rosen, L. A., G. Anhalt, et al. (1994). "Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes." J Exp Med **179**(4): 1317-1330.

Cash, H., M. Relle, et al. (2010). "Interleukin 6 (IL-6) deficiency delays lupus nephritis in MRL-Faslpr mice: the IL-6 pathway as a new therapeutic target in treatment of autoimmune kidney disease in systemic lupus erythematosus." J Rheumatol **37**(1): 60-70.

Chan, A. C. and P. J. Carter (2010). "Therapeutic antibodies for autoimmunity and inflammation." Nat Rev Immunol **10**(5): 301-316.

Chan, O. and M. J. Shlomchik (1998). "A new role for B cells in systemic autoimmunity: B cells promote spontaneous T cell activation in MRL-lpr/lpr mice." J Immunol **160**(1): 51-59.

Chen, C., H. Li, et al. (2006). "Selection of anti-double-stranded DNA B cells in autoimmune MRL-lpr/lpr mice." J Immunol **176**(9): 5183-5190.

Chen, Y., S. Khanna, et al. (2009). "Regulation of dendritic cells and macrophages by an anti-apoptotic cell natural antibody that suppresses TLR responses and inhibits inflammatory arthritis." J Immunol **183**(2): 1346-1359.

Christensen, S. R., M. Kashgarian, et al. (2005). "Toll-like receptor 9 controls anti-DNA autoantibody production in murine lupus." J Exp Med **202**(2): 321-331.

Christensen, S. R. and M. J. Shlomchik (2007). "Regulation of lupus-related autoantibody production and clinical disease by Toll-like receptors." Semin Immunol **19**(1): 11-23.

Christensen, S. R., J. Shupe, et al. (2006). "Toll-like receptor 7 and TLR9 dictate autoantibody specificity and have opposing inflammatory and regulatory roles in a murine model of lupus." Immunity **25**(3): 417-428.

Cinamon, G., M. A. Zachariah, et al. (2008). "Follicular shuttling of marginal zone B cells facilitates antigen transport." Nat Immunol **9**(1): 54-62.

Clarke, S. H. and L. W. Arnold (1998). "B-1 cell development: evidence for an uncommitted immunoglobulin (Ig)M+ B cell precursor in B-1 cell differentiation." J Exp Med **187**(8): 1325-1334.

Cooper, G. S., K. M. Gilbert, et al. (2008). "Recent advances and opportunities in research on lupus: environmental influences and mechanisms of disease." Environ Health Perspect **116**(6): 695-702.

Crispin, J. C., M. Oukka, et al. (2008). "Expanded double negative T cells in patients with systemic lupus erythematosus produce IL-17 and infiltrate the kidneys." J Immunol **181**(12): 8761-8766.

Crispin, J. C. and G. C. Tsokos (2010). "IL-17 in systemic lupus erythematosus." J Biomed Biotechnol **2010**: 943254.

Dauphinee, M., Z. Tovar, et al. (1988). "B cells expressing CD5 are increased in Sjogren's syndrome." Arthritis Rheum **31**(5): 642-647.

David, K., S. Heiligtag, et al. (2001). "Initial characterization of the apoptosis-inducing receptor for natural human anti-neuroblastoma IgM." Med Pediatr Oncol **36**(1): 251-257.

De Lorenzo, B. H., R. R. Brito, et al. (2007). "Tolerogenic property of B-1b cells in a model of allergic reaction." Immunol Lett **114**(2): 110-118.

Decker, P., I. Kotter, et al. (2006). "Monocyte-derived dendritic cells over-express CD86 in patients with systemic lupus erythematosus." Rheumatology (Oxford) **45**(9): 1087-1095.

DiLillo, D. J., T. Matsushita, et al. (2010). "B10 cells and regulatory B cells balance immune responses during inflammation, autoimmunity, and cancer." Ann N Y Acad Sci **1183**: 38-57.

Dong, C. (2006). "Diversification of T-helper-cell lineages: finding the family root of IL-17-producing cells." Nat Rev Immunol **6**(4): 329-333.

Dong, C. (2008). "TH17 cells in development: an updated view of their molecular identity and genetic programming." Nat Rev Immunol **8**(5): 337-348.

Dong, G., R. Ye, et al. (2003). "IL-17 induces autoantibody overproduction and peripheral blood mononuclear cell overexpression of IL-6 in lupus nephritis patients." Chin Med J (Engl) **116**(4): 543-548.

Dorshkind, K. and E. Montecino-Rodriguez (2007). "Fetal B-cell lymphopoiesis and the emergence of B-1-cell potential." Nat Rev Immunol **7**(3): 213-219.

Duan, B. and L. Morel (2006). "Role of B-1a cells in autoimmunity." Autoimmun Rev **5**(6): 403-408.

Dunkelberger, J. R. and W. C. Song (2010). "Complement and its role in innate and adaptive immune responses." Cell Res **20**(1): 34-50.

Edgerton, C., J. C. Crispin, et al. (2009). "IL-17 producing CD4+ T cells mediate accelerated ischemia/reperfusion-induced injury in autoimmunity-prone mice." Clin Immunol **130**(3): 313-321.

Ehlers, M., H. Fukuyama, et al. (2006). "TLR9/MyD88 signaling is required for class switching to pathogenic IgG2a and 2b autoantibodies in SLE." J Exp Med **203**(3): 553-561.

Ehlers, M. and J. V. Ravetch (2007). "Opposing effects of Toll-like receptor stimulation induce autoimmunity or tolerance." Trends Immunol **28**(2): 74-79.

Ehrenstein, M. R., H. T. Cook, et al. (2000). "Deficiency in serum immunoglobulin (Ig)M predisposes to development of IgG autoantibodies." J Exp Med **191**(7): 1253-1258.

Ehrenstein, M. R., D. R. Katz, et al. (1995). "Human IgG anti-DNA antibodies deposit in kidneys and induce proteinuria in SCID mice." Kidney Int **48**(3): 705-711.

Ehrenstein, M. R. and C. A. Notley (2010). "The importance of natural IgM: scavenger, protector and regulator." Nat Rev Immunol **10**(11): 778-786.

Ehrenstein, M. R., T. L. O'Keefe, et al. (1998). "Targeted gene disruption reveals a role for natural secretory IgM in the maturation of the primary immune response." Proc Natl Acad Sci U S A **95**(17): 10089-10093.

El Shikh, M. E., R. M. El Sayed, et al. (2009). "T-independent antibody responses to T-dependent antigens: a novel follicular dendritic cell-dependent activity." J Immunol **182**(6): 3482-3491.

Enghard, P., J. Y. Humrich, et al. (2010). "Class switching and consecutive loss of dsDNA-reactive B1a B cells from the peritoneal cavity during murine lupus development." Eur J Immunol **40**(6): 1809-1818.

Enghard, P., J. Y. Humrich, et al. (2010). "Class switching and consecutive loss of dsDNA reactive B1a B cells from the peritoneal cavity during murine lupus development." Eur J Immunol.

Fagarasan, S., N. Watanabe, et al. (2000). "Generation, expansion, migration and activation of mouse B1 cells." Immunol Rev **176**: 205-215.

Ferguson, A. R., M. E. Youd, et al. (2004). "Marginal zone B cells transport and deposit IgM-containing immune complexes onto follicular dendritic cells." Int Immunol **16**(10): 1411-1422.

Ferry, H., P. K. Potter, et al. (2007). "Increased positive selection of B1 cells and reduced B cell tolerance to intracellular antigens in c1q-deficient mice." J Immunol **178**(5): 2916-2922.

Fillatreau, S., D. Gray, et al. (2008). "Not always the bad guys: B cells as regulators of autoimmune pathology." Nat Rev Immunol **8**(5): 391-397.

Fillatreau, S., C. H. Sweenie, et al. (2002). "B cells regulate autoimmunity by provision of IL-10." Nat Immunol **3**(10): 944-950.

Finck, B. K., B. Chan, et al. (1994). "Interleukin 6 promotes murine lupus in NZB/NZW F1 mice." J Clin Invest **94**(2): 585-591.

Fischer, M. and M. Ehlers (2008). "Toll-like receptors in autoimmunity." Ann N Y Acad Sci **1143**: 21-34.

Forger, F., T. Matthias, et al. (2004). "Clinical significance of anti-dsDNA antibody isotypes: IgG/IgM ratio of anti-dsDNA antibodies as a prognostic marker for lupus nephritis." Lupus **13**(1): 36-44.

Franchi, L., C. McDonald, et al. (2006). "Nucleotide-binding oligomerization domain-like receptors: intracellular pattern recognition molecules for pathogen detection and host defense." J Immunol **177**(6): 3507-3513.

Fukata, M., K. Breglio, et al. (2008). "The myeloid differentiation factor 88 (MyD88) is required for CD4+ T cell effector function in a murine model of inflammatory bowel disease." J Immunol **180**(3): 1886-1894.

Gaipl, U. S., L. E. Munoz, et al. (2007). "Clearance deficiency and systemic lupus erythematosus (SLE)." J Autoimmun **28**(2-3): 114-121.

Garrett-Sinha, L. A., S. John, et al. (2008). "IL-17 and the Th17 lineage in systemic lupus erythematosus." Curr Opin Rheumatol **20**(5): 519-525.

Genestier, L., M. Taillardet, et al. (2007). "TLR agonists selectively promote terminal plasma cell differentiation of B cell subsets specialized in thymus-independent responses." J Immunol **178**(12): 7779-7786.

George, J., Y. Adler, et al. (1995). "Apoptosis and its association with autoimmunity." Clin Rheumatol **14**(5): 505-509.

Giorgini, A., H. J. Brown, et al. (2008). "Fc gamma RIII and Fc gamma RIV are indispensable for acute glomerular inflammation induced by switch variant monoclonal antibodies." J Immunol **181**(12): 8745-8752.

Glik, A. and A. Douvdevani (2006). "T lymphocytes: the "cellular" arm of acquired immunity in the peritoneum." Perit Dial Int **26**(4): 438-448.

Godaly, G., G. Bergsten, et al. (2001). "Neutrophil recruitment, chemokine receptors, and resistance to mucosal infection." J Leukoc Biol **69**(6): 899-906.

Goodnow, C. C. (1996). "Balancing immunity and tolerance: deleting and tuning lymphocyte repertoires." Proc Natl Acad Sci U S A **93**(6): 2264-2271.

Goodnow, C. C., S. Adelstein, et al. (1990). "The need for central and peripheral tolerance in the B cell repertoire." Science **248**(4961): 1373-1379.

Haas, K. M., J. C. Poe, et al. (2005). "B-1a and B-1b cells exhibit distinct developmental requirements and have unique functional roles in innate and adaptive immunity to S. pneumoniae." Immunity **23**(1): 7-18.

Hardy, R. R. (2006). "B-1 B cell development." J Immunol **177**(5): 2749-2754.

Hardy, R. R. (2006). "B-1 B cells: development, selection, natural autoantibody and leukemia." Curr Opin Immunol **18**(5): 547-555.

Harty, J. T., A. R. Tvinnereim, et al. (2000). "CD8+ T cell effector mechanisms in resistance to infection." Annu Rev Immunol **18**: 275-308.

Hayakawa, K., R. R. Hardy, et al. (1985). "Progenitors for Ly-1 B cells are distinct from progenitors for other B cells." J Exp Med **161**(6): 1554-1568.

Hemmi, H., O. Takeuchi, et al. (2000). "A Toll-like receptor recognizes bacterial DNA." Nature **408**(6813): 740-745.

Herzenberg, L. A. (2000). "B-1 cells: the lineage question revisited." Immunol Rev **175**: 9-22.

Hsu, H. C., P. Yang, et al. (2008). "Interleukin 17-producing T helper cells and interleukin 17 orchestrate autoreactive germinal center development in autoimmune BXD2 mice." Nat Immunol **9**(2): 166-175.

Hsu, H. C., T. Zhou, et al. (2006). "Production of a novel class of polyreactive pathogenic autoantibodies in BXD2 mice causes glomerulonephritis and arthritis." Arthritis Rheum **54**(1): 343-355.

Ishikawa, S. and K. Matsushima (2007). "Aberrant B1 cell trafficking in a murine model for lupus." Front Biosci **12**: 1790-1803.

Ishikawa, S., T. Sato, et al. (2001). "Aberrant high expression of B lymphocyte chemokine (BLC/CXCL13) by C11b+CD11c+ dendritic cells in murine lupus and preferential chemotaxis of B1 cells towards BLC." J Exp Med **193**(12): 1393-1402.

Ito, T., S. Ishikawa, et al. (2004). "Defective B1 cell homing to the peritoneal cavity and preferential recruitment of B1 cells in the target organs in a murine model for systemic lupus erythematosus." J Immunol **172**(6): 3628-3634.

Jacob, J., G. Kelsoe, et al. (1991). "Intraclonal generation of antibody mutants in germinal centres." Nature **354**(6352): 389-392.

Jaiswal, A. I. and M. Croft (1997). "CD40 ligand induction on T cell subsets by peptide-presenting B cells: implications for development of the primary T and B cell response." J Immunol **159**(5): 2282-2291.

Jin, D., L. Zhang, et al. (2008). "The inflammatory Th 17 subset in immunity against self and non-self antigens." Autoimmunity **41**(2): 154-162.

Kabelitz, D. (2007). "Expression and function of Toll-like receptors in T lymphocytes." Curr Opin Immunol **19**(1): 39-45.

Kantor, A. B. (1991). "The development and repertoire of B-1 cells (CD5 B cells)." Immunol Today **12**(11): 389-391.

Katsiari, C. G., S. N. Liossis, et al. (2010). "The pathophysiologic role of monocytes and macrophages in systemic lupus erythematosus: a reappraisal." Semin Arthritis Rheum **39**(6): 491-503.

Kawahara, T., H. Ohdan, et al. (2003). "Peritoneal cavity B cells are precursors of splenic IgM natural antibody-producing cells." J Immunol **171**(10): 5406-5414.

Kimbrell, D. A. and B. Beutler (2001). "The evolution and genetics of innate immunity." Nat Rev Genet **2**(4): 256-267.

Kisielow, P. and H. von Boehmer (1995). "Development and selection of T cells: facts and puzzles." Adv Immunol **58**: 87-209.

Klein, U. and R. Dalla-Favera (2008). "Germinal centres: role in B-cell physiology and malignancy." Nat Rev Immunol **8**(1): 22-33.

Koenders, M. I. and W. B. van den Berg (2010). "Translational mini-review series on Th17 cells: are T helper 17 cells really pathogenic in autoimmunity?" Clin Exp Immunol **159**(2): 131-136.

Koffler, D., P. H. Schur, et al. (1967). "Glomerular localization of DNA and antibodies to nuclear constituents." Trans Assoc Am Physicians **80**: 149-155.

Koren, E., M. Koscec, et al. (1995). "Murine and human antibodies to native DNA that cross-react with the A and D SnRNP polypeptides cause direct injury of cultured kidney cells." J Immunol **154**(9): 4857-4864.

Korn, T., E. Bettelli, et al. (2009). "IL-17 and Th17 Cells." Annu Rev Immunol **27**: 485-517.

Kotzin, B. L. (1996). "Systemic lupus erythematosus." Cell **85**(3): 303-306.

Kubo, T., Y. Uchida, et al. (2009). "Augmented TLR9-induced Btk activation in PIR-B-deficient B-1 cells provokes excessive autoantibody production and autoimmunity." J Exp Med **206**(9): 1971-1982.

Kyttaris, V. C., Z. Zhang, et al. (2010). "Cutting edge: IL-23 receptor deficiency prevents the development of lupus nephritis in C57BL/6-lpr/lpr mice." J Immunol **184**(9): 4605-4609.

Lajoie, S., I. P. Lewkowich, et al. (2010). "Complement-mediated regulation of the IL-17A axis is a central genetic determinant of the severity of experimental allergic asthma." Nat Immunol **11**(10): 928-935.

Lampropoulou, V., E. Calderon-Gomez, et al. (2010). "Suppressive functions of activated B cells in autoimmune diseases reveal the dual roles of Toll-like receptors in immunity." Immunol Rev **233**(1): 146-161.

Lampropoulou, V., K. Hoehlig, et al. (2008). "TLR-activated B cells suppress T cell-mediated autoimmunity." J Immunol **180**(7): 4763-4773.

Lanzavecchia, A. and F. Sallusto (2007). "Toll-like receptors and innate immunity in B-cell activation and antibody responses." Curr Opin Immunol **19**(3): 268-274.

Lartigue, A., P. Courville, et al. (2006). "Role of TLR9 in anti-nucleosome and anti-DNA antibody production in lpr mutation-induced murine lupus." J Immunol **177**(2): 1349-1354.

Lau, C. M., C. Broughton, et al. (2005). "RNA-associated autoantigens activate B cells by combined B cell antigen receptor/Toll-like receptor 7 engagement." J Exp Med **202**(9): 1171-1177.

Leadbetter, E. A., I. R. Rifkin, et al. (2002). "Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors." Nature **416**(6881): 603-607.

Lee, J., J. M. Gonzales-Navajas, et al. (2008). "The "polarizing-tolerizing" mechanism of intestinal epithelium: its relevance to colonic homeostasis." Semin Immunopathol **30**(1): 3-9.

Lefkowith, J. B. and G. S. Gilkeson (1996). "Nephritogenic autoantibodies in lupus: current concepts and continuing controversies." Arthritis Rheum **39**(6): 894-903.

Lemaitre, B., E. Nicolas, et al. (1996). "The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults." Cell **86**(6): 973-983.

Lewis, M. J., T. H. Malik, et al. (2009). "Immunoglobulin M is required for protection against atherosclerosis in low-density lipoprotein receptor-deficient mice." Circulation **120**(5): 417-426.

Lobo, P. I., K. H. Schlegel, et al. (2008). "Naturally occurring IgM anti-leukocyte autoantibodies (IgM-ALA) inhibit T cell activation and chemotaxis." J Immunol **180**(3): 1780-1791.

Louten, J., K. Boniface, et al. (2009). "Development and function of TH17 cells in health and disease." J Allergy Clin Immunol **123**(5): 1004-1011.

Luster, A. D. (2002). "The role of chemokines in linking innate and adaptive immunity." Curr Opin Immunol **14**(1): 129-135.

Mak, T. W. (1995). "Gaining insights into the ontogeny and activation of T cells through the use of gene-targeted mutant mice." J Inflamm **45**(2): 79-84.

Mandik-Nayak, L., S. J. Seo, et al. (1999). "MRL-lpr/lpr mice exhibit a defect in maintaining developmental arrest and follicular exclusion of anti-double-stranded DNA B cells." J Exp Med **189**(11): 1799-1814.

Manson, J. J., C. Mauri, et al. (2005). "Natural serum IgM maintains immunological homeostasis and prevents autoimmunity." Springer Semin Immunopathol **26**(4): 425-432.

Martin, F. and J. F. Kearney (1999). "CD21high IgMhigh splenic B cells enriched in the marginal zone: distinct phenotypes and functions." Curr Top Microbiol Immunol **246**: 45-50; discussion 51-42.

Martin, F. and J. F. Kearney (2000). "B-cell subsets and the mature preimmune repertoire. Marginal zone and B1 B cells as part of a "natural immune memory"." Immunol Rev **175**: 70-79.

Martin, F. and J. F. Kearney (2001). "B1 cells: similarities and differences with other B cell subsets." Curr Opin Immunol **13**(2): 195-201.

Martin, F., A. M. Oliver, et al. (2001). "Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens." Immunity **14**(5): 617-629.

Masmoudi, H., T. Mota-Santos, et al. (1990). "All T15 Id-positive antibodies (but not the majority of VHT15+ antibodies) are produced by peritoneal CD5+ B lymphocytes." Int Immunol **2**(6): 515-520.

McHeyzer-Williams, L. J., L. P. Malherbe, et al. (2006). "Helper T cell-regulated B cell immunity." Curr Top Microbiol Immunol **311**: 59-83.

McHeyzer-Williams, M. G., L. J. McHeyzer-Williams, et al. (2000). "Antigen-specific immunity. Th cell-dependent B cell responses." Immunol Res **22**(2-3): 223-236.

Meffre, E., A. Schaefer, et al. (2004). "Surrogate light chain expressing human peripheral B cells produce self-reactive antibodies." J Exp Med **199**(1): 145-150.

Mihara, M., N. Takagi, et al. (1998). "IL-6 receptor blockage inhibits the onset of autoimmune kidney disease in NZB/W F1 mice." Clin Exp Immunol **112**(3): 397-402.

Mitsdoerffer, M., Y. Lee, et al. (2010). "Proinflammatory T helper type 17 cells are effective B-cell helpers." Proc Natl Acad Sci U S A **107**(32): 14292-14297.

Mizoguchi, A. and A. K. Bhan (2006). "A case for regulatory B cells." J Immunol **176**(2): 705-710.

Mogensen, S. C. (1979). "Role of macrophages in natural resistance to virus infections." Microbiol Rev **43**(1): 1-26.

Montecino-Rodriguez, E. and K. Dorshkind (2006). "New perspectives in B-1 B cell development and function." Trends Immunol **27**(9): 428-433.

Mosmann, T. R., H. Cherwinski, et al. (1986). "Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins." J Immunol **136**(7): 2348-2357.

Mullis, K., F. Faloona, et al. (1986). "Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction." Cold Spring Harb Symp Quant Biol **51 Pt 1**: 263-273.

Murakami, M., H. Yoshioka, et al. (1995). "Prevention of autoimmune symptoms in autoimmune-prone mice by elimination of B-1 cells." Int Immunol **7**(5): 877-882.

Nalbandian, A., J. C. Crispin, et al. (2009). "Interleukin-17 and systemic lupus erythematosus: current concepts." Clin Exp Immunol **157**(2): 209-215.

Nemazee, D. and K. Buerki (1989). "Clonal deletion of autoreactive B lymphocytes in bone marrow chimeras." Proc Natl Acad Sci U S A **86**(20): 8039-8043.

Nemazee, D. A. and K. Burki (1989). "Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes." Nature **337**(6207): 562-566.

Nimmerjahn, F., P. Bruhns, et al. (2005). "FcgammaRIV: a novel FcR with distinct IgG subclass specificity." Immunity **23**(1): 41-51.

Nimmerjahn, F. and J. V. Ravetch (2005). "Divergent immunoglobulin g subclass activity through selective Fc receptor binding." Science **310**(5753): 1510-1512.

Nimmerjahn, F. and J. V. Ravetch (2006). "Fcgamma receptors: old friends and new family members." Immunity **24**(1): 19-28.

Nimmerjahn, F. and J. V. Ravetch (2008). "Analyzing antibody-Fc-receptor interactions." Methods Mol Biol **415**: 151-162.

Nimmerjahn, F. and J. V. Ravetch (2008). "Fcgamma receptors as regulators of immune responses." Nat Rev Immunol **8**(1): 34-47.

Nossal, G. J. and B. L. Pike (1980). "Clonal anergy: persistence in tolerant mice of antigen-binding B lymphocytes incapable of responding to antigen or mitogen." Proc Natl Acad Sci U S A **77**(3): 1602-1606.

Ollert, M. W., K. David, et al. (1996). "Normal human serum contains a natural IgM antibody cytotoxic for human neuroblastoma cells." Proc Natl Acad Sci U S A **93**(9): 4498-4503.

Ono, M., S. Bolland, et al. (1996). "Role of the inositol phosphatase SHIP in negative regulation of the immune system by the receptor Fc(gamma)RIIB." Nature **383**(6597): 263-266.

Peng, Y., R. Kowalewski, et al. (2005). "The role of IgM antibodies in the recognition and clearance of apoptotic cells." Mol Immunol **42**(7): 781-787.

Pepper, M., J. L. Linehan, et al. (2010). "Different routes of bacterial infection induce long-lived TH1 memory cells and short-lived TH17 cells." Nat Immunol **11**(1): 83-89.

Pereira, J. P., L. M. Kelly, et al. (2010). "Finding the right niche: B-cell migration in the early phases of T-dependent antibody responses." Int Immunol **22**(6): 413-419.

Pernis, A. B. (2009). "Th17 cells in rheumatoid arthritis and systemic lupus erythematosus." J Intern Med **265**(6): 644-652.

Pisitkun, P., J. A. Deane, et al. (2006). "Autoreactive B cell responses to RNA-related antigens due to TLR7 gene duplication." Science **312**(5780): 1669-1672.

Pozdnykova, O. O., A. P. Prodeus, et al. (1998). "Deficiency in complement C4 or receptors CD21/CD35 leads to increased anti-nuclear and -dsDNA antibodies and disease in lpr mice." Molecular Immunology **35**(6-7): 338-338.

Prodeus, A. P., S. Goerg, et al. (1998). "A critical role for complement in maintenance of self-tolerance." Immunity **9**(5): 721-731.

Quartier, P., P. K. Potter, et al. (2005). "Predominant role of IgM-dependent activation of the classical pathway in the clearance of dying cells by murine bone marrow-derived macrophages in vitro." Eur J Immunol **35**(1): 252-260.

Rabe, B., A. Chalaris, et al. (2008). "Transgenic blockade of interleukin 6 transsignaling abrogates inflammation." Blood **111**(3): 1021-1028.

Rahman, A. and D. A. Isenberg (2008). "Systemic lupus erythematosus." N Engl J Med **358**(9): 929-939.

Randall, T. D., J. W. Brewer, et al. (1992). "Direct evidence that J chain regulates the polymeric structure of IgM in antibody-secreting B cells." J Biol Chem **267**(25): 18002-18007.

Reap, E. A., E. S. Sobel, et al. (1993). "Conventional B cells, not B-1 cells, are responsible for producing autoantibodies in lpr mice." J Exp Med **177**(1): 69-78.

Roark, J. H., C. L. Kuntz, et al. (1995). "Breakdown of B cell tolerance in a mouse model of systemic lupus erythematosus." J Exp Med **181**(3): 1157-1167.

Romagnani, S. (1999). "Th1/Th2 cells." Inflamm Bowel Dis **5**(4): 285-294.

Roncarolo, M. G., M. Battaglia, et al. (2003). "The role of interleukin 10 in the control of autoimmunity." J Autoimmun **20**(4): 269-272.

Ruprecht, C. R. and A. Lanzavecchia (2006). "Toll-like receptor stimulation as a third signal required for activation of human naive B cells." Eur J Immunol **36**(4): 810-816.

Santiago-Raber, M. L., I. Dunand-Sauthier, et al. (2010). "Critical role of TLR7 in the acceleration of systemic lupus erythematosus in TLR9-deficient mice." J Autoimmun **34**(4): 339-348.

Seder, R. A. and R. Ahmed (2003). "Similarities and differences in CD4+ and CD8+ effector and memory T cell generation." Nat Immunol **4**(9): 835-842.

Segal, A. W. (2005). "How neutrophils kill microbes." Annu Rev Immunol **23**: 197-223.

Seidl, K. J., J. D. MacKenzie, et al. (1997). "Frequent occurrence of identical heavy and light chain Ig rearrangements." Int Immunol **9**(5): 689-702.

Serra, P. and P. Santamaria (2006). "To 'B' regulated: B cells as members of the regulatory workforce." Trends Immunol **27**(1): 7-10.

Shibuya, A. and S. Honda (2006). "Molecular and functional characteristics of the Fcalpha/muR, a novel Fc receptor for IgM and IgA." Springer Semin Immunopathol **28**(4): 377-382.

Shibuya, A., N. Sakamoto, et al. (2000). "Fc alpha/mu receptor mediates endocytosis of IgM-coated microbes." Nat Immunol **1**(5): 441-446.

Shimizu, I., T. Kawahara, et al. (2007). "B-cell extrinsic CR1/CR2 promotes natural antibody production and tolerance induction of anti-alphaGAL-producing B-1 cells." Blood **109**(4): 1773-1781.

Sindhava, V., M. E. Woodman, et al. (2010). "Interleukin-10 mediated autoregulation of murine B-1 B-cells and its role in Borrelia hermsii infection." PLoS One **5**(7): e11445.

Smith, H. R. and R. R. Olson (1990). "CD5+ B lymphocytes in systemic lupus erythematosus and rheumatoid arthritis." J Rheumatol **17**(6): 833-835.

Snapper, C. M. and W. E. Paul (1987). "Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production." Science **236**(4804): 944-947.

Stall, A. M., S. Adams, et al. (1992). "Characteristics and development of the murine B-1b (Ly-1 B sister) cell population." Ann N Y Acad Sci **651**: 33-43.

Stavnezer, J. (1996). "Antibody class switching." Adv Immunol **61**: 79-146.

Stavnezer, J. (1996). "Immunoglobulin class switching." Curr Opin Immunol **8**(2): 199-205.

Strom, T. B. and M. Koulmanda (2009). "Recently discovered T cell subsets cannot keep their commitments." J Am Soc Nephrol **20**(8): 1677-1680.

Sze, D. M., K. M. Toellner, et al. (2000). "Intrinsic constraint on plasmablast growth and extrinsic limits of plasma cell survival." J Exp Med **192**(6): 813-821.

Takai, T., M. Ono, et al. (1996). "Augmented humoral and anaphylactic responses in Fc gamma RII-deficient mice." Nature **379**(6563): 346-349.

Takeda, K. and S. Akira (2005). "Toll-like receptors in innate immunity." Int Immunol **17**(1): 1-14.

Tomlinson, S. (1993). "Complement defense mechanisms." Curr Opin Immunol **5**(1): 83-89.

Turner, M. W. and R. M. Hamvas (2000). "Mannose-binding lectin: structure, function, genetics and disease associations." Rev Immunogenet **2**(3): 305-322.

van de Veerdonk, F. L., M. S. Gresnigt, et al. (2009). "Th17 responses and host defense against microorganisms: an overview." BMB Rep **42**(12): 776-787.

Varambally, S., Y. Bar-Dayan, et al. (2004). "Natural human polyreactive IgM induce apoptosis of lymphoid cell lines and human peripheral blood mononuclear cells." International Immunology **16**(3): 517-524.

Vassilev, T., N. Mihaylova, et al. (2006). "IgM-enriched human intravenous immunoglobulin suppresses T lymphocyte functions in vitro and delays the activation of T lymphocytes in hu-SCID mice." Clin Exp Immunol **145**(1): 108-115.

Viau, M. and M. Zouali (2005). "B-lymphocytes, innate immunity, and autoimmunity." Clin Immunol **114**(1): 17-26.

Viglianti, G. A., C. M. Lau, et al. (2003). "Activation of autoreactive B cells by CpG dsDNA." Immunity **19**(6): 837-847.

Wan, Y. Y. (2010). "Multi-tasking of helper T cells." Immunology **130**(2): 166-171.

Ward, E. S. and V. Ghetie (1995). "The effector functions of immunoglobulins: implications for therapy." Ther Immunol **2**(2): 77-94.

Wardemann, H., S. Yurasov, et al. (2003). "Predominant autoantibody production by early human B cell precursors." Science **301**(5638): 1374-1377.

Warrington, A. E., A. J. Bieber, et al. (2007). "A recombinant human IgM promotes myelin repair after a single, very low dose." J Neurosci Res **85**(5): 967-976.

Wells, S. M., A. B. Kantor, et al. (1994). "CD43 (S7) expression identifies peripheral B cell subsets." J Immunol **153**(12): 5503-5515.

Wen, X., D. Zhang, et al. (2004). "Transgene-mediated hyper-expression of IL-5 inhibits autoimmune disease but increases the risk of B cell chronic lymphocytic leukemia in a model of murine lupus." Eur J Immunol **34**(10): 2740-2749.

Werwitzke, S., D. Trick, et al. (2005). "Inhibition of lupus disease by anti-double-stranded DNA antibodies of the IgM isotype in the (NZB x NZW)F1 mouse." Arthritis Rheum **52**(11): 3629-3638.

Whipple, E. C., R. S. Shanahan, et al. (2004). "Analyses of the in vivo trafficking of stoichiometric doses of an anti-complement receptor 1/2 monoclonal antibody infused intravenously in mice." J Immunol **173**(4): 2297-2306.

Williams, M. A. and M. J. Bevan (2007). "Effector and memory CTL differentiation." Annu Rev Immunol **25**: 171-192.

Wong, C. K., L. C. Lit, et al. (2008). "Hyperproduction of IL-23 and IL-17 in patients with systemic lupus erythematosus: implications for Th17-mediated inflammation in auto-immunity." Clin Immunol **127**(3): 385-393.

Wu, X. and S. L. Peng (2006). "Toll-like receptor 9 signaling protects against murine lupus." Arthritis Rheum **54**(1): 336-342.

Yanaba, K., J. D. Bouaziz, et al. (2008). "A regulatory B cell subset with a unique CD1dhiCD5+ phenotype controls T cell-dependent inflammatory responses." Immunity **28**(5): 639-650.

Yanaba, K., J. D. Bouaziz, et al. (2009). "The development and function of regulatory B cells expressing IL-10 (B10 cells) requires antigen receptor diversity and TLR signals." J Immunol **182**(12): 7459-7472.

Yap, D. Y. and K. N. Lai (2010). "Cytokines and their roles in the pathogenesis of systemic lupus erythematosus: from basics to recent advances." J Biomed Biotechnol **2010**: 365083.

Yung, S. and T. M. Chan (2008). "Anti-DNA antibodies in the pathogenesis of lupus nephritis--the emerging mechanisms." Autoimmun Rev **7**(4): 317-321.

Zhu, J., H. Yamane, et al. (2010). "Differentiation of effector CD4 T cell populations (*)." Annu Rev Immunol **28**: 445-489.

Acknowledgment

Arriving in Berlin in December 2007, I was not sure what was expecting me. What I found at the DRFZ were three years of exciting research in the Laboratory of Tolerance and Autoimmuntity and three amazing years with newly gained friends.

Though only my name appears on the cover of this dissertation, a great many people have contributed to its completion.

Foremost, I want to express my special gratitude to Prof. Dr. Marc Ehlers. Marc not only gave me the opportunity to work in such an interesting research context and laid out the theoretical and methodological foundations of my research; he was also a constructive critic in the best sense. His guidance, patience, encouragement and support helped me to successfully finish this dissertation.

I am grateful to Prof. Roland Lauster, who kindly agreed to supervise my thesis on behalf of the TU Berlin and to review my dissertation.

Furthermore, I am very thankful to all present and past members of the Laboratory of Tolerance and Autoimmunity for the fantastic time I had at the DRFZ. In particular I thank Alexander Stöhr, for his non-constraining help and teamwork in huge experiments, our lab-angel Vivien Holecska, Andre Winkler for numerous valuable discussions, Maria Mertes for introducing me into the topic of murine lupus, Alexandra Lorenz, Carolin Oefner and Felix Oden. Especially, I want to thank Susanne Eiglmeier, Constanze Heß and Felix Lorenz, not only for their any time help and supportive discussions in the lab. Various coffee breaks, BBB "meetings" and weekend trips made the last three years unforgettable also on a personal level.

I want to acknowledge Dr. Hedda Wardemann and her group at the MPI for infection biology for the exceedingly friendly collaboration throughout my entire time at the DRFZ.

I want to thank our lab neighbors, the AG Löhning for their unlimited help in answering technical questions, for numerous discussions and practical advice.

For helpful remarks and for carefully reading and commenting on preliminary versions of this work I want to particularly thank Marko Knoll, Susanne Eiglmeier, Dr. Anja Fröhlich and Dr. Ahmed Hegazy.

I am grateful to the entire DRFZ staff, in particular the "Heidis", for their help in the matter of antibody purification and the team of the FACS core facility, for solving any problems regarding FACS analysis and cell sorting.

Many friends have helped me stay sane throughout the last three years. I greatly value their friendship and I deeply appreciate their belief in me.

Most importantly, none of this would have been possible without the encouragement and patience of my family. My parents, my two little sisters Sophie and Amelie and my boyfriend Stephan have been a constant source of love, concern, support and strength.