The role of CD4 T helper cells and sialylated antigen-specific IgG antibodies in rheumatic autoimmune diseases

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Abstract

Systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) are rheumatic autoimmune diseases characterized by IgG autoantibody production. The systemic inflammation observed in SLE patients causes damage to multiple organs due to autoantibodies specific for various nuclear antigens. Otherwise, autoantibodies found in RA patients are mainly directed against the Fc-part of IgG (rheumatoid factor) and collagen type II causing inflammation primarily involving the joints and therefore resulting in destruction of cartilage.

T cells contribute to autoimmunity by stimulating the activation and functional differentiation of autoreactive B cells in germinal centers (GCs), which secrete high-affinity, isotype-switched autoantibodies. Furthermore, T cells facilitate and enhance autoimmunity by secretion of cytokines that promote recruitment and activation of innate immune cells such as macrophages and neutrophils into target organs. The aim of this study was to investigate different T cell subsets involved in the pathogenesis of SLE and RA using lupus prone RIIB-/-TLR9-/- mice and the collagen-induced arthritis (CIA) mouse model, respectively.

Analysis of RIIB-/-TLR9-/- mice showed an increase of Th1 and Th17 cell differentiation compared to wild-type mice. Deleting IFNγR1 or IL-17RA in RIIB-/-TLR9-/- mice made clear that both $CD4^+$ T helper cell subsets contribute to the pathogenesis of lupus nephritis. Th1 cells probably prevent disease onset by complete inhibition of IgG2c autoantibody development. Th17 cells influence germinal center formation in secondary lymphoid organs and macrophage infiltration into renal tissues. Thus, a reagent targeting both Th1 and Th17 cells might be a promising tool to treat SLE.

Investigation of the role of Th17 cells in the CIA mouse model showed reduced disease severity and disease incidence. This was due to impaired macrophage and neutrophil differentiation upon inhibition of Th17 cell differentiation by administration of an anti-IL-23R antibody.

Here, I used sialylated CII-specific IgG antibodies to investigate its effect on Th17 differentiation in the CIA mouse model. Recent studies showed that de-glycosylated autoantibodies mediate pro-inflammatory responses during the pathogenesis of RA. Furthermore, sialylated IgGs found in IVIG mediate anti-inflammatory responses antigen-unspecifically.

In this work, I compared the effect of sialylated and non-sialylated antigen-specific IgGs to IVIG regarding the cellular and humoral immune response in the CIA mouse model. My data show for the first time that low dose of antigen-specific sialylated IgG antibodies are sufficient to inhibit the development of CIA by inhibiting Th17 cell differentiation and pathogenic IgG autoantibody development. The data provide evidence that enzymatic glycosylation of antigen-specific antibodies is crucial to switch their effector function triggering anti-inflammatory effects and thus protecting from inflammatory immune responses. Furthermore, I provide evidence that sialylated antigen-specific IgG antibodies could substitute the immunosuppressive effect of IVIG in the future.

Zusammenfassung

Systemischer Lupus Erythematodes (SLE) und Rheumatoide Arthritis (RA) sind rheumatische Autoimmunerkrankungen, die durch die Entstehung von pathogenen IgG Autoantikörper charakterisiert sind. SLE ist eine systemische Autoimmunerkrankung, die sich durch die Entstehung von IgG Autoantikörper spezifisch für anti-nukleäre Antigene auszeichnet. Diese lagern sich in Form von Immunkomplexen in verschiedenen Organen ab, was zu Organversagen führt. Pathogene IgG Autoantikörper gegen den Fc-Teil von IgGs (Rheumafaktor IgGs) und Kollagen Typ 2 findet man bei Patienten der RA, wobei hier spezifische Entzündungen der Gelenke charakteristisch sind.

In Autoimmunerkrankungen wie SLE und RA stimulieren T Zellen autoreaktive B Zellen zur Differenzierung in Keimzentren, welche daraufhin hoch-affine IgG Antikörper sekretieren. Darüber hinaus können T Zellen zur Autoimmunität beitragen indem sie Zytokine produzieren, welche die Aktivierung von Immunzellen des angeborenen Immunsystems wie Makrophagen und Neutrophile und deren Rekrutierung in die Zielorgane fördern. Das Ziel dieser Arbeit war es, die Rolle von T Zellen, die an der Pathogenese von SLE und RA beteiligt sind, zu untersuchen. Hierfür wurden Lupus-anfällige RIIB-/-TLR9-/- Mäuse bzw. das Kollagen-induzierte Arthritis (CIA) Mausmodell verwendet.

Die Analyse der RIIB-/-TLR9-/- Mäuse zeigte eine Akkumulation von Th1 und Th17 Zellen im Vergleich zu Wildtyp-Mäusen. Die zusätzliche Deletion der Rezeptoren IFNγR1 oder IL-17RA in RIIB-/-TLR9-/- Mäusen zeigte deutlich, dass sowohl Th1 als auch Th17 Zellen an der Pathogenese der Lupusnephritis beteiligt sind. Th1 Zellen verhindern die Entstehung von Nephritis durch eine vollständige Hemmung der IgG2c Autoantikörperentstehung und eine sigifikante Reduktion von IgG2b. Th17 Zellen beeinflussen die Differenzierung von B Zellen in Kemzentren in sekundären lymphoiden Organen und die Infiltration von Makrophagen ins Nierengewebe.

In dem CIA Mausmodel für RA reduzierte ich die Th17 Differenzierung mit Hilfe eines IL-23R blockierenden Antikörpers. Dies führte zu verminderten Arthritissymptomen und reduzierter Anzahl an erkrankten Mäusen.

Die Daten aus beiden Krankheitsmodellen zeigen, dass ein Präparat, welches die Entstehung von Th17 und/oder Th1 Zellen verhindert, ein vielverprechendes Medikament zur Behandlung von Autoimmunerkrankungen darstellen könnte.

Daraufhin habe ich antigen-spezifische sialylierte IgGs als therapeutischen Ansatz zur Modifikation der Th1 und Th17 Differenzierung untersucht. Es ist bekannt, dass deglykosylierte Autoantikörper von RA-Patienten pro-inflammatorische Effekte vermitteln. Außerdem vermitteln hochsialylierte IgGs im IVIG entzündungshemmende Reaktionen auf antigen-unspezifische Weise.

In dieser Arbeit wurde die Wirkung von sialylierten und nicht-sialylierten kollagenII-spezifischen IgGs mit IVIG hinsichtlich der zellulären und humoralen Immunantwort im CIA Mausmodell verglichen. Meine Daten zeigen, dass eine niedrige Dosis von sialylierten kollagenII-spezifischen IgG-Antikörpern ausreicht, um die Entstehung von Th17 Zellen und pathogenen IgG Autoantikörpern zu vermindern und damit die Entstehung von CIA zu verhindern. Die hier gewonnenen Daten belegen, dass die Glykosylierung von antigenspezifischen Antikörpern entscheidend ist, um anti-inflammatorische Effekte auslösen zu können und damit Schutz vor entzündlichen Immunreaktionen zu bieten. Außerdem zeigt diese Arbeit, dass sialylierte antigen-spezifische IgG-Antikörper in Zukunft die immunsuppressive Wirkung von IVIG ersetzen könnten.

Diese Arbeit entstand im Deutschen Rheuma-Forschungszentrum (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 Januar 2008 bis Januar 2011 angefertigt.

Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die 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.

Susanne Eiglmeier Berlin, 27. Januar 2011

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1 Introduction

1.1 The innate and adaptive immune system - an overview

The immune system can be broadly classified into the adaptive and the innate immune systems (Fearon and Locksley 1996; Janeway 2001). Innate immunity develops an immediate response following exposure to immune stimuli in a non-specific manner while adaptive immunity mediates a delayed, specific response to foreign antigen (Janeway and Medzhitov 2002). Adaptive immune responses are essential for controlling pathogens that escape elimination by the innate immune response and are important for the development of immunological memory (Fearon and Locksley 1996; Schwartz 2000; Janeway 2001). The innate immune response is generated within seconds to minutes following invasion of a pathogen, whereas the adaptive immune response requires several days to respond. Both the adaptive and the innate immune system use cell receptors to bind foreign antigen and initiate an immune response to control and eliminate pathogens. The adaptive immune response recognizes details of molecular structures, such as proteins, peptides or carbohydrates. It utilizes clonal B cell receptors (BCR or soluble antibody) or T cell receptors (TCR) that have undergone germline rearrangement and recognize antigenic sequences that are specific to each individual pathogen (Fearon and Locksley 1996; Schwartz 2000; Janeway 2001). In contrast, the innate immune system uses pattern recognition receptors (PRRs) to recognize conserved pathogen-associated molecular patterns (PAMPS), such as LPS, bacterial CpG DNA or glycans, that are shared by classes of pathogens. The PRRs are fixed in the genome and no rearrangement is necessary, while B and T cell receptors are encoded in gene segments and reach their specificity and great variability through rearrangment and addition of p and n nucleotides. Macrophages, dendritic cells (DCs), mast cells, neutrophils, eosinophils, and natural killer cells (NK) feature germline-encoded recognition receptors. These cells become activated during an inflammatory response and rapidly differentiate into short-lived effector cells to eliminate the pathogen and to abort the infection (Fearon and Locksley 1996; Banchereau and Steinman 1998).

The innate immune system can instruct the adaptive immune system about the nature of the pathogenic challenge. Antigen presenting cells (APCs), such as macrophages and DCs recognize PAMPs and efficiently internalize antigen by phagocytosis or receptor-mediated endocytosis on the site of infection. PAMPs induce endogenous signals: (1) cytokine and

chemokine secretion, (2) expression of costimulatory molecules (e.g. CD80 and CD86), and (3) presentation of processed antigenic peptides in form of MHC-peptide complexes. Subsequently APCs migrate to the secondary lymphoid organs, where they present processed antigenic peptides in complex with MHC molecules to naive T cells. The appropriate clonal T cell of the adaptive immune system becomes activated, undergoes cell division and expansion, and travels to the site of infection. Thus, the innate immune system controls the initiation of the adaptive immune system and is necessary for its induction (June, Bluestone et al. 1994; Banchereau and Steinman 1998; Janeway and Medzhitov 2002).

Finally, another difference between the innate and adaptive immune system involves immunological memory. Upon secondary exposure to a pathogen, the innate immune system remains unaltered. However, the adaptive immune system can now react more rapidly and robustly due to the presence of memory T and B cells. Overall, the adaptive immune system is able to eliminate a specific existing infection, while the innate immune system provides a directed but broader response to control a pathogen before it is able to establish an infection.

1.2 T lymphocytes and the cellular immune response

Activation of naive T cells occurs in secondary lymphoid organs where they encounter their adequate antigen and costimulatory signals presented by the same APC. Antigen recognition occurs through the TCR, which recognizes antigen bound to major histocompatibility complex I or II (MHC I or MHC II) on APCs. Most T cells express a TCR composed of an alpha and a beta chain. Alpha/beta T cells are further categorized into two major subsets, called CD8 T cells and CD4 T cells, based on distinct cell surface proteins. CD8 T cells express the CD8 co-receptor, which associates with the T cell receptor (TCR) to interact with MHC I molecules expressed by APCs. CD8 T cells are often referred to as cytotoxic T lymphocytes (CTL) because their major function is to kill virally-infected cells or tumor cells that express abnormal peptides within their MHC I molecules. CD4 T cells express the CD4 co-receptor, which associates with the TCR to interact with MHC II molecules expressed mainly by professional APC, such as DCs, macrophages, and B cells. CD4 T cells, or T helper cells, secrete cytokines that orchestrate the adaptive immune response by "helping" other cells such as CD8 T cells or B cells to function appropriately.

Signals transduced by the TCR trigger a cascade of intracellular pathways that result in gene expression and synthesis of cytokines (IL-2) and cell surface molecules (IL-2 receptor, CD25) and therefore enabling the effector functions. Acivation of T cells by TCR ligation with MHC molecules is facilitated by CD3, a complex of cell surface molecules associated with the TCR.

Additional costimulatory molecules are necessary to specify and initiate proper T cell activation. One typical costimulatory event in T cell activation involves the interaction of CD28 on T cells with CD80 or CD86 on APCs. CD28 transduces signals into the cell, which promote and maintain T cell activation and proliferation, including cytokine and cytokine receptor expression. Mice deficient for CD28 or CD80/CD86 display impaired T cell proliferation, T cell priming by APCs, and T cell dependent antibody production (Linsley, Brady et al. 1991). While the costimulation of T cell activation via CD28/CD80/CD86 interaction is clearly discovered, the role for a second ligand for CD80/CD86, called cytotoxic lymphocyte antigen 4 (CTLA-4), is more controversial. CTLA-4 is only expressed upon T cell activation and found at much lower levels. Recent studies consider CTLA-4 to be the inhibitory counterpart to CD28 (Liu 1997; Wu, Guo et al. 1997). Mice deficient for CTLA-4 develop massive lymhoproliferative disorder: multi-organ inflammation, spontaneous proliferation of lymphocytes, increased numbers of B cells and serum immunogobulins (Igs) of all isotypes (Tivol, Borriello et al. 1995; Waterhouse, Penninger et al. 1995).

The interaction between CD40, expressed on B cells, DCs, macrophages and hematopoietic progenitor cells, and CD40L (CD154), expressed on activated T cells, is of great importance during cellular and humoral immune responses. CD154 expression is induced when B cells present peptide:MHC II complexes and simultaneously costimulate via CD28 T helper cells. The interaction between CD40 and CD154 results in clonal expansion of B cells, germinal center formation, isotype switching, affinity maturation, and plasma cell differentiation (Liu, Johnson et al. 1992; Lederman, Yellin et al. 1994). The CD40-CD154 system offers great potential for therapeutic immune modulation to treat autoimmune diseases or tolerance induction after transplantations (Quezada, Jarvinen et al. 2004).

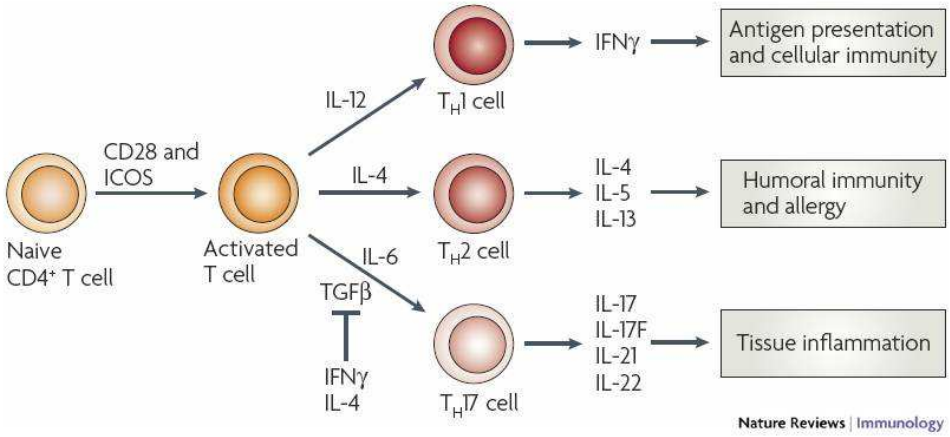
1.2.1 T helper cell differentiation

In 1986 the existence of two distinct subsets of T helper (Th) cells was postulated: Th1 and Th2 cells, which can be characterized by distinct cytokine secretion (Mosmann, Cherwinski et al. 1986). Th1 cells secrete Tumor necrosis factor (TNF)-α and IFNγ, thereby promoting cell-mediated immunity and induction of complement-fixing antibodies of the IgG2a isotype. Th1 cell response is often accompanied by cytokine induction in macrophages, activation of natural killer cells and cytotoxic CD8 T cells. If uncontrolled, Th1 cells can mediate immunopathology and have been implicated in autoimmune diseases. The transcription factor T-bet is crucial for Th1 differentiation. IL-12 is a dominant factor inducing the development of Th1 cells from a naive CD4 T cell precursor, and Th1 commitment is enhanced by IFN-γ which upregulates the IL-12 receptor while inhibiting the development of Th2 cells. IL-12 induced activation of the transcription factor STAT4 through the IL-12 receptor is crucial for Th1 responses (O'Garra and Arai 2000).

Th2 cells produce IL-4, IL-5 and IL-13, thereby supporting humoral immunity and counteracting Th1 cell responses. The transcription factor GATA-3 is essential for Th2 differentiation and *in vitro* Th2 cells can be induced by IL-4. Moreover they can eliminate extracellular parasites by activating mast cells and eosinophils. However, these cells also mediate allergic diseases by induction of IgE. Both Th1- and Th2-specific cytokines can promote growth or differentiation of their own respective T cell subset, but additionally might inhibit the development of the opposing subset. This might explain why Th1 and Th2 responses are often mutually exclusive (Sher and Coffman 1992; Urban, Madden et al. 1992; O'Garra and Arai 2000).

Besides effector T helper cells immune responses are kept in balance by regulatory T cells (Tregs). $CD4^+CD25^+Foxp3^+$ Tregs develop in the thymus and control effector T cell responses in the periphery. Hence, depletion of naturally occurring Treg cells results in hyperprolifertation of effector T cells and the development of autoimmunity (Sakaguchi 2000; Kim, Rasmussen et al. 2007). Treg cell differentitation is induced by transforming growth factor-β (TGFβ) in *vitro* and *in vivo*. During steady state, when the immune system is not activated, TGFβ favors the induction of Treg cells, which suppress inflammation and prevent autoimmune responses. However, after activation of the immune system by a patho-

gen, innate immune cells secrete IL-6 which together with TGFβ induces Th17 cells, a third effector T cell subset (Figure 1.1) (Bettelli, Carrier et al. 2006).



(Dong 2008)

Figure 1.1: CD4 T helper cell differentiation. Activation of naive CD4 T helper cells by antigen presenting cells (APCs) in the presence of co-stimulatory signals (CD28, ICOS) leads to expression and secretion of different cytokines and its receptors. The cytokine environment determines the terminal lineage commitment and cytokine expression profiles. Three different lineages of effector CD4 T helper cells can arise: Th1, Th2 and Th17 cells. Interferon-γ (IFNγ) produced by TH1 cells is important in the regulation of antigen presentation and cellular immunity. The TH2-cell cytokines IL-4, IL-5 and IL-13 regulate B-cell responses and anti-parasite immunity and are crucial mediators of allergic diseases. TH17 cells have been shown to express IL-17A, IL-17F, IL-21 and IL-22 and to regulate inflammatory responses. TGF β, transforming growth factor β.

1.2.1.1 Function and characterization of Th17 cells and their cytokines

From 2003 to 2005 several papers were published that led to the discovery of a new CD4 T helper cell subset, defined by the production of IL-17A and thus named Th17 cells (Aggarwal, Ghilardi et al. 2003; Murphy, Langrish et al. 2003; Harrington, Hatton et al. 2005; Langrish, Chen et al. 2005; Park, Li et al. 2005). Additional studies have shown that Th17 cells also express IL-17F, IL-23, IL-21 and IL-22 (Langrish, Chen et al. 2005; Liang, Tan et al. 2006). Th17 cells can be further characterized by expression of chemokine receptor CCR6 and its ligand CCL20 (Hirota, Yoshitomi et al. 2007). The current model suggests that Th1 cells are most important for clearance of intracellular pathogens, Th2 cells are

important for clearance of parasites and some extracellular pathogens, and Th17 cells are important for extracellular pathogens, including bacteria and funghi (summarized in Table 1.1 and Figure 1.1). The picture is undoubtly more complex, and the roles of the individual cytokines are still emerging (Bettelli, Korn et al. 2008).

The cytokines IL-17A and IL-17F are able to form IL-17A and IL-17F homodimers as well as IL-17A/F heterodimer *in vitro* and *in vivo*. Whereupon the heterodimer has intermediate biological activity compared to the homodimers, with IL-17A homodimer having the most activity. All three forms of IL-17 signal through the same receptor subunits, IL-17receptor A (IL-17RA) and IL-17receptor C (IL-17RC), which together form a heteromeric complex. However, it is suggested that the two homodimers and IL-17A/F heterodimer have different effects on different cell types (Fouser, Wright et al. 2008). Studies comparing IL-17A-/- mice with IL-17F-/- mice indicate that IL-17A has a more important role in driving autoimmunity than IL-17F, probably owing to its more potent strength of signaling (Yang, Chang et al. 2008; Ishigame, Kakuta et al. 2009).

The unique IL-17 receptor family consists of five receptor subunits, IL-17RA-IL-17RE (Aggarwal 2002). All of these receptor subunits are single transmembrane domain proteins. IL-17RA is a common signaling subunit used by at least four ligands. IL-17RA also binds IL-17F although weaker than IL-17A. The precise IL-17R binding complex has not been determined, but data indicate the existence of a trimeric complex containing two IL-17RA and one IL-17RC subunit. IL-17RA is expressed ubiquitously, with particularly high levels in hematopoietic tissues. High amounts of IL-17RA were detected in spleen, thymus, $B220^+$, $CD11c^+$, $MacI^+$, and macrophages although the widespread expression, IL-17RA can be dynamically regulated. This could be of great importance as proper IL-17 signaling is dependent on high IL-17RA expression, which is in contrast with most cytokine receptors. Another function of IL-17RA might be to limit signaling by receptor-mediated internalization of the ligand. Indeed, surface expression of IL-17RA rapidly decreases after IL-17 binding, theoretically internalizing IL-17A and clearing it from the inflammatory milieu (Lindemann, Hu et al. 2008).

Differentiation of Th17 cells is induced by IL-6 and TGFβ in mice (Cua and Kastelein 2006; Zhou, Ivanov et al. 2007, veldhoen 2006). IL-23 and IL-21 are important for maintenance and expansion of Th17 cells *in vitro* and *in vivo* (Langrish, Chen et al. 2005; Korn, Bettelli et al. 2007). Retinoid-related orphan receptor γt (RORγt) has been implicated as a

key transcription factor in Th17 cells (Ivanov, McKenzie et al. 2006). STAT 3 is an additional key transcription factor downstream of IL-6, IL-23, and IL-21 and important during Th17 cell development. STAT3 knock out mice have impaired Th17 cells differentiation (Harris, Grosso et al. 2007). Traditional Th1 and Th2 signature cytokines induce the activation of signaling pathways mediated by Janus kinas (JAK) and STAT proteins. By contrast IL-17A and IL-17F produced by Th17 cells additionally induce signaling mediated by ACT, TNFR-associated factor 6 (TRAF6) and nuclear factor-κB, which are much more reminiscent of receptors associated with innate immunity. Thus, regarding the unusual signaling properties of IL-17, Th17 cells may act as a bridge between adaptive and innate immunity (Yu and Gaffen 2008). Moreover, IL-17 stimulates granulocyte colony-stimulating factor (G-CSF) and GM-CSF, IL-6, matrix metallopeptidases and neutrophil-attracting CXC chemokines (reviewed in (Shen and Gaffen 2008)). Therefore, IL-17 is involved in immune cell recruitment and pro-inflammatory activities. Th17 cell cytokines and their target genes are promising targets for new therapeutic approaches in autoimmunity and inflammatory diseases.

Table 1.1: Subsets of murine $CD4^+$ T helper cells

	Th1	**Th2**	**Th17**
Signature cytokines	IFNγ, TNFα	IL-4, IL-5, IL-13, IL-25	IL-17A+F, IL-21, IL-22, CCL20, CCR6
Inductive cytokines	IL-12 (p35+p40), IFNγ	IL-4	TGFβ, IL-6
Key transcription factors	T-bet, STAT2, STAT4	GATA-3, STAT-6	RORγt, RORα, STAT3
Inhibitors of development	IL-4	IFNγ	IL-4, IFNγ
Main immune functions	Activation of macrophages, IgG2a/c production, autoimmunity and cellular immunity	Activation of mast cells and basophils, allergy humoral immunity	Activation of neutrophils, autoimmunity and tissue inflammation
Main microbial targets	Intracellular bacteria, viruses	Extracellular pathogens	Extracellular bacteria, fungi

1.3 B lymphocytes and the humoral immune response

Humoral immunity is mediated by B lymphocytes and involves the binding of antibodies to antigens. Together with the cellular immunity humoral immunity establish specific immunity. T cell progenitors migrate to the thymus to further mature whereas B cells reside in the bone marrow. During the maturation of B cells the stepwise rearrangement of the Ig loci takes place. This genetic process allows an extreme and random diversity of Ig molecules that may then recognize a large diversity of antigens. During development, the expression of a rearranged B cell receptor is important for the positive selection of B cells so that only successfully rearranged, non-self specific B cells can survive and migrate to the periphery. Follicular and marginal zone (MZ) B cells are the two main types of mature B cells found in the spleen, B1 cells predominantly reside in the peritoneal cavity. Follicular B cells circulate through the blood and lymph and can be found in spleen and lymph nodes, whereas MZ B cells only reside in the marginal zone of follicles in the spleen in rodents. Both MZ and B1 B cells are considered as the main source of IgM in response to T cell independent antigens and have the capacity of self-renewal (Martin and Kearney 2000). Mature naive B cells remain in a resting state until they encounter their cognate antigen or die within three days if they do not encounter their antigen. Depending on the nature of the antigen there are two general mechanisms of B cell activation.

Crosslinking of the B cell receptor via T cell independent (TI) antigens (e.g. repetitive structures such as bacterial polysaccharides) leads to clustering of the receptor and thus signaling via the Igα and Igβ. Such activated B cells may then proliferate to form extrafollicular foci in secondary lymphoid organs and differentiate into plasma cells that produce low affinity antibodies, mainly of the IgM isotype. This type of activation is commonly found in MZ B and B1 cells.

T cell dependent (TD) antigens on the other hand bind specifically to the BCR and are then presented on MHC II complexes after internalization. CD4 T cells that have been activated through binding of the antigen presented on a DC may then interact with the antigen-presenting B cells at the T-B-cell-zone border within follicles. In addition to the antigen-derived signal from the BCR T cells deliver costimulatory signals to B cells. One of these signals is mediated by the CD40 receptor that binds to the CD40 ligand (CD154)

expressed on activated CD4 T cells. In concert action, these signaling pathways stimulate B cells to either form extrafollicular foci or germinal centers (GCs).

GCs are structures in follicles of secondary lymphoid organs that arise four to seven days after administration of antigen. A network of follicular dendritic cells (FDC) and CD4 T cells can be found in between these B cell clones. Within these structures proliferating B cells are found in the dark zone (centroblasts), whereas the emerging B cell clones are selected in the light zone (centrocytes). Two important processes take place in germinal center B cells: somatic hypermutation (SHM) and class switch recombination (CSR). Both require the action of a specific enzyme, activation induced cytidine deaminase (AID) and of general DNA repair mechanisms. During somatic hypermutation, the variable (V) region of the Ig locus accumulates point mutations as well as small deletions or duplications. Due to this diversification process B cell clones that show a higher affinity to their cognate antigen are positively selected by FDCs. The second process, class switch recombination, leads to the expression of different antibody classes and thus influences the effector function of these molecules when they are secreted into the serum. Antibody classes are defined by the Fc (constant) region of the Ig heavy chain and can be altered genetically by class switch recombination, whereas the V region of the antibody molecule keeps the same antigen specificity. In response to different cytokines B cells can switch from IgM to IgG1, IgG2a, IgG2b, IgG3, IgE or IgA. B cells that have undergone these processes and have been positively selected by FDCs and CD4 T cells in the GC may then differentiate into plasma or memory B cells. Plasma cells function as effector cells that migrate to the bone marrow where they produce and secrete antibodies that bind to the specific pathogen. This leads to neutralization, opsonization and phagocytosis by macrophages or complement mediated lysis of the pathogen. Memory B cells on the other hand remain in a resting state in the body for long periods of time but can be activated promptly upon a second antigen challenge leading to a fast and specific secondary immune response.

1.4 Autoimmunity

Autoimmune disorders result from failure of central and peripheral tolerance mechanisms. Tolerance mechanisms are necessary to distinguish between self and foreign structures. Central tolerance occurs during B and T lymphocyte development in the bone marrow or

thymus, respectively. Thereby cells binding self-antigens are deleted before they develop into mature immune cells. Peripheral tolerance develops after B and T cell maturation in the periphery by inducing anergy or deletion of lymphocytes (Goodnow 1996). If one of these tolerance mechanism breaks down self-reactive lymphocytes are not eliminated and autoimmune disorders develop. Development of autoimmune diseases requires genetic defects, environmental factors, or infections to result in a loss of tolerance and induction of antibodies reactive to self-antigens. Autoimmune diseases can be broadly divided into systemic and organ-specific autoimmune disorders, depending on the principal pathologic features of each disease. Systemic lupus erythematosus (SLE), Sjörgen´s syndrom, and rheumatoid arthritis are systemic autoimmune diseases and are associated with autoantibodies to self-antigens, which are not tissue specific. Whereas diabetes mellitus type 1 is an organ-specific autoimmune disease and tissue-specific autoantibodies are secreted.

Traditional treatments for autoimmune diseases are immunosuppressive, anti-inflammatory, or palliative, such as rituximab or intravenous IgG purified from healthy donors (IVIG). Until now no cure has been found for autoimmune disorders. Hence, extensive research is necessary to identify and understand mechanisms, immune cells and signaling pathways involved in autoimmunity.

1.4.1 Systemic lupus erythematosus (SLE)

Systemic lupus erythematosus (SLE) is a chronic systemic inflammatory disease and is associated with a wide range of autoantibody production against nuclear antigens (ANA). ANA antibodies are present in more that 95% of the patients (Jimenez, Cervera et al. 2003). Additional lupus-associated antibodies are anti-ribonuclear (anti-RNP-70), anti-SSA (Ro), anti-SSB (La), anti-histone, and anti-Smith antibodies. Autoantibodies can form immune complexes (ICs) and trigger inflammation, which results in multiple organ damage. Cell death and therefore the increase of extracellular DNA are connected with SLE. Increased levels of extra-cellular DNA could occur either by an increase in cell death or by an impaired clearance of dying cells (Su and Pisetsky 2009).

Furthermore, impaired B cell tolerance contributes to the pathogenesis of SLE. It is estimated that more than half of all newly generated BCRs are capable of binding autoantigen

(Nemazee 1995). But not all BCRs that bind an autoantigen are potentially dangerous; they can either bind the antigen with too low affinity to trigger an autoimmune response or bind several antigens and are called polyreactive (Haspel, Onodera et al. 1983). However, many polyreactive cells and some but not all autoreactive cells are lost during the transition from pre-BII to immature B cells. In patients with SLE and rheumatoid arthritis these autoreactive and polyreactive BCRs are not eliminated at this late checkpoint, providing strong evidence of the role of this checkpoint in preventing autoimmune diseases. To identify genes associated with SLE various genetic studies in murine models of SLE have elucidated loci that confer susceptibility to systemic autoimmunity. Transgenic mice for B lymphocyte activating factor (BAFF) developed autoimmune symptoms (Mackay, Woodcock et al. 1999). Moreover, the alleles for Sle1 impair B cell anergy and Sle2 impacts the production of autoantibodies and lupus nephritis (Morel, Rudofsky et al. 1994; Vyse and Kotzin 1998; Kono and Theofilopoulos 2000; Liu, Li et al. 2007). On B cells the inhibitory FcγReceptor IIB (RIIB) contributes to the maintenance of tolerance during affinity maturation by inducing apoptosis, which results in deletion of low affinity, self-reactive B cells from the GC. Indeed, mice deficient for FcγRIIB develop ANA autoantibodies and display symptoms of lupus nephritis (Bolland and Ravetch 2000). In accordance, genetic studies in human have correlated SLE with a polymorphism in the gene for FcγRIIB amongst other genes (Liu and Ahearn 2009). Another study investigated the expression of FcγRIIB on different B cell subsets of SLE patients and found FcγRIIB downregulated on plasma blast whereas healthy individuals showed increased FcγRIIB expression on their memory B cells (Mackay, Stanevsky et al. 2006).

Recently, it has been reported that genetic variations of TLR9 predispose to SLE (Tao, Fujii et al. 2007). TLR9 binds CpG-rich DNA and is widely expressed on innate and adaptive immune cells and is an important costimulatory factor. Costimulation of BCR and TLR9, which resides in endosomes, induces DNA-specific antibodies (Leadbetter, Rifkin et al. 2002; Christensen, Shupe et al. 2006; Ehlers, Fukuyama et al. 2006). To examine the role of TLR9 in autoimmunity different mouse models for SLE were crossed to TLR9 deficient mice (Christensen, Kashgarian et al. 2005; Ehlers, Fukuyama et al. 2006; Lartigue, Courville et al. 2006; Marshak-Rothstein 2006). All studies reported immune complex (IC) deposition in the kidney and renal tissue destruction indicating a tolerogenic role of TLR9 in SLE. However, Christensen et al. claimed anti-DNA and anti-nucleosome antibody generation to

be TLR9 dependent whereas Ehlers et al only found anti-nucleosome antibodies diminished in TLR9 deficient lupus prone mice (FcγRIIB-/-). The exact mechanism how TLR9 mediates an anti-inflammatory response in lupus mouse models remains unclear.

The variety of genes involved in B cell tolerance, activation, differentiation, and apoptosis displays the complexity of SLE. To discover a treatment for SLE we first have to understand the pathogenesis of SLE. In this study FcγRIIB-/-TLR9-/- mice exhibiting symptoms of SLE were used for further investigations.

1.4.2 Rheumatoid arthritis (RA)

Rheumatoid arthritis (RA) is a chronic autoimmune disease that targets the joints of the hands, wrists, and feet. Classical symptoms include swollen and painful joints, morning stiffness, fatigue and occasionally moderate fever. RA affects approximately 0.5-1% of the population of Western Europe and North America (Harvey, Lotze et al. 1981). The most pathological changes associated with RA occur within the joint, i.e. inflammation occurs in the lining of the joints, called synovial membrane. In healthy state, the synovium is a relatively acellular structure with a fine intimal lining. In RA the synovium becomes hyperplastic with infiltration of CD4 T helper cells, B cells and macrophages. Macrophage-like cells and fibroblast-like cells express degradative enzymes, including metalloproteinase's and serine proteases, digesting the extracellular matrix and resulting in bone erosion. Neutrophils expressing pro-inflammatory cytokines, such as IL-1, IL-6, and TNFα further promote the inflammation. B cells and DCs aggregate with T cells resulting in autoantibody production and formation of ICs (Ritchlin, Dwyer et al. 1994). The first autoantibody defined in RA was the rheumatoid factor (RF); it recognizes the Fc portion of IgG immunoglobulins. Anti-citruillinated antibodies are antibodies recognizing peptides that were citrullinated post-translationally by converting the amino acid arginine to citrulline in an inflammatory environment. Anti-RF and anti-citrullinated antibodies are the autoantibodies with the highest specificity and sensitivity in RA (Rantapaa-Dahlqvist 2005). Other autoantibodies recognize collagen II (CII) and proteoglycans (Sweeney and Firestein 2004). However their pathological role is not fully elucidated. It is believed that ICs formed by rheumatoid factors and other autoantibodies fix complement and release chemotactic factors. Inflam-

matory cells are subsequently recruited to the rheumatoid joint along a chemotactic gradient where they are activated and contribute to local destruction. Neutrophils, in particular, accumulate in synovial fluid where they engulf ICs and release proteolytic enzymes (Zvaifler 1973).

Although autoantibodies and IC formation are important for acute inflammation, infiltrated T cells, macrophages and neutrophils as well are key participants in the pro-inflammatory response in RA. Animal models, such as collagen-induced arthritis (CIA), clearly identified RA to be T cell dependent (Fox 1997). However, cytokine profiling in RA revealed relatively low concentration of typical T cell cytokines, whereas macrophage and fibroblast cytokines, including IL-1, IL-6, IL-15, TNFα, and GM-CSF and many others were produced by the rheumatoid synovium (Firestein, Alvaro-Gracia et al. 1990).

Collagen-induced arthritis (CIA) is perhaps the most well characterized mouse model for RA. In this model, mice immunized subcutaneously with Complete Freund's Adjuvant (CFA, a mixture of oil and *M. tuberculosis* products) and chicken collagen II (the major form of collagen present in joint cartilage) develop a monophasic, progressive polyarthritis that ultimately resolves (Courtenay, Dallman et al. 1980).

Several strategies have been attempted to treat RA patients, such as the depletion of T lymphocytes using antibodies directed against cell surface markers, oral tolerization protocols with collagen II, and cytokine directed therapies (Cush and Kavanaugh 1995; Olsen and Stein 2004). Unfortunately, with the exception of the TNFα inhibitors (De Vita, Zaja et al. 2002), these immunological approaches have been largely unsuccessful due to lack of efficacy or unacceptable adverse side effects. Furthermore, many of these biological therapies suppress the whole immune system and do not target cells or mechanisms responsible for RA directly.

1.4.3 Th1 and Th17 cells in autoimmunity

The ability of autoreactive T cells to induce autoimmunity by autoreactive B cell activation and tissue inflammation is mediated not only by their specificity for self-antigen but, more importantly, by their effector functions. Upon self-antigen recognition, autoreactive T helper cells become activated, expand and differentiate into diverse effector T cell subsets,

such as Th1, Th2 and Th17 helper cells. As mentioned before, their distinct differentiation is dependent on cytokine production and transcription factor regulation. How these different T cell subsets induce or regulate autoimmunity is widely discussed (Crispin, Kyttaris et al. ; Damsker, Hansen et al. ; Jager and Kuchroo ; Dardalhon, Korn et al. 2008) (Figure 1.2).

Traditionally, Th1 cells were described to be the pathogenic subset in T cell driven autoimmune diseases, whereas Th2 cells were reported to exert inhibitory effects (Liblau, Singer et al. 1995). Several observations led to this conclusion: (1) Adoptive transfer of IFNγ-producing T cell lines can induce EAE, a model for human multiple sclerosis (MS) (Pettinelli and McFarlin 1981). (2) In mice susceptible to EAE, IFNγ-levels in the central nervous system (CNS) correlate with disease severity (Merrill, Kono et al. 1992). (3) Infiltrating CD4 T cells were identified as the source of this IFNγ, and (4) treatment of mice with IL-12 exacerbates CIA, a mouse model for RA (Germann, Hess et al. 1996). Furthermore, Th1 cells can attract and activate macrophages by producing cytokines including IFNγ, MIP-1a, MIP-1b and TCA-3 (Kuchroo, Martin et al. 1993), and activated macrophages infiltrating the CNS can contribute to demyelination in EAE. The important role of Th1 cells was further confirmed by the fact that mice deficient for the main transcription factors T-bet or STAT4 were resistant to EAE (Bettelli, Sullivan et al. 2004). Moreover, administration of IFNγ exacerbates disease symptoms in MS patients (Panitch, Hirsch et al. 1987). However, mice lacking IFNγ are not resistant but more susceptible to multiple autoimmune diseases, including EAE and CIA (Ferber, Brocke et al. 1996; Matthys, Vermeire et al. 1999). Therewith, the concept of Th1 cells being exclusively responsible for driving autoimmune diseases was challenged.

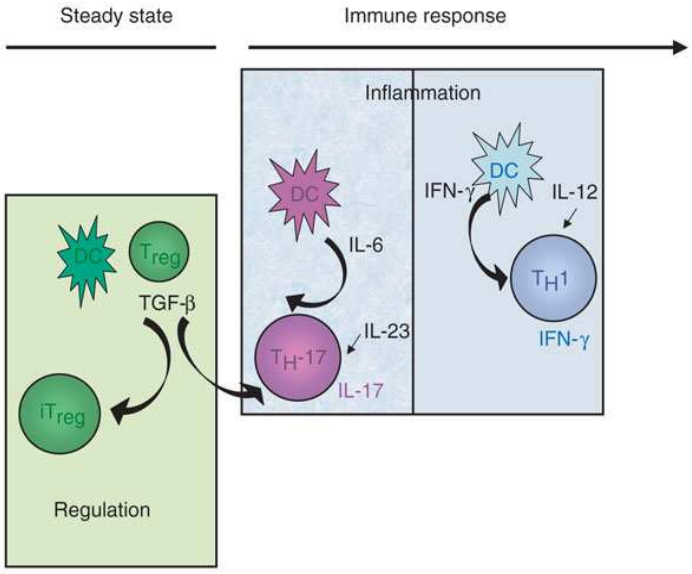
Mice deficient for the two subunits of IL-12, p35 and p40, shed light on the paradoxon of Th1 cells and autoimmunity. p35 knock out mice are susceptible to EAE and CIA, whereas p40 knock out mice are resistant to autoimmunity (Becher, Durell et al. 2002; Gran, Zhang et al. 2002; Cua, Sherlock et al. 2003; Murphy, Langrish et al. 2003). The subunit p40 can not only form IL-12 with p35, but can also bind to another subunit, called p19, to form IL-23 (Oppmann, Lesley et al. 2000). Until now, it has been shown that rather IL-23 than IL-12 is the critical cytokine to induce inflammation in EAE and that IL-23 is produced by a distinct T cell subset (Cua, Sherlock et al. 2003; Langrish, Chen et al. 2005). In 2006 Bettelli reported that IL-23 may has an important role during differentiation of a unique T cell

subset, which were named Th17 cells, due to IL-17 production (Bettelli, Carrier et al. 2006). However, IL-23 cannot induce de novo Th17 cell differentiation, but can act on memory T cells that already express IL-23 receptor and is required for stabilization and expansion of Th17 cells. To date the importance of Th17 cells in various autoimme mouse models, such as CIA, EAE, colitis, and lupus nephritis has been implicated (Kyttaris, Zhang et al. ; Bush, Farmer et al. 2002; Nakae, Nambu et al. 2003; Komiyama, Nakae et al. 2006; Hsu, Yang et al. 2008; Kelchtermans, Schurgers et al. 2009). Indeed, it has been shown that Th17 cells are more potent than Th1 cells in transferring EAE to naive mice (Langrish, Chen et al. 2005). In addition, in chronic inflammatory bowel disease, Th17 cells seem to be essential in inducing the break down of the intestinal barrier (Schwartz, Beaulieu et al. 2005). The cytokine IL-17 induce secretion of other proinflammatory cytokines, such as IL-6, GM-CSF or matrix-metalloproteinases, and neutrophil-attractant chemokines that promote inflammation and tissue destruction (Shen and Gaffen 2008). In fact, IL-17 is directly involved in cartilage and bone destruction shown in *in vitro* and in an experimental mouse model (Sato, Suematsu et al. 2006). In addition, IL-17 expression is upregulated in patients with multiple sclerosis, SLE, inflammatory bowel disease and RA (Kotake, Udagawa et al. 1999; Lock, Hermans et al. 2002; Fujino, Andoh et al. 2003; Crispin, Oukka et al. 2008). Besides IL-17 and IL-23, other cytokines produced by Th17 cells, such as IL-21 and IL-22 are involved in autoimmunity. IL-21 regulates Th17 cell differentiation, enhances B cell proliferation and regulates T follicular helper cell differentiation and GC formation reviewed in (Fouser, Wright et al. 2008). In autoimmunity IL-21 has the potential to enhance T cell proliferation, autoantibody production and affinity maturation of B cells in GCs (Kuchen, Robbins et al. 2007). Blockade of IL-21 in CIA and in a lupus mouse model clarified that IL-21 contributes to the pathogenesis of autoimme diseases (Fouser, Wright et al. 2008). Il-22 in contrast does not target immune cells but mediates crosstalk between infiltrated immune cells after inflammation and non-immune cells, such as fibroblasts or endothelial cells (Zheng, Valdez et al. 2008). Expression of IL-22 is upregulated in many autoimmune diseases and correlates with disease activity (Brand, Beigel et al. 2006; Schmechel, Konrad et al. 2008).

Although Th17 cells are potent inducers of autoimmunity, involvement of Th1 cells in autoimmune pathogenesis has been proven as well. However, the distinct role or function of each T helper cell subset has not been clarified so far. Furthermore, Th1 and Th17 seem to regulate each other. In CIA IFNγ deficient mice display increased Th17 response, whe-

reas Th1 response is elevated in IL-17 knock out mice (Damsker, Hansen et al. ; Luger, Silver et al. 2008). Several differences have been identified during Th1 and Th17 immune response: (1) order of infiltration into the inflammatory organ depending on the disease (Khader, Bell et al. 2007; O'Connor, Prendergast et al. 2008), (2) expression of different cytokines and chemokines and therefore recruitment of distinct inflammatory cells (Su, Grajewski et al. 2007; Kelchtermans, Billiau et al. 2008; Kroenke, Carlson et al. 2008), (3) histopathological and symptomal disease differences (Jager and Kuchroo), (4) site of infiltration inside the target organ (Kroenke, Carlson et al. 2008) and (5) Th1 cells might be more easily controlled by Tregs than Th17 cells (Li, Wan et al. 2007). Treg cells might act as a source of TGFβ and even induce Th17 cell differentiation (Bettelli, Carrier et al. 2006; Veldhoen, Hocking et al. 2006). Commonly both Th1 and Th17 cells colocalize within the region of inflammation and require each other for recruitment and/or entry to this region. Moreover, there is evidence that the line between Th17 and Th1 lineage is not as clear as it was initially thought. The existence of cells secreting both IL-17 and IFNγ has been detected *in vivo*. Their exact role has not been characterized yet but Th1/Th17 cells may have a physiological advantage over Th1 and Th17 cells because of their combined effector repertoire on the single-cell level, coexpressing IFN-γ and IL-17, but also chemokine receptors of both Th1 and Th17 cells (Boniface, Blumenschein et al. ; Kebir, Ifergan et al. 2009).

In the last years it has become clear that the impact of Th1 and Th17 cells in autoimmunity is more complex than initially anticipated. Although their differentiation is antagonistic they can cooperate with each other or might even depend on each other. Both T cell subsets can induce different pathological symptoms, as it is seen in patients suffering from autoimmune diseases. Thus, every subset probably employs different mechanisms for induction of tissue inflammation, because each subset produces different cytokines and chemokines recruiting different inflammatory cell types into the target organ. One patient might have a Th1-dominated or a Th17-dominated disease course, while in others both Th1 and Th17 cells might drive the disease together. To efficiently treat Th1 and Th17 related autoimmune diseases we have to understand the exact function of each T cell subset on its own and their relationship to each other.



(Bettelli, Oukka et al. 2007)

Figure 1.2 T cells in the circle of immunity and autoimmunity. Cytokines critically determine the development of the immune response. At steady state, TGFβ produced by many cell types, including dendritic cells (DCs) and naturally occurring T_{reg} cells, promotes immunosuppression and regulation. Additionally, TGFβ induces the differentiation of induced T_{reg} (iT_{reg}). However, during inflammatory conditions, IL-6 in combination with TGFβ induces Th17 cells, whereas IFNγ induces the differentiation of Th1 cells. These two subsets further act independently or together in inducing inflammation (Bettelli, Oukka et al. 2007).

1.5 Fcγ-receptors and their functions

The interaction of the Fc domain of antibodies with Fc receptors on immune cells results in a wide range of responses that can be activating or inhibiting on both innate and adaptive immune cells. Activating signals trigger cell activation and pro-inflammatory responses, followed by elimination of the pathogen. Absence of such signals inhibits cell activation and anti-inflammatory responses can occur. The exact regulation of these pro- or anti-inflammatory responses is necessary to allow complete pathogen elimination or prevent from autoimmune reactions. Fcγ receptors (FcγRs) are key components during this regulation to guarantee a well-balanced immune response. Indeed, in diverse autoimmune diseases, such as RA and SLE, aberrant expression or the presence of allelic variants of FcγRs

with altered functionality that contribute to the pathogenesis of the disease have been observed (Nimmerjahn 2006).

FcγRs expressed on innate immune cells, such as basophiles, mast cells, neutrophils, monocytes and macrophages, trigger release of chemoattractants and pro-inflammatory mediators, phagocytosis and cytotoxicity upon IC binding. Binding of ICs to FcγRs on APCs results in antigen presentation on MHC I and II molecules, therefore activation of T cells, which can provide T cell help for antigen-specific B cells. B cells only express the inhibitory low-affinity FcR for IgG: FcγRIIB (RIIB). On B cells RIIB contributes to the maintenance of tolerance by a negative feedback mechanisms during affinity maturation by inducing apoptosis and thus deletion of self-reactive B cells from the GC (Smith and Clatworthy 2010).

Mouse FcγRs can be distinguished by their affinity for the antibody Fc fragment and by the signaling pathway they induce. FcγRI is the only high affinity FcγR; all other FcγRs have low to medium affinity for the antibody Fc fragment. The activating FcγRs (FcγRI, FcγRIII and FcγRIV) consist of a ligand-binding α–chain and a signal-transducing γ-chain dimer, which carries immunoreceptor tyrosine based activating motifs (ITAMs) that are necessary to recruit proteins involved in triggering activating signaling proteins. RIIB is the only known inhibitory FcγR and consists of a single α-chain, which carries an immunoreceptor tyrosine based inhibitory motif (ITIM) that is necessary to recruit other negative regulatory signaling proteins (Nimmerjahn and Ravetch 2008). The four IgG subclasses in mice (IgG1, IgG2a, IgG2b and IgG3) bind with varying affinity and specificity to different FcγRs (reviewed in (Nimmerjahn and Ravetch 2005). In mice IgG2a and IgG2b are the most pro-inflammatory IgG subclasses and show a greater activity than mouse IgG1 and IgG3 in many in vivo mouse model systems (Nimmerjahn and Ravetch 2006).

When RIIB is cross-linked with the BCR by ICs the B cell activation threshold is increased, antibody production is decreased and apoptosis in plasma cells is induced. When RIIB is cross-linked with activating FcγRs on DCs antigen-presentation and maturation of DCs is inhibited. In fact, DCs derived from RIIB knock out mice generate a stronger and longer-lasting immune response in vitro and in vivo. This implies that the inhibitory RIIB prevents spontaneous DC maturation under non-inflammatory steady-state conditions (Boruchov, Heller et al. 2005; Dhodapkar, Kaufman et al. 2005). On macrophages and neutrophils

RIIB can influence the innate immune system by cross-linking with activating FcγRs that results in inhibiting phagocytosis and cytokine release.

As mentioned earlier failure of peripheral and central tolerance leads to autoimmune diseases. Many genes contribute to disease susceptibility; one of these genes is riib. In SLE proper B cell function and clearance are impaired, which leads to autoantibody production and chronic inflammation. As RIIB is a key regulator of B cells it might be involved in the pathogenesis of SLE. Indeed, RIIB deficient mice backcrossed to C57BL/6 backround spontaneously develop autoantibodies and IC-mediated glomerulonephritis (Bolland and Ravetch 2000). Moreover, modest transgenic overexpression of RIIB on B cells markedly reduces the development of SLE in MRL-lpr mice (Brownlie, Lawlor et al. 2008). This study and unpublished data (Alexander Stöhr) implicate RIIB in the maintenance of B cell tolerance and therefore in the pathogenesis of autoimmunity. In other nephritis mouse models RIIB may also have an important role: the major locus contributing to SLE susceptibility in the NZB x NZW F1 and BXSB mouse models contains the FcR region (Wakeland, Liu et al. 2001). Thus there is consistent association between RIIB and SLE, determined by genetic association or cell surface expression.

Patients with RA show an association between a polymorphism encoding the $RIIB^{T232}$ variant with increased radiological joint damage and DCs from RA patients with the $RIIB^{T232}$ variant display increased IC-mediated-inflammation *in vitro* (Radstake, Franke et al. 2006; Wenink, Santegoets et al. 2009). RIIB deficiency increases collagen II-specific IgG titers and disease severity in CIA (Yuasa, Kubo et al. 1999). Reduced disease severity and CII-specific IgG titers was observed in RIIB B cell transgenic mice, but not in RIIB macrophage transgenic mice (Brownlie, Lawlor et al. 2008). Furthermore, a direct effect of RIIB on cartilage destruction was reported by Blom et al (Blom, van Lent et al. 2003).

1.6 Fc glycosylation in autoimmunity

IgG antibodies consist of two identical heavy (H) and light (L) chains which are linked by disulfide bonds. The light chains consist of a variable (V_L) and a constant (C_L) region and the heavy chains assemble of variable (V_H) and three constant regions (C_H1, C_H2 and C_H3). The variable regions of the heavy and light chain make up the antigen-binding site of the

antibody (Fab fragment), whereas the C-domains determine the Ig isotype and the corresponding functional properties (Fc fragment).

There are two N-linked glycosylation sites on IgG antibodies. One can be attached to the variable region of the Fab fragment and is highly sialylated. The other N-glycan is present at the asparagine 297 (Asn^{297}) in the C_H2 domain of the Fc fragment and is highly conserved (Figure 1.3). It has a biantennary structure whose core consists of 2 α-mannosyl residues attached to a β-mannosyl-di-N-acetylglucosamine unit. To this core structure, N-acetylglucosamine, galactose, fucose and sialic acid can be added via enzymes to form a mature glycoform. Different glycoforms can be generated: ranging from no galactose and no sialic acid (G0S0) to two galactoses to two sialic acids (G2S2) (Huhn, Selman et al. 2009). These Fc glycans point to each other influencing the conformation of the Fc effector region and therefore regulating the interaction with IgG FcγRs. Deletion of the whole sugar moiety changes the structure of the antibody Fc fragment resulting in impaired binding to FcγRs (Arnold, Wormald et al. 2007; Nimmerjahn and Ravetch 2008). The lack of fucose increases the affinity of all IgG subclasses to murine FcγRIV 10-50 fold and enhances antibody-dependent cytotoxicity (Shields, Lai et al. 2002; Shinkawa, Nakamura et al. 2003; Ferrara, Stuart et al. 2006).

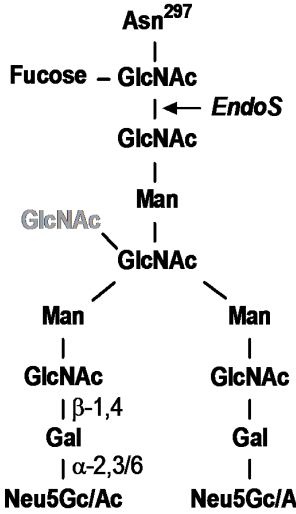


Figure 1.3 Fc-glycan structure. The IgG Fc glycan coupled to Asn 297 consists of a core structure of four N-acetyl-glucosamines (GlcNAc) and three mannoses (Man), which can be modified by Fucose, bisecting GlcNAc, galactose (Gal) and sialic acid (murine Neu5Gc or human Neu5Ac). Cleavage site of EndoS is indicated by an arrow.

The residues galactose and sialic acid have been implicated in autoimmune disorders. Patients with RA have an increased level of IgG antibodies without terminal galactose and sialic acid (Parekh, Dwek et al. 1985; Arnold, Wormald et al. 2007). Recently, this has been confirmed by another study reporting abberant IgG galactosylation ratios in RA patients. A correlation of the G0:G1 ratio of total serum IgG and IgG autoantibodies with disease activity was further shown (Ercan, Cui et al. 2010). In addition, the MRL/lpr/lpr autoimmune mouse model displays changes in the glycosylation pattern of IgGs (Bond, Cooke et al. 1990; Mizuochi, Hamako et al. 1990). In healthy individuals around 60% of IgGs are glycosylated on Asn^{297} which decreases with age. In contrast, patients with RA generate IgGs with short Fc glycan structures, more than 60% of all IgGs are of the G0 glycan type meaning no galactose bound to the core glycan. Moreover, in healthy individuals up to 15% of the serum IgGs possess a terminal sialic acid molecule whereas RA patients carry no terminal sialic acid (Scherer, van der Woude et al. ; Parekh, Dwek et al. 1985; Huhn, Selman et al. 2009). The knowledge achieved by the group of Ravetch has clarified that the immunosuppressive property of intravenously injected immunoglobulin (IVIG) in autoimmune diseases is dependent on sialylation of the Fc N-glycan. Sialylation of IgG antibodies is associated with the maintenance of an anti-inflammatory environment, reduced cytotoxicity and if challenged by immunization desialylation of antigen-specific antibodies occurs (Nimmerjahn and Ravetch 2005; Kaneko, Nimmerjahn et al. 2006; Nimmerjahn and Ravetch 2008).

1.6.1 Intravenous Immunoglobulin G (IVIG) therapy

Administration of pooled serum IgG from healthy donors (IVIG) is used as a therapeutic agent to treat viral infections (HIV), B cell malignancies or after bone marrow transplantation. Today the two major clinical indications for which IVIG is used are IgG replacement therapy and treatment of acute and chronic autoimmune diseases (Sullivan, Kopecky et al. 1990; Olopoenia, Young et al. 1997; Negi, Elluru et al. 2007; Siberil, Elluru et al. 2007). For IgG replacement therapy only 300-500mg/kg body weight IVIG is needed to increase IgG serum levels to 500mg/dl. To treat autoimmune disorders, however, a very high dose IVIG (1-3g/kg body weight) is required to induce an anti-inflammatory effect (Nimmerjahn and Ravetch 2008). The anti-inflammatory effect of IVIG could be demon-

strated in various autoimmune mouse models, such as autoantibody induced thrombocytopenia (Samuelsson, Towers et al. 2001), serum transfer arthritis (Bruhns, Samuelsson et al. 2003), and nephrotoic nephritis (Kaneko, Nimmerjahn et al. 2006). Recently, Ravetch and colleagues identified the Fc fragment of the injected IgGs as absolutely required to mediate an anti-inflammatory effect. More precisely, they identified a N-linked glycan terminated in sialic acid as the minor fraction responsible for the anti-inflammatory effect observed after IVIG administration in autoimmune patients. Only 1-3% of IVIG contain sialylated antibodies, which explains the high dose of IVIG required to promote an anti-inflammatory effect in autoimmune diseases (Kaneko, Nimmerjahn et al. 2006; Anthony, Nimmerjahn et al. 2008).

Side effects after treatment with IVIG can be headache, nausea, fever, cough, and sore throat. Moreover, a strict quality check is obligatory to avoid these side effects that may trigger activating FcγRs unspecifically. Replacement of IVIG with recombinant IgG antibodies could diminish side effects, prevent supply shortages and diminish costs. To achieve this goal the molecular mechanism of IVIG activity has to be clarified.

1.6.2 Mechanisms of IVIG

The explanation of the anti-inflammatory activity of IVIG is complicated and a matter of much debate (Figure 1.4). How a mixture of IgGs can suppress the activity of the very same class of molecules, i.e. other IgG antibodies recognizing autoantigens, remains to be elucidated. Many studies demonstrated that the Fc fragments are responsible for mediating the anti-inflammatory effect of IVIG. However, there are reports showing that the Fab fragment is also involved in IVIG activity.

Two main mechanisms showing the Fab fragment is involved in the anti-inflammatory activity have been described. First, the Fab fragment could bind and neutralize the pro-inflammatory activity of C3a and C5a in a mouse model of asthma. However, allergic diseases differ from autoimmune diseases and no beneficial connection between IVIG treatment and asthma has been reported (Basta, Van Goor et al. 2003; Orange, Hossny et al. 2006). Second, in the IVIG preparation antibodies specific to TCRα/β, Siglec-9, CD5, anti-integrin, Fas, cytokines or cytokine receptors were detected. Indeed, there are studies con-

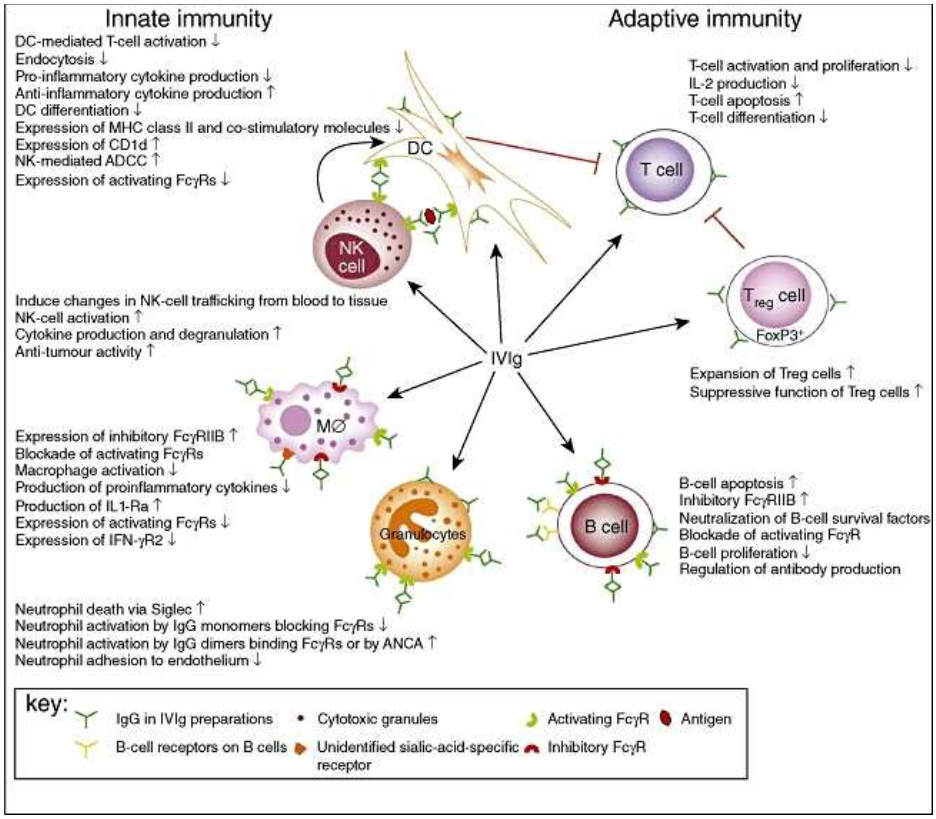
firming the activity of certain antigen-specific IgGs contained in IVIG by blocking interactions. However, more research has to be done on this subject to clarify the role of each antibody found in the IVIG preparation (reviewed in Nimmerjahn and Ravetch 2008).

In contrast, several studies clearly identified the Fc fragment of IgGs to be responsible for the anti-inflammatory activity of IVIG. In autoimmune mouse models, such as arthritis and nephritis, and in ITP patients the Fc fragment was clearly as protective as the whole IVIG fraction (Samuelsson, Towers et al. 2001; Bruhns, Samuelsson et al. 2003; Kaneko, Nimmerjahn et al. 2006). There are four Fc fragment-dependent mechanisms hypothesized so far: (1) IVIG-mediated saturation of the neonatal Fc receptor (FcRn), which regulates IgG half-life. The very high dose of IVIG may compete with autoantibodies for binding to the FcRn and induce autoantibody clearance. (2) ICs present in IVIG bind to activating FcγRs and thereby prevent binding of autoantibody ICs, which would trigger pro-inflammatory immune responses. (3) Upregulation of the inhibitory FcγRIIB on effector macrophages, which leads to an increased threshold for cell activation and thereby reduces pro-inflammatory immune responses. And (4) differential antibody glycosylation on the Fc fragment, whereas deglycosylated IgGs lost their therapeutic activity. In 2006 Kaneko et al. demonstrated that the anti-inflammatory response mediated by IVIG is clearly dependent on the small fraction of sialylated IgGs contained in IVIG explaining the requirement of such high doses IVIG to treat autoimmune diseases (Kaneko, Nimmerjahn et al. 2006). Later, a specific macrophage population in the spleen was identified to be required for mediating IVIG activity. Furthermore, they associated the C-type lectin receptor SIGN-R1 expressed on splenic marginal zone macrophages with binding of sialic acid. Upon binding of sialylated IgGs to SIGN-R1 an anti-inflammatory immune response is triggered caused by RIIB upregulation on effector macrophages. The homologous pathway in the human differs in that the receptor is DC-SIGN, which is expressed on DCs and is thus not restricted to the spleen (Anthony, Wermeling et al. 2008).

Others reported an affect of IVIG on T cells and DCs, showing that IVIG inhibits *in vitro* and *in vivo* antigen-dependent T cell responses by decreasing the ability of APCs to present antigens in an MHC II-restricted manner. Furthermore, it was demonstrated that IVIG was able to inhibit B cell-mediated antigen presentation. Interestingly, MacMillan et al. showed a direct effect of IVIG in primary human T cells by inhibiting proliferation. These studies suggest that such inhibitions would result in decreased presentation of self-antigens to au-

toreactive T cells or less proliferation of self-reactive T cells, which would influence autoantibody generation and therefore diminish pro-inflammatory effects in autoimmune diseases (Bayry, Lacroix-Desmazes et al. 2003; MacMillan, Lee et al. 2009; Aubin, Lemieux et al. 2010; Paquin Proulx, Aubin et al. 2010).

Investigating the mechanism of IVIG opens new possibilities for innovative therapies using sialylated antigen-specific antibodies with an anti-inflammatory potential to treat autoimmune diseases such as RA.



(Durandy, Kaveri et al. 2009)

Figure 1.4 Proposed mechanisms of action of intravenous immunoglobulin (IVIG) on cellular immunity. Besides the many proposed interactions of sialylated IgGs with different cell types of the adaptive and innate immune system depicted in this figure, we suggest novel unidentified modes of action of antigen-specific sialylated IgG antibodies, because our results have excluded the inhibitory Fc▪ -receptor IIB as regulatory downstream signal of sialylated IgGs. Up to now there exist several proposed modes of action, but only few publications have suggested direct or indirect interactions and regulation mechanisms of sialylated IgGs with B-cells, T-cells, peripheral macrophages and other components of the immune system (Durandy, Kaveri et al. 2009).

1.7 Aim of this thesis

Autoimmune diseases occur in up to 3-5% of the population and therefore represent a serious global issue. Considerable heterogeneity characterizes human autoimmune disorders, both within a disease and among the different disorders. The heterogeneity contributes to the difficulty in determining the underlying cause of disorders and in developing beneficial treatments. T cells, especially Th1 and Th17 cells have been implicated in the development of various autoimmune diseases. However, changing the balance between Th1 and Th17 cell differentiation by genetically modifying mice or administration of neutralizing antibodies revealed mixed results.

The aim of this study was to determine the distinct role of Th1 and Th17 cells in a spontaneous model of murine lupus characterized by the formation of immune complexes containing anti-nuclear antibodies and their deposition in the kidney resulting in glomerulonephritis. Thereby, the impact of Th1 cells on the development of autoantibodies, a prerequisite for lupus nephritis, and the impact of Th17 on the development of autoantibodies and on the recruitment and activation of pro-inflammatory immune cells should be investigated.

Furthermore, I aimed to verify the importance of Th17 during the development of arthritis on innate immune cell expansion and antibody production in a collagen-induced arthritis (CIA) mouse model.

Finally, the purpose of this study was to investigate the function of antigen-specific sialylated IgGs on the development of arthritis in the CIA mouse model and in particular their effect on pro-inflammatory Th17 cells. In detail, I tried to identify immune cells directly targeted by antigen-specific sialylated IgGs.

2 Materials and Methods

2.1 Mice

Table 2.1 lists all mice used in this dissertation. All mice used were either generated on the C57BL/6 backround or backrossed for at least eight generations. FcγRIIB-/- mice have been described previously (Bolland and Ravetch 2000; Ehlers, Fukuyama et al. 2006). TLR9-/- (Hemmi, Takeuchi et al. 2000) mice were crossed with FcγRIIB-/- mice to produce FcγRIIB-/-TLR9-/- double-deficient mice. IFNγR1-/- or IL-17RA-/- were crossed with FcγRIIB-/-TLR9-/- double-deficient mice. For induction of CIA exclusively 8 to 12 weeks old RIIB-/- mice were used.

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*.

Table 2.1: Mice

Abbreviation	General remarks & references
C57BL/6	purchased from Charles River Laboratories
TLR9-/-	(Hemmi, Takeuchi et al. 2000)
IFNγR1-/-	obtained from Max-Planck-Institut f. Infektionsbiologie
FcγRIIB-/-	(Takai et al.1996, Bolland and Ravetch 2000)
IL-17RA-/-	(Ye, Rodriguez et al. 2001)

2.2 Materials

2.2.1 Technical devices

Used technical devices are listed in table *2.2*.

2.2.2 Consumables

All used consumables are summed up in table *2.3*.

2.2.3 Chemicals

All chemicals used in this work are listed in table *2.4*.

Table 2.2: 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 2.3: 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 2.4: 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 *2.5*.

Table 2.5: 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
MOPS buffer	50 mM 3-morpholinopropanesulfonic acid (Roth); pH 7.2
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
IMDM (complete) (GIBCO/Invitrogen)	IMDM; 2mM L-Guanine; 10% FCS; 20 µM 2-mercaptoethanol (both Sigma-Aldrich); 25 mM HEPES; non essential amino acids; 100 U/l Penicillin; 100 U/l Streptomycin (both Seromed)
LB Media Capsules	purchased from MPBIO
TAE (50×)	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

2.2.5 Kits

The kits listed in table *2.6* were applied according to the manufacturer's instructions.

Table 2.6: Kits

Name	Supplier
anti-Nucleosome-ELISA	Orgentec
ANA-detect-ELISA	Orgentec
anti-RNP-70 ELISA	Orgentec
IL-6 ELISA kit	BD Bioscience
IL-10 ELISA kit	BD Bioscience
IL-22 ELISA kit	R&D
NucleoBond Xtra Maxi	Macherey-Nagel
QIAprep Spin Miniprep-Kit	Quiagen

2.2.6 Enzymes, antigens, adjuvants and other biological derivatives

All used enzymes and other biological derivatives are summed up in table *2.7*.

Table 2.7: Enzymes, antigens, adjuvants 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
Age I	NEB
CIP	NEB
Proteinase K	Sigma
FCS	Sigma

Primatone RL	MP Biomedicals
e.coli DNA	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
β-1,4-galactosyltransferase	Calbiochem
UDP-α-D-Galactose, disodium salt	Calbiochem
Human α2,6-(N)-sialyltransferase	Calbiochem
CMP-sialic acid, disodium salt	Calbiochem
Chicken collagen type II	SIGMA-Aldrich
Freund´s adjuvant, complete (Pettinelli and McFarlin)	SIGMA-Aldrich
Freund´s adjuvant, incomplete (IFA)	SIGMA-Aldrich
Mycobacterium tuberkulosis H37 RA	DIFCO laboratories
Beriglobin (IVIG)	Behringer Ingelheim

2.2.7 Cells

A human embryonic kidney cell line stably expressing the large T-antigen of SV40 (HEK 293T), which was supplied by the DRFZ house facility was used to produce monoclonal IgG antibodies.

2.2.8 Primer

All primers used for genotyping are listed in table *2.8*.

Table 2.8: 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
IL-17RA KS35.20	AGCTGCTGTTAGCACTTTGC
IL-17RA TGX53.18	CTTGTGTAGCGCCAAGTG
IL-17RA KS37.19	CGTACGCACACACTCTCGA
IFNγR1-S	CCCATTTAGATCCTACATACGAAACATACG
IFNγR1-AS	TTTCTGTCATCATGGAAAGGAGGGATACAG
IFNγR1-neo34	TCCCGCTTCAGTGACAACGTC

2.2.9 Antibodies

All used antibodies were either produced by the DRFZ in-house facility or purchased from commercial suppliers and are listed in table *2.9* and *2.10*.

Table 2.9: 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 CD3	145-2C11	Alexa 488	House Facility
mouse CD8	53-6.72	Cy5	House Facility
mouse CD138	N418	Pe	BD Bioscience
mouse IL17	TC11-18H10	Pe	BD Bioscience
mouse IL17	TC11-18H10	FITC	BD Bioscience
mouse IFN-γ	XMG1.2	APC	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 Gr-1	RB6-8C5	FITC	House Facility
mouse GL-7	GL-7	FITC	BD biosience
mouse Fas	Jo2	Pe	BD bioscience
mouse IgM	M41	Cy5	House Facility
mouse B220	RA3-6B2	PerCP	BD bioscience
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 CXCR3	220803	APC	R&D Systems
mouse IgG2c	polyclonal goat	FITC	Bethyl
mouse IgG2b	polyclonal goat	FITC	Bethyl
mouse IgG1	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 2.10: Unconjugated Antibodies

Specificity	Clone	Supplier
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
mouse CD4	GK1.5	House Facility
mouse CD154	MR-1	House Facility

2.2.10 Software

The software programs used in this work are listed in table *2.11*.

Table 2.11: 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 Anti-collagen type II antibody sequences

We used sequences of antibodies specific for collagen type II (CII) of different species that have been described in the literature. However, articles describing nucleotide sequences of the variable region (V-region) of anti-CII IgG´s usually focus on the complementary determining region (CDR) sequences, which are of main interest when studying the interaction of antibodies with antigens. To obtain the fulllength nucleotide sequences of the V(D)J-regions of IgH (heavy) and IgL (Liu, Li et al.) chain genes, which were necessary for cloning, we completed V(D)J-region sequences of anti-CII antibodies (Iribe, Kabashima et al. 1988; Ito, Ueda et al. 1997; Uysal, Bockermann et al. 2009) (Table 2.12) with their corresponding germ line gene sequences that were determined by IgBLAST comparison with GenBank database (http://www.ncbi.nlm.nih.gov/igblast/) and sequences of the IMGT database (http://imgt.cines.fr) (Uysal, Bockermann et al. 2009) (Table 2.13). Additionally we corrected mismatches between the anti-CII IgH and IgL chain sequences and their germ line genes that appeared upstream of the CDR1. Sequencing errors could have been the reason for these mismatches. In case of doubt we used the germ line sequence. If IgH or IgL chains were described in part as amino acid sequence in the literature (Uysal, Bockermann et al. 2009), the triplet codon of the corresponding germ line gene was corrected by single base exchange according to the germ line gene segment. To ensure segre-

gation of the antibody chains we added a leader sequence (GenBank accession number DQ407610) also containing an AgeI restriction site for cloning. At the end of the heavy chain VDJ DNA sequence a SalI restriction site was introduced to later combine the VDJ sequence with the constant murine IgH chain in frame. Accordingly at the end of the light chain VJ DNA sequence a BsiWI restriction site was introduced to later combine the VJ sequence with the constant murine Igκ sequence in frame. The final anti-CII antibody sequences of the IgH and IgL chain V-regions were ordered at MrGene (https://mrgene.com).

Table 2.12: GenBank accession numbers of the clones CII-specific antibodies

Name	IgH	IgL	Mouse strain
aCII 1-5	U69538	U69539	DBA/1J
M2139	Z2462	Z72463	DBA/1

Table 2.13: Corresponding germ line VDJ IgH gene segments and VJ IgL kappa gene segments used to fill up incomplete sequences. Mutations far upstream of the CDR1 region have been reverted according to their germ line V-genes. Germ line gene segments were determined by IgBLAST and IMGT database.

Name		Orig. isotype	V-gene	D-gene	J-gene		V-gene	J-gene
aCII 1-5	Ig heavy chain	IgG2a	J558.45	DFL16.1	JH2	Ig kappa chain	12-44	JK1
M2139		IgG2b	J558.45	DSP2.8	JH2		21-1	JK2

2.3.1.2 Expression Vector

To co-express the IgH and the IgL chain of each antibody in cell culture we used expression vectors for murine IgG1 heavy chains (mIgG1) and murine kappa light chains (Igκ) (Wardemann, Yurasov et al. 2003; Tiller, Meffre et al. 2008; Tiller, Busse et al. 2009). The vectors (Figure 2.1; GenBank accession number DQ407610) were a kind gift of Dr. Hedda Wardemann (Max-Planck-Institute for Infection Biology, Berlin). Upstream of the mIgG1 and the mIgκ sequences there is a multiple cloning sites (MCS) containing AgeI and SalI (IgG1) and AgeI and BsiWI (Igκ) restriction sites to insert the V(D)J region sequences.

The human cytomegalovirus (HCMV) promoter provides a strong transcriptional activity. Selection occurs via an ampicillin resistance cassette.

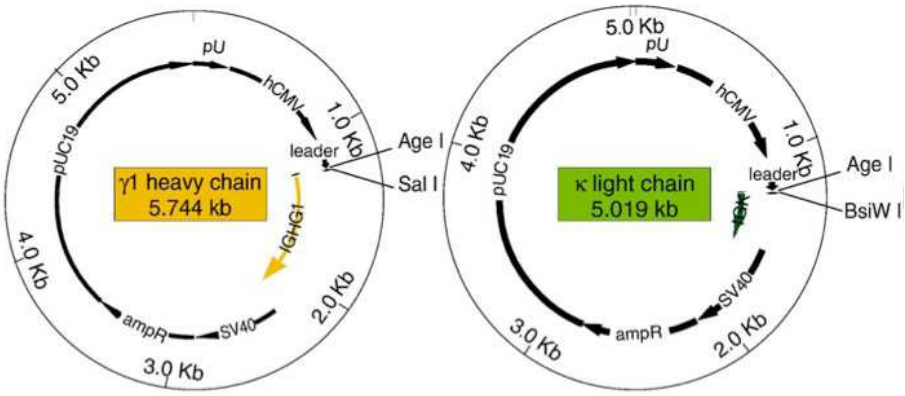


Figure 2.1 Expression vector maps. The human cytomegalovirus promoter is followed by a leader sequence, a MCS for inserting the V gene sequences and an IgG1 (γ1 heavy chain) or an Igκ (κ light chain) constant region. The eukaryotic expression vectors are promoted in bacterial clones based on ampicillin resistance.

2.3.1.3 Restriction Digest

The VDJ region sequences of the IgH chains were cut out of the vector backbone delivered by MrGene with AgeI and SalI restriction enzymes. The VJ regions of the IgL chains were cut with AgeI and BsiWI (all enzymes by NEB – New England Biolabs). IgG1 heavy chain and Igκ light chain expression vectors were digested analogous to create sticky DNA ends, where the V(D)J sequences could be inserted. The reaction conditions were set up according to the manufacturer's instructions (NEB). Just in brief, 3µg DNA were digested with 3U restriction enzyme in 30µl total reaction volume containing 3µl of the proper 10x enzyme buffer (AgeI and SalI (IgH) in NEB-4 buffer, AgeI and BsiWI (IgL) in NEB-1 buffer). IgH (AgeI, SalI) sequences were double digested at 37°C for 1h. Igκ sequences were digested sequentially. After AgeI digest for 1h at 37°C, 3U BsiWI were added and temperature was raised to 55°C for 1h.

2.3.1.4 Fragment Purification

Prior to ligation the digested V-region fragments were seperated from the vector backbones on a 0,9% agarose gel (1xTAE buffer (Tris-acetate-EDTA), 1µl ethidium bromide per 20ml agarose gel; Orange G loading dye) for 30 minutes at 140V. Fragments were cut out of the gel with a scalpel and purified with NucleoSpin® Extract II kit (Macherey-Nagel) according to manufacturer's instructions.

2.3.1.5 Ligation

Reaction volumes for ligation of the V-region fragments into expression vector backbones were calculated with GENtle software. The total reaction volume was 10µl containing 2µl 5x T4 DNA-Ligase reaction buffer (Invitrogen). 100ng DNA with a molar insert to vector ratio of 3:1 was ligated by 1U of T4 DNA-Ligase (Invitrogen) for 20 minutes at room temperature.

2.3.1.6 Transformation

Competent E. coli DH10B bacteria (Clontech) were thawed on ice and incubated with 100ng ligated DNA for 5 minutes on ice. The transfer of the expression vector into the bacterial cells – called transformation – was carried out for 30 seconds on 42°C. After 1 minute on ice 250µl pre-warmed SOC-medium were added and transformed bacteria recovered for 30 minutes shaking at 37°C. 100µl transformed bacteria were plated onto lysogeny broth (LB) ampicillin (100µg/ml) plates and incubated over night at 37°C. The antibiotic ampicillin was used to select for ampicillin resistant clones carrying our expression plasmid with an ampicillin resistance cassette, which expresses the enzyme beta-lactamase to cleave cell wall synthesis inhibiting ampicillin in the medium. Only bacteria expressing beta-lactamase could proliferate to from colonies in the presence of ampicillin.

2.3.1.7 Plasmid Preparation

Colony forming units (CFU) of single bacterial clones carrying the transformed plasmid were picked and grown in 4ml liquid LB-medium over night shaking at 37°C. Preparation of plasmids (NucleoSpin® Plasmid, NucleoBond® Xtra Maxi Plus, all by Macherey-Nagel) was done due to instruction guidelines. Just in brief, after centrifugation the cell pellets were resuspended and cells were lysed in an SDS/alkaline buffer to set the plasmids free. Genomic DNA, which is attached to the bacterial cell membrane, is washed away after neutralization. Clarified lysates are transferred to a silica membrane where plasmid DNA keeps bound throughout several washing steps until elution with 50µl elution buffer (5mM Tris/HCl, pH8.5).

2.3.1.8 Sequencing

To screen V-region inserts of the IgG1 and Igκ chains for correct insertion into the vector and for mutations we delivered the final constructs containing the aCIIantibody IgH and IgL chains to MWG (http://www.eurofinsdna.com/de/) for sequencing. Primers were binding upstream of the insert in the leader sequence (Ab-sense) of the aCII-antibody chains and in the respective murine IgG and murine Igκ C-region (Tiller, Meffre et al. 2008) (Table 2.14).

Table 2.14: Primer sequences for the insert screen. 5´ Absense binds in the leader. 3´primers bind in the C-region of IgH (IgG internal) and IgL (Cκ 494). Sequencing products reveal mutations and correct insertion of the V(D)J-region fragment.

Primer name	5´→ 3´sequence
5´ absense	GCTTCGTTAGAACGCGGCTAC
3´IgG (internal)	GTTCGGGGAAGTAGTCCTTGAC
3´Cκ 494	GTGCTGTCCTTGCTGTCCTGCT

A former diploma student Felix K.M. Lorenz designed and cloned these antibodies.

2.3.2 Cell culture

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

HEK 293T Cells were cultured in DMEM 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%. After removal of the medium and washing with 15ml 1xPBS cells were trypsinized with 1ml of trypsin-EDTA. Next, cells were centrifuged (1200rpm; 5 min), resuspended in new DMEM medium and distributed to new culture dishes at a ratio of 1:3-1:10.

2.3.2.2 Production of collagenII-specific IgG antibodies in HEK 293T cells

Recombinant collagenII-specific IgG antibodies were produced by polyethylenimine-mediated co-transfection of HEK 293T cells with the respective IgH and IgL chain encoding plasmid DNA. Best transfection efficiency could be achieved at a cell confluency of about 70% at the time of transfection. Cells should be in exponential growth phase (log phase) with least contact growth inhibition.

Cell culture dishes were washed with 1xPBS before adding transfection medium containing 22ml DMEM per plate with 2,5% primatone in PBS (Kerry Primatone RL/UF 5X59057) cell culture additives and 10ml/L penicillin/streptomycin but no FCS to avoid IgG contamination. 10µg heavy chain and 10µg light chain plasmid DNA (per plate) are vortexed for 30s with 0,6µg/µl of branched polyethylenimine (PEI, Sigma 408727) in a final volume of 3,000µl PBS. 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% CO2.

2.3.2.3 Cultivation of primary mouse cells

Splenocytes or bone marrow (BM) cells were cultured in RPMI or IMDM supplemented with 10% FCS and 1% penicillin/streptomycin (PS) at 37°C and 5% CO_2, respectively. Cells were cultured for 48h up to 10 days and then analyzed by flow cytometry or the supernatant was analyzed by ELISA.

2.3.2.3.1 Cultivation and stimulation of bone marrow derived dendritic cells (BMDCs) *ex vivo*

After isolating BM cells from femur and tibia and preparing a cell suspension (see 2.3.7.2) concentration of BM cells was adjusted to $1x10^6$/ml and cell suspension was supplemented with additional 10ng/ml recombinant IL-4 (R&D Systems) and GM-CSF (supernatant, MPI, Prof. Dr. Fritz Melchers), diluted 1:33. The dilution was elucidated in a preliminary experiment. $1x10^6$ cells were plated per well and ml in a six well plate (Greiner). BMDCs were generated over 9 days in complete IMDM (Gibco/Invitrogen) (see table 2.5) containing IL-4 and GM-CSF. Every other day, the medium was changed by removing half of the volume and replacing it with IMDM containing 10ng/ml IL-4 and GM-CSF, diluted 1:66.

On day 9, BMDCs were stimulated with the indicated immune complexes (ICs) for 16-24 hours, in each well with 1ml IMDM, containing non-essential amino acids, (Gibco/Invitrogen), 10% FCS* (IgG-free FCS, house preparation), 1% PS (Seromed) and 50µM 2-mercaptoethanol (Sigma-Aldrich). IC-formation was performed incubating antigen and antibody in a ratio of 1:4 at 37°C for 1 hour. In detail, CII-ICs were prepared incubating 10µg CII with 40 µg of the indicated anti-CII IgG1 antibody (M2139). IVIG was added to the BMDCs in a concentration of 10 mg/ml. IL-6 concentrations in the culture supernatants were detected by IL-6 ELISA as described by the manufacturer's instructions.

2.3.3 Purification of IgG antibodies

To analyze monoclonal antibodies produced in HEK 293T cells the IgG antibodies were purified via affinity chromatography using Protein G sepharose. Protein G is a protein that

is located in the bacterial cell wall and shows a high binding affinity to the Fc part of IgG molecules. This protein is coupled to sepharose and therefore enables purification via affinity chromatography. The binding capacity of Protein G accounts 20 mg IgG pro ml gel.

Harvested supernatant of transfected HEK 293T cells 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 a Protein G Sepharose® (GE Healthcare) column. Binding to protein-G in the column retained the antibodies. Elution was performed by adding 5x the column bed volume of 0,1 glycin buffer (pH3). The fractions were collected and neutralized with 1/20 of the elution volume of 1M Tris/HCl buffer (pH 8,5). Antibody concentration, which was usually high in fractions 2 and 3, was determined by photometric analysis at a wavelength of 280nm (NanoDrop). Before storage at -80°C the antibody solution was dialysed over night against 1xPBS.

2.3.4 Glycosylation modification of IgG antibodies

Purified monoclonal anti-collagenII antibodies have Fc-glycans lacking terminal sialic acid. About two-thirds of the bisecting Fc-glycans are also lacking galactose. In order to check the impact of terminal sialic acid of the antibodies on the immune response, we performed an enzymatic glycosylation of the IgG molecules *in vitro*. Enzymatic glycosylation was performed in a two-step procedure by first galactosylating and second sialylating the antibodies, and has been described previously (Anthony et al., 2008).

Galactosylation

Antibodies were first dialysed against 50 mM MOPS (Sigma-Aldrich) pH 7.2 overnight at 4°C and then concentrated to 5 mg/ml. To 5 mg/ml antibodies 10 μM UDP-Galactose (Calbiochem), 75 mU β1,4-galactosyltransferase (Calbiochem) and 20 mM $MnCl_2$ were added and incubated for 48 hours at 37°C while constantly shaking at 350 rpm. A buffer exchange to 50 mM MES (Sigma-Aldrich) pH 6.0 overnight at 4°C was followed by the sialylation step.

Sialylation

25 mU human α2,6-(N)-sialyltransferase (Calbiochem) and 0.5 mM CMP-sialic acid (CMP-Neu5Ac, Calbiochem) was added to the galctosylated antibodies and incubated for 48 h at 37°C while constantly shaking at 350 rpm. Finally a two-step buffer exchange to 100 mM NaCl (Sigma-Aldrich) and afterwards to PBS was done using a centrifugation device with a MWCO of 50 kDa. Concentrations of differentially glycosylated IgG1 antibodies were determined by Nanodrop spectrophotometer.

Efficiency of enzymatic glycosylation and antibody Fc-N-glycosylation pattern were analysed by MALDI-TOF analysis. Reactivity to CII of antibodies was verified via ELISA.

2.3.5 Biochemical methods

2.3.5.1 Enzyme-linked immunosorbent Assay (ELISA)

ELISA is a biochemical technique used to detect the presence and specificity 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 temperature or overnight at 4°C. The reactivity pattern of the anti-CII antibodies was determined via reactivity ELISA. Collagen type II (2mg/ml solubilized in 0,05M acetic acid over night at 4°C) of chicken and mouse origin was coated at 2µg/ml in 100µl per well on a high binding microtiter 96-well plate over night 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, IgG1, 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 turn-

over 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-22 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-dsDNA 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.

2.3.5.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The expression of both, antibody heavy and light chain in the HEK293T cells was verified via denaturating polyacrylamid gel electrophoresis. 10µg of each purified antibody sample mixed with protein loading buffer was heated for 10min at 94°C. Loading buffer contained sodium dodecyl sulfate (SDS), which is an amphiphilic detergent where the non-polar carbohydrate chain (C-12) wraps around the heat denatured protein backbone. The anionic sulfate head of the molecule provides charge to the SDS-protein complex. The larger a protein the more SDS molecules can attach, which provides every protein chain with the same mass to charge ratio of 1,4µg SDS per 1µg protein. Protein migration in an electric field occurs due to size in a polyacrylamid gel. The intrinsic charge of the protein becomes negligible. Loading buffer (0,25M Tris-HCl) additionally contains glycerin to give the samples more density for optimal loading conditions. β-mercaptoethanol reduces disulfide bridges for complete denaturation of the protein. Bromphenol blue is used as a tracking dye.

A protein ladder (Kaleidoscope; BioRad) with recombinant prokaryotic proteins of known size was used as molecular weight marker. 10% AA (acrylamid/bisacrylamid) resolving gel was made with 5,9ml H_2O, 5,0ml acryl-bisacrylamide mix (30% AA), 3,8ml 1,5M Tris (pH 8,8) and 150µl SDS. 150µlammonium persulfate (10% APS) initiates gel formation when finally 6µl of TEMED are added to start polymerization. 5% AA stacking gel was formed

by mixing 5,5ml H_2O, 1,3ml AA (30%), 1ml 1,5M Tris (pH 6,8) and 80µl SDS (10%). Polymerization was started with 80µl APS (10%) and 8µl TEMED.

The gel was run at 140V for 3h.

2.3.5.3 Matrix-assisted laser desorption/ionization (MALDI-TOF) Analysis

MALDI-TOF mass spectrometry was used to resolve Fc-oligosaccharide structures. IgG antibody samples were digested with endoglycosidase S (EndoS) purified from Streptococcus pyogenes. EndoS specifically cleaves the N-linked oligosaccharide at the Fc-fragment exclusively from IgG antibodies between the first and second Nacetylglucosamine (GlcNAc). Cleaved oligosaccharides were purified by solid phase extraction using reversed-phase C18 and graphitized carbon columns. Oligosaccharide samples were permethylated according to standard protocols and further investigated by MALDI-TOF mass spectrometry. Spectra were recorded on an Ultraflex III mass spectrometer (Bruker Daltonics) equipped with a Smartbeam laser. Calibration was performed on a glucose ladder and DHB was used as a matrix. Spectra were recorded in reflector positive ionization mode and mass spectra from 3000 laser shots were accumulated.

2.3.6 Immunological methods

2.3.6.1 Lymphocyte stimulation

Lymphocyte stimulation amplifies the secretion and enrichment of cytokines in the cell. In detail, prepared lymphocytes (see *2.3.7.2*) 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.

2.3.6.2 Flow cytometric analysis

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 staining 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 2.9) 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 incubation, 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 intracellular proteins

For intracellular staining of proteins, cells were fixed with 50-100 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 2.9) in a total volume of 50 µl Perm/wash buffer for 30 minutes at 4°C.

2.3.6.3 Fluorescence immunhistological stainings

Immunhistological staining with the help of fluorophore-coupled antibodies enables 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/IgG2b (IgG2c and IgG2b 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).

2.3.6.4 Immunohistochemistry

The feet of RIIB-/- after induction of CIA were put in . Paraffin-sections (2 µm) of formalin-fixed and decalcified tissue were either subjected to H&E staining or a heat-induced epitope retrieval step prior to incubation with anti-CD3 antibody (#N1580, Dako, Hamburg, Germany, dilution 1:10) Followed by biotinylated donkey anti-rabbit secondary antibody (Dianova, Hamburg, Germany) and the streptavidinAP kit (Dako). Nuclei were counterstained with hematoxylin. Negative controls were performed omitting the primary antibody. Images were aquired using an AxioImager Z1 microscope equipped with a CCD camera (AxioCam MRm) and processed with Axioviosion software (Carl Zeiss MicroImaging, Jena, Germany).

2.3.7 Mouse experimental methods

2.3.7.1 Blood withdrawal

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 5min. Serum was transferred to a 1,5ml tube and stored at -20°C.

For flow cytometric analysis of peripheral lymphocytes from blood samples, blood was collected in tubes containing 5µl of heparin and further prepared as described in organ preparation (2.3.7.2).

2.3.7.2 Organ preparation

For the removal of organs, mice were sacrificed by cervical dislocation. Organs and cells were processed as described in the preparation protocols below.

Preparation of lymphocytes from spleen and lymph nodes

For the generation of lymphocytes, draining lymph nodes or spleens were homogenized in PBS/BSA. Afterwards, cells were treated with 600 µl lysis buffer in order to remove all erythrocytes from the suspension. Subsequent to 5 minutes of incubation, lysis was stopped by adding 600 µl PBS/BSA, cells were filtrated by using a 30 µm pre-separation filter (Miltenyi Biotec) and centrifuged. Then, cells were washed with 1 ml PBS/BSA and finally resuspended in the appropriate volume of PBS/BSA or medium.

Preparation of bone marrow

For the generation of BMDCs, bone marrow cells were first washed out from the femur and tibia of B6 or RIIB-/- mice. Then, BM cells were treated with 600 µl Lysis buffer in order to remove all erythrocytes from the suspension. Subsequent to 5 minutes of incubation, lysis was stopped by adding 600 µl PBS/BSA, cells were filtrated by using a 30 µm preseparation filter (Miltenyi Biotec). After washing the cells with 1 ml PBS/BSA, cells were finally resuspended in 1 ml IMDM (Invitrogen) supplemented with nonessential amino acids (Gibco/Invitrogen), 10% FCS* (Gibco/Invitrogen), 1% PS (Gibco/Invitrogen),

and 50 μM 2-mercaptoethanol. Subsequent, cell numbers were determined using the Neubauer counting-chamber.

2.3.7.3 Depletion of CD4 T cells

In order to deplete existing CD4 T cells and inhibit new CD4 T cell expansion, RIIB-/-TLR9-/- mice were treated with 500μg anti-mouse CD4 antibody (clone GK1.5) once a week. Anti-CD4 antibody was first injected i.p. by the age of three months and treatment was continued for six months. Efficiency of CD4 T cell depletion was verified by flow cytometry using FITC-coupled anti-CD4 antibody (clone YTS191).

2.3.7.4 Inhibition of CD40/CD40L interaction

In order to inhibit interaction between CD40 and its ligand CD154 (CD40L), RIIB-/-TLR9-/- mice were treated with 500μg anti-mouse CD154 antibody (clone MR-1) once a week. Anti-CD154 antibody was first injected i.p. by the age of three months and treatment was continued for six months. Efficiency of the CD154 antibody was verified by staining for germinal center formation (Fas^+GL7^+) using flow cytometry.

2.3.7.5 Determination of proteinurea

Proteinurea 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 negative, 1 <75 mg/dl, 2 <125 mg/dl, 3 ≥125 mg/dl.

2.3.8 Collagen induced arthritis mouse model (CIA)

The induction of autoimmune diseases like collagen-induced arthritis is commonly elicited in genetically susceptible mouse strains like DBA/1 mice. Susceptibility is restricted by the class II molecules of the MHC haplotype H-2_q and H-2_r (Campbell, Hamilton et al. 2000). Nevertheless C57BL/6 mice, which were usually regarded as being resistant to collagen-induced arthritis, became a target of studying autoimmune diseases, because most genetically modified mouse strains are on a C57BL/6 background (H-2_b) and recent publications have provided protocols for reproducibly induce arthritis in H-2_b haplotypes (Inglis, Simelyte et al. 2008).

It has been shown that RIIB-/- knock out mice on C57BL/6 background are more susceptible to autoimmune disease (Yuasa, Kubo et al. 1999). Exclusively 8-12 weeks old RIIB$^{-/-}$ mice were used for induction of CIA.

Reagent setup

Chicken collagen type II (chCII) was dissolved at a concentration of 2mg/ml in 0,05M acetic acid.

1vial (100mg) grinded Mycobacterium tuberculosis (H37Ra, Difco Laboratories) was added to 25ml incomplete Freund´s adjuvants (IFA, Sigma) to increase H37Ra content up to 5mg/ml (enriched CFA). Effective immunization with sufficient amount of *M. tuberculosis* as immunogenic stimulus is necessary to overcome tolerance in H-2_b haplotypes (Inglis, Simelyte et al. 2008).

Immunization

Chicken CII emulsified in an equal volume of *M.tuberculosis*-enriched CFA. 8-10 week-old RIIB-/- mice on a C57BL/6 background were immunized subcutaneously (s.c.) with 100µl emulsion (100 µg of CII). On day 21 a booster s.c. injection with 100 µg of chicken CII in IFA was given. Mice were monitored for onset and disease severity in a blinded manner. Swelling of each foot was scored from 0 (healthy) to 3 (severe swelling of the entire foot), so that each mouse could achieve a maximum score of 12. The mean clinical score was calculated by summing up the total score of all mice in a group divided by the number of mice per group. The incidence of arthritis indicates the percentage of arthritic mice in a group.

2.4 Statistical analysis

Statistical analyses were done using GraphPad Prism 4. P-values were calculated using unpaired two-tailed Student´s t test or logrank test for survival curves: ns $P>0.05$, *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$

3 Characterization of RIIB-/-TLR9-/- mice

The characterization of RIIB-/- and RIIB-/-TLR9-/- was done by Carolin Schön, Alexander Stöhr, and Maria Mertes from the group Tolerance and Autoimmunity (DRFZ).

In 2000 Bolland and Ravetch reported high levels of IgG autoantibodies and spontaneous development of glomerulonephritis in mice deficient for the only inhibitory FcγReceptor IIB (RIIB) (Bolland and Ravetch 2000). High levels of IgG autoantibodies lead to immune complex (IC) formation, which accumulate in the kidneys. Macrophages and other innate immune cells are recruited to the kidneys causing damage of renal glomeruli, which subsequently leads to death due to nephritis. In our mouse facility about 20% of lupus prone RIIB-/- mice display proteinurea and die from nephritis by the age of nine months (Figure 3.1A+B). Lupus nephritis and mortality were significantly increased to 80% in RIIB-/- mice additionally lacking Toll-like receptor 9 (RIIB-/-TLR9-/-). RIIB-/- as well as RIIB-/-TLR9-/- mice start to develop nephritis by the age of 5-6 months. As observed for other lupus mouse models loss of TLR9 was associated with low levels of anti-nucleosome but increased levels of anti-RNA-related (RNP-70), anti-nuclear (ANA) and anti-IgG rheumatoid factor IgG2c (IgG2a, haplotype b) and IgG2b serum autoantibodies in 5-6 month-old RIIB-/-TLR9-/- mice compared to age-matched RIIB-/- control mice (Figure 3.1C+D).

As RIIB-/-TLR9-/- mice exhibited highly exacerbated development of lupus disease compared to RIIB-/- mice, only RIIB-/-TLR9-/- mice were used to analyze the role of inflammatory Th1 and Th17 cells.

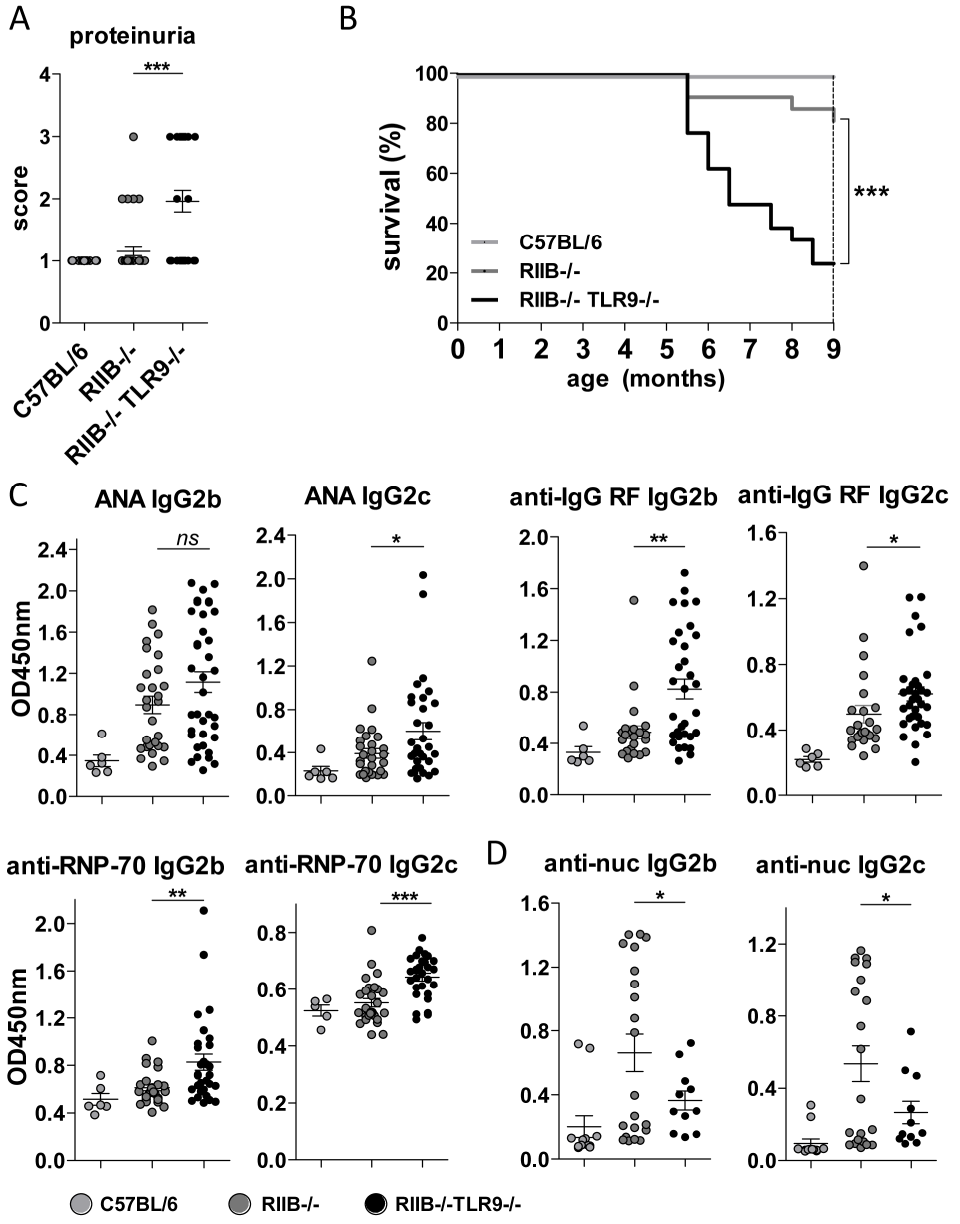


Figure 3.1 RIIB-/-TLR9-/- mice exhibit exacerbated development of lupus nephritis and enhanced levels of IgG autoantibodies. (A) Proteinurea scores in 5-6 month-old C57BL/6 (n=15), FcγRIIB-/- (RIIB-/-; n=39) and FcγRIIB-/-TLR9 (RIIB-/-TLR9-/-, n=29) mice. Symbols represent data from individual mice. Horizontal lines show mean values with standard error of the mean (SEM). (B) Kaplan-Meier survival curves for wild-type C57BL/6 (n=30), RIIB-/- (n=21) and RIIB-/-TLR9 (n=21) mice. (C) IgG2b and IgG2c anti-hu IgG RF, ANA and anti-RNP-70 serum autoantibody levels of 5-6 month-old wild-type C57BL/6 (n=6), RIIB-/- (n=24) or RIIB-/-TLR9-/- (n=33) mice were analyzed by ELISA. Symbols represent data from individual mice. Horizontal lines show mean values with SEM. (D) IgG2b and IgG2c anti-nucleosome (nuc) serum autoantibody levels of 5-6 month-old wild-type C57BL/6 (n=12), RIIB-/- (n=21) or RIIB-/-TLR9-/- (n=11) mice were analyzed by ELISA. Symbols represent data from individual mice. Horizontal lines show mean values with SEM. Data are representative for two independent ELISA.

4 Results

4.1 Function of CD4 T helper cells in a murine model of systemic lupus erythematosus (SLE)

4.1.1 Absence of CD4 T helper cells inhibits autoantibody production and disease manifestation in RIIB-/-TLR9-/- mice

Production of pathogenic IgG autoantibodies against nuclear antigens, e.g. DNA and ribonuclear proteins is a main feature in systemic lupus erythematosus (SLE). Activation of naive B cells occurs via help of CD4 T cells. Upon interaction, B cells proliferate and differentiate into plasma cells, which secrete immunoglobulins. To investigate whether CD4 T helper cells contribute to the production of pathogenic autoantibodies and initiation of SLE in the lupus prone mouse model RIIB-/-TLR9-/-, we injected an anti-CD4 antibody in RIIB-/-TLR9-/- mice (n=6) to deplete existing CD4 T helper cells and prevent the differentiation of new CD4 T helper cells. 500μg anti-CD4 antibody was administered intraperitoneally (i.p.) once a week starting at the age of three months for a period of six months. After one month no CD4 T cells could be detected in the blood of anti-CD4-treated mice (Figure 4.1A). Autoantibodies specific for double-stranded DNA, nuclear antigens (ANA), and to RNA related antigens (RNP-70) were analyzed using enzyme linked immunosorbent assay (ELISA). Anti-DNA and anti-nuclear (ANA) antibody production was abrogated completely in anti-CD4-treated RIIB-/-TLR9-/- mice probably due to impaired B cell class switching. The level of anti-RNP-70 autoantibodies was not significantly reduced compared to untreated RIIB-/-TLR9-/- (Figure 4.1B).

Anti-CD4 treatment was continued until the age of nine months. At the age of six to nine months the incident of proteinurea was analyzed. Manifestation of proteinurea is a sign for late damage of the renal glomeruli and subsequently leads to death. In the mouse facility of the DRFZ about 80% of the lupus prone RIIB-/-TLR9-/- mice develop nephritis and die from disease symptoms by the age of nine months. The anti-CD4-treated RIIB-/-TLR9-/- mice neither developed proteinurea nor died from lupus nephritis until the age of nine months (Figure 4.1C+D).

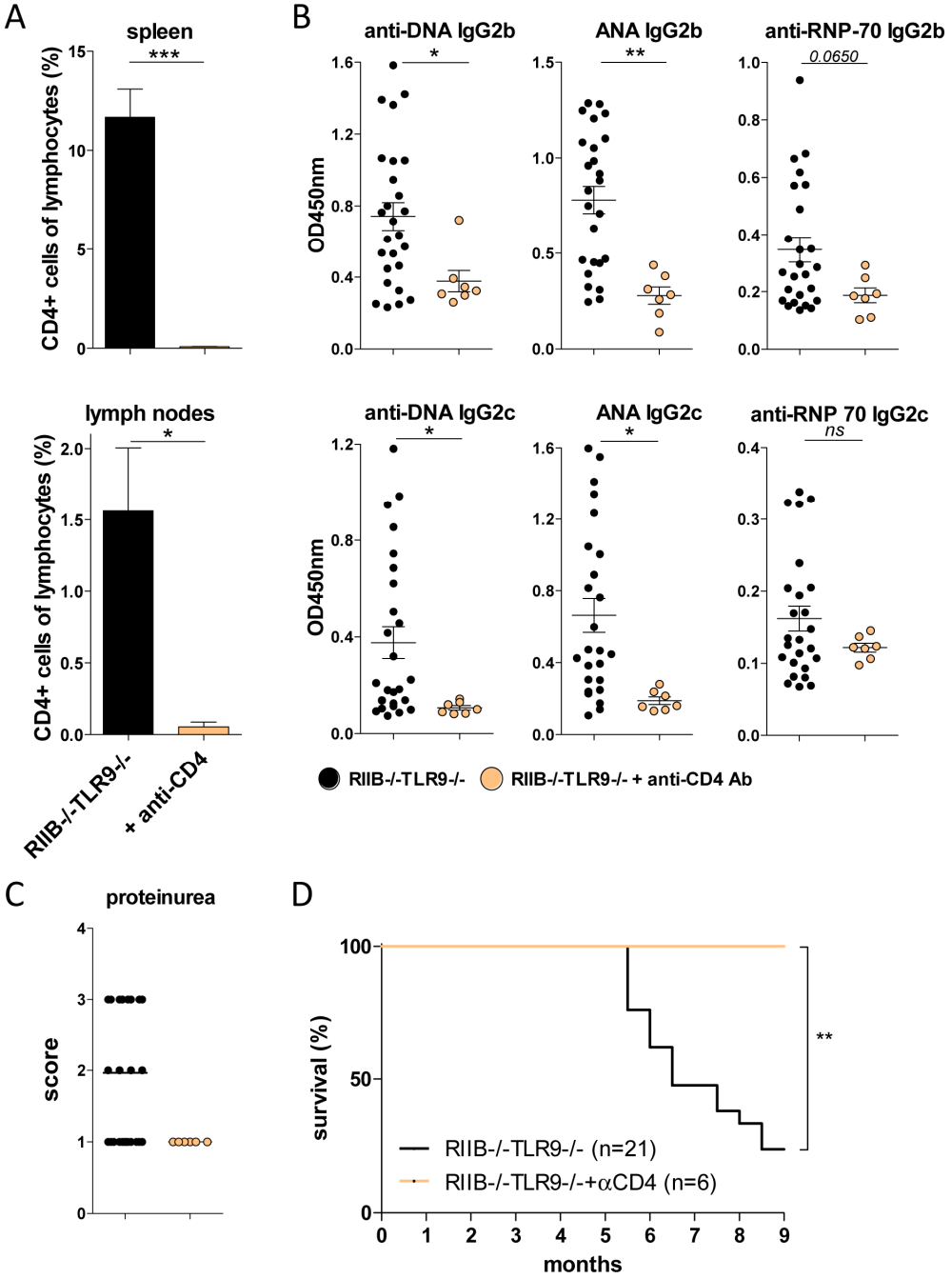


Figure 4.1 Autoantibody production and disease progression in lupus prone RIIB-/-TLR9-/- mice is CD4 T cell dependent. (A) Frequencies of $CD4^+$T cells in spleen and lymph nodes of non-treated (n=20) and anti-CD4-treated (n=6) RIIB-/-TLR9-/- mice were determined by flow cytometry. Bar graphs show the mean value with standard error of the mean (SEM) for each group of mice. (B) IgG2b and IgG2c anti-DNA, ANA and anti-RNP-70 serum autoantibody levels of 6 month-old non-treated (n=20) and anti-CD4-treated (n=6) RIIB-/-TLR9-/- mice were analyzed by ELISA. Symbols represent data from individual mice. Horizontal lines show mean values with SEM. (C) Proteinurea scores and (D) Kaplan-Meier survival curves of 6 month-old non-treated (n=29) and anti-CD4-treated (n=6) RIIB-/-TLR9-/- mice. ns $P>0.05$, * $P\leq0.05$, ** $P<0.01$, ***$P<0.001$

Thus, the level of autoreactive antibodies observed in the lupus prone RIIB-/-TLR9-/- mice is clearly CD4 T helper cell dependent. Importantly, the abrogation of autoantibody production is sufficient to inhibit the development of lupus nephritis.

4.1.2 Inhibition of T and B cell interaction abrogates autoantibody production and disease manifestation in RIIB-/-TLR9-/- mice

During T cell dependent B cell activation co-receptors of B cells play an essential role. The CD40L-CD40 interaction stimulates B cells and leads to an upregulation of receptors for various cytokines and chemokines which are essential for differentiation, proliferation, and migration of B cells (Burstein and Abbas 1991). The CD40-transduced signal also prevents apoptosis of germinal center (GC) B cells, which are characterized by Fas and GL7 expression (Lederman, Yellin et al. 1994).

To verify the importance of interaction between pathogenic T cells and B cells in our lupus mouse model, we tested the effect of an antibody reactive to the CD40-ligand (CD154 or CD40L) expressed on activated T cells. 500µg of anti-CD154 antibody was administered i.p. in RIIB-/-TLR9-/- once a week starting at the age of three months. At the age of six months serum autoantibody levels reactive to DNA, ANA and RNP-70 were analyzed using ELISA. Antibody-treated RIIB-/-TLR9-/- mice displayed significantly decreased autoantibody titers compared to untreated RIIB-/-TLR9-/- mice (Figure 4.2A).

The treatment of RIIB-/-TLR9-/- mice with an anti-CD154 antibody was performed over a period of six months until the age of nine months. The anti-CD154-treated RIIB-/-TLR9-/- mice neither developed proteinurea nor died of lupus nephritis (Figure 4.2B+C). As mentioned earlier the interaction between CD40-CD154 prevents apoptosis of GC B cells. GCs are defined areas within the spleen and lymph nodes where B cells undergo proliferation and maturation and are characterized by GL7 and Fas expression. To verify the effect of the anti-CD154 antibody on GC B cells *in vivo* we sacrificed anti-CD154-treated RIIB-/-TLR9-/- mice by the age of nine months, stained GC B cells (Fas^+GL7^+) in the spleen and analyzed GC formation by flow cytometry. Analysis revealed a complete abrogation of GC formation in the spleen of anti-CD154-treated RIIB-/-TLR9-/- mice (Figure 4.2D), proofing the functionality of the used antibody.

These data demonstrated that the interaction of CD40-CD154 is essential for the development of nephritis in RIIB-/-TLR9-/- mice. Thus, I conclude that autoantibody production, and therefore disease development are dependent on B and T cell interaction in RIIB-/-TLR9-/- mice.

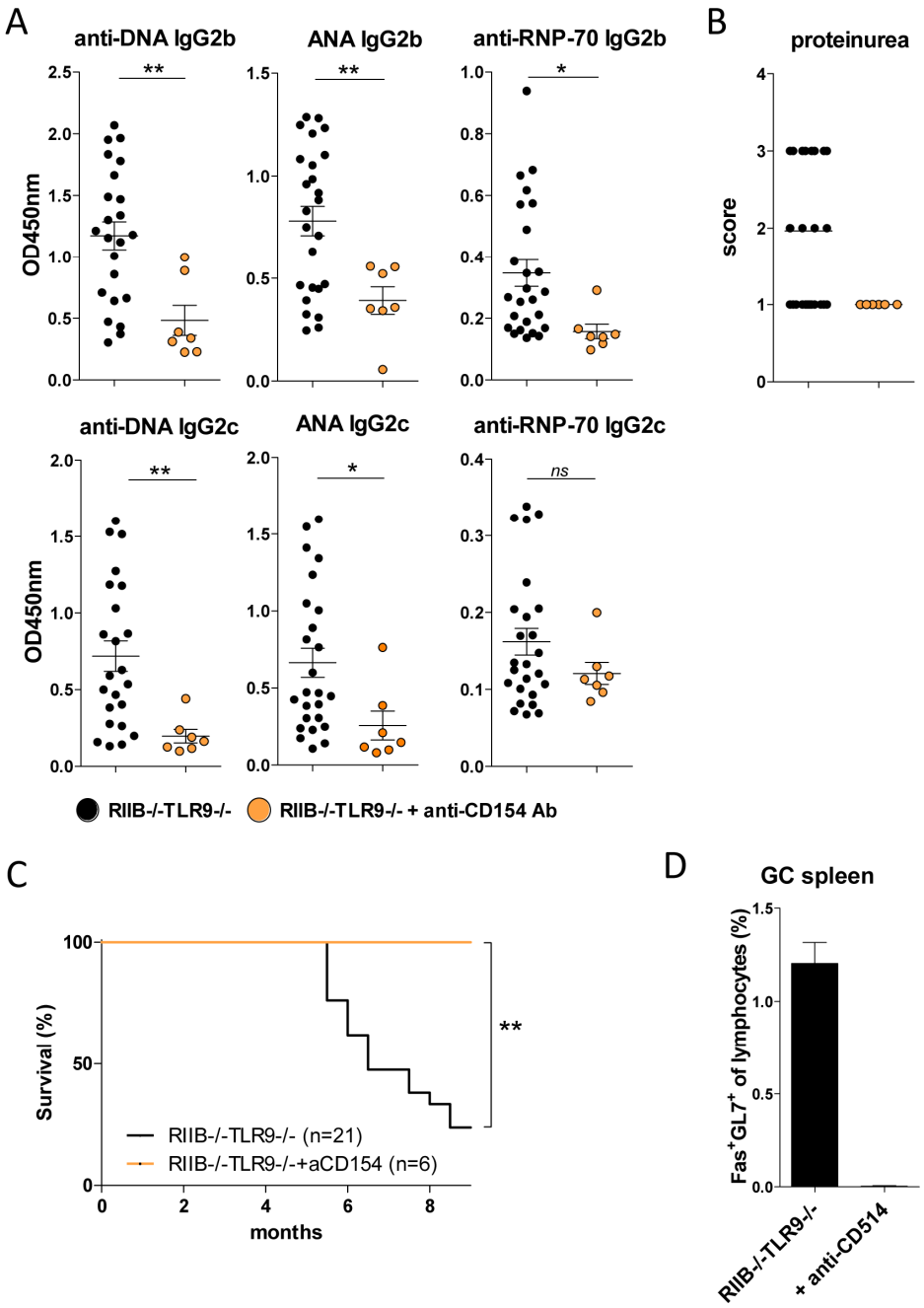


Figure 4.2 Autoantibody production and disease progression in lupus prone RIIB-/-TLR9-/- mice is dependent on T and B cell interaction. (A) IgG2b and IgG2c anti-DNA, ANA and anti-RNP-70 serum autoantibody levels of 6 month-old non-treated (n=20) and anti-CD154-treated (n=7) RIIB-/-TLR9-/- mice were analyzed by ELISA. Symbols represent data from individual mice. Horizontal lines show mean values with standard error of the mean (SEM). (B) Proteinurea scores and (C) Kaplan-Meier survival curves of 6 month-old non-treated (n=29) and anti-CD154-treated (n=7) RIIB-/-TLR9-/- mice. (D) Frequencies of $GL7^{+}Fas^{+}$ GC cells in the spleen of non-treated (n=20) and anti-CD154-treated (n=7) RIIB-/-TLR9-/- mice were determined by flow cytometry. Bar graphs show the mean value with SEM for each group of mice. ns $P>0.05$, * $P\leq0.05$, ** $P<0.01$, ***$P<0.001$

4.1.3 Increased percentages of IFNγ- and IL-17-producing CD4 T cells are associated with exacerbated lupus symptoms in RIIB-/-TLR9-/- mice

The data presented in this section were obtained together with Carolin Schön, Alexander Stöhr, and Maria Mertes from the group Tolerance and Autoimmunity (DRFZ).

So far, I confirmed the importance of CD4 helper T cells on B cell activation and subsequent autoantibody development. Particularly, I verified the influence of CD4 helper T cells and T-B cell interaction on the onset of lupus nephritis in RIIB-/-TLR9-/- mice.

To date, three distinct effector CD4 helper T cell subsets have been identified: T helper type 1 (Th1), Th2, and Th17 (Mosmann, Cherwinski et al. 1986; Bettelli, Carrier et al. 2006; Veldhoen, Hocking et al. 2006). Both IFNγ-producing CD4 T helper cells type 1 (Th1) and IL-17-producing CD4 T helper cells (Th17) are associated with autoimmunity (Crispin, Kyttaris et al. ; Damsker, Hansen et al. ; Jager and Kuchroo ; Dardalhon, Korn et al. 2008). To quantify Th1 and Th17 differentiation in RIIB-/-TLR9-/- mice flow cytometry analysis of IFNγ-and IL17-producing $CD4^+$ T helper cells, respectively, was performed. Six month-old C57BL/6 and RIIB-/-TLR9-/- were sacrificed and blood, spleen and lymph nodes were stained for CD4, IL17A and IFNγ. Frequencies of Th1 as well as Th17 cells were increased in RIIB-/-TLR9-/- compared to C57BL/6 wild-type mice. Hence, exacerbated lupus disease in RIIB-/-TLR9-/- can be associated with increased Th1 and Th17 cell frequencies (Figure 4.3A+B). Th1 cells are involved in the development of antibodies, especially of the subclasses IgG2b and IgG2c, which are generally considered as the pathogenic subclasses in murine lupus (Snapper and Paul 1987; Kotzin 1996). Here, augmented autoantibody levels in RIIB-/-TLR9-/- mice could be linked to highly increased Th1 cell frequencies. The effect of Th17 cells on B cell proliferation and isotype class switching has only recently been started to investigate. Mitsdoerffer et al just reported that IL-17 can induce class switch to IgG2a and IgG3, but not to IgG1 and only to a low level to IgG2b (Mitsdoerffer, Lee et al. 2010). Thus, also increased Th17 cell differentiation in RIIB-/-TLR9-/- mice could promote increased IgG2b and IgG2c autoantibody generation.

Th17 cells induce intense inflammatory responses by secreting IL-17, IL-21, and IL-22 (Crispin, Kyttaris et al. 2010). To determine whether IL-17 can be detected in target organs such as renal tissues, immunhistological stainings of kidney sections were performed. Kidney sections of C57BL/6 and RIIB-/-TLR9-/- mice were stained for MacI (blue), CD4

and IL-17 (green). Kidney sections of RIIB-/-TLR9-/- mice depict increased numbers of IL-17-producing CD4 T cells compared to C57BL/6 wild-type mice (Figure 4.3C). Additionally, kidneys of RIIB-/-TLR9-/- mice exhibit increased infiltration of macrophages (blue) compared to wild-type mice.

To analyze cytokine production in lupus prone mice, splenocytes from B6 and RIIB-/-TLR9-/- mice were isolated and cultured for 48h in RPMI (+FCS, P/S) medium. Supernatants were analyzed for IL-6 and IL-22 production using ELISA. Both cytokines were significantly increased in RIIB-/-TLR9-/- mice compared to C57BL/6 wild-type mice (Figure 4.3D+E). Therefore, severe inflammation and elevated Th17 cells in RIIB-/-TLR9-/- mice can be associated with increased production of the pro-inflammatory cytokines IL-6 and IL-22.

These data suggest that Th1 as well as Th17 cells are involved in the pathogenesis of nephritis in our mouse model.

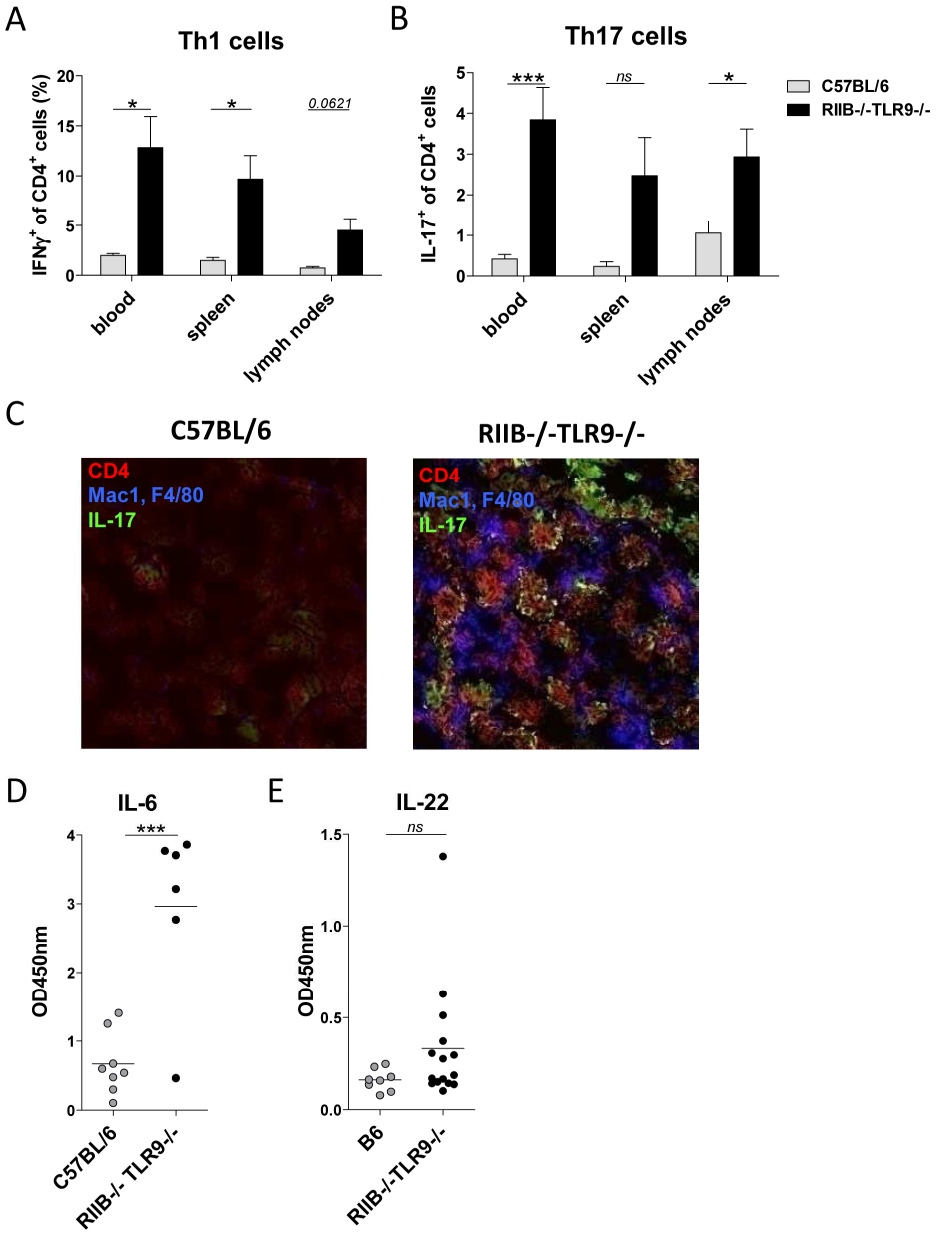


Figure 4.3 RIIB-/-TLR9-/- mice show increased expansion of pro-inflammatory Th1 and Th17 cells. Frequencies of (A) $CD4^+IFN\gamma^+$ Th1 cells and (B) $CD4^+IL\text{-}17^+$ Th17 cells were determined by flow cytometry in blood, spleen and lymph nodes of 5-6 month-old C57BL/6 (n=5) and RIIB-/-TLR9-/- (n=6) mice upon stimulation with PMA/Ionomycin. Bar graphs show the mean value with standard error of the mean (SEM) for each group of mice. (C) Immunofluorescence staining of frozen sections of kidneys from 6 month-old C57BL/6 mice or RIIB-/-TLR9-/- (Proteinurea positive) mice stained with anti-MacI (blue), anti-CD4 (Baudino, Nimmerjahn et al.) and anti-IL17 (green). Images are representative of 3 different mice per group. (D+E) ELISA of (D) IL-6 and (E) IL-22 in the supernatant of 48h cultured splenocytes of 5-6 month-old C57BL/6 (n=8) and RIIB-/-TLR9-/- (n=6 in D; n=15 in E) mice. ELISA are representative of two independent experiments. ns $P>0.05$, * $P\leq0.05$, ** $P<0.01$, ***$P<0.001$

4.1.4 Role of Th1 and Th17 in a murine model of SLE

So far, I showed increased Th1 and Th17 cell frequencies in RIIB-/-TLR9-/- mice compared to wild-type C57BL/6 mice. To better understand the distinct role of both CD4 T helper cell subsets we crossed lupus prone RIIB-/-TLR9-/- with either IFNγR1-/- or IL-17RA-/- mice. IFNγR1 encodes the ligand-binding α-chain of the heterodimeric gamma interferon receptor. Binding of IFNγ to its receptor is necessary to promote and maintain Th1 cell differentiation properly (O'Garra 1998). IL-17RA binds four different types of IL-17, in particular IL-17A, which has been associated with autoimmunity (Yang, Chang et al. 2008; Ishigame, Kakuta et al. 2009). Neither single IFNγR1-/- nor single IL17RA-/- showed symptoms of lupus nephritis, they were fertile and showed normal development.

4.1.4.1 IFNγR1 deficiency in RIIB-/-TLR9-/- mice inhibits autoantibody production and protects from lupus nephritis

IFNγ is the hallmark cytokine of Th1 cells and is one important mediator of immunity and inflammation. In the last years, it has been shown that IFNγ can either augment or suppress autoimmunity depending on the specific disease and the timing, location and intensity of IFNγ action (Kelchtermans, Billiau et al. 2008). To investigate the role of IFNγ in our lupus prone mice we crossed RIIB-/-TLR9-/- mice with IFNγR1-/- mice.

Since the Th1 cell subset is predominantly linked with the development of antibodies of the IgG2b and IgG2c isotype (Snapper and Paul 1987; Kotzin 1996), we analyzed autoantibody production in six month-old RIIB-/-TLR9-/- deficient for IFNγR1 using ELISA. As expected, IFNγR1 deficiency in RIIB-/-TLR9-/- mice completely inhibited the production of IgG2c ANA and anti-RNP-70 autoantibodies, and significantly decreased the production of IgG2b autoantibodies (Figure 4.4A). Flow cytometric analysis of the frequencies of plasma cells ($CD138^+$) within splenocytes of B6, RIIB-/-TLR9-/-, and RIIB-/-TLR9-/-IFNγR1-/- mice further confirmed the ELISA data. Deficiency in IFNγR1 in RIIB-/-TLR9-/- mice completely abrogated plasma cell differentiation and therefore secretion of immunoglobulins (Figure 4.4B).

Since production of autoantibodies is a prerequisite for the development of lupus nephritis, none of the lupus prone RIIB-/-TLR9-/-IFNγR1-/- developed nephritis and died until the age of 12 months (Figure 4.4C).

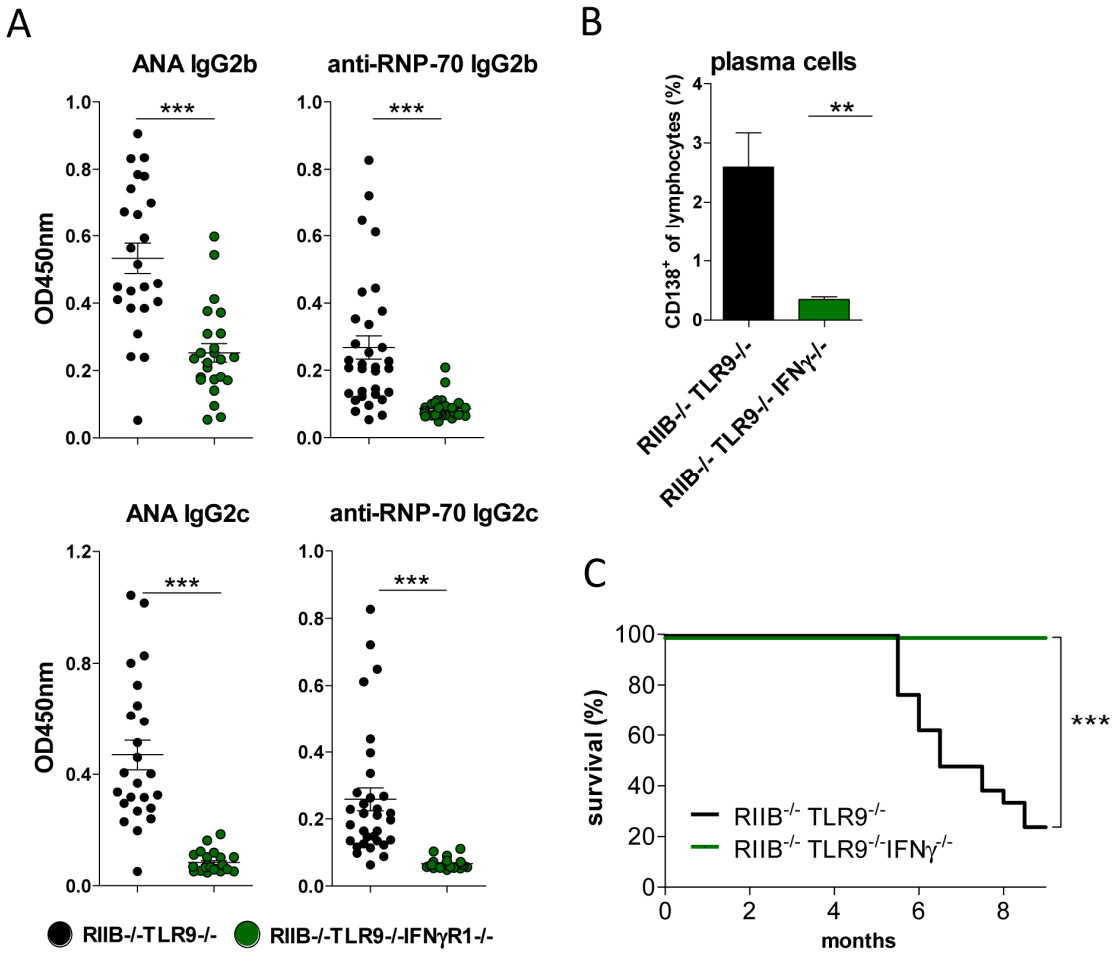


Figure 4.4 Autoantibody production and disease progression in RIIB-/-TLR9-/- mice is dependent on IFNγR1 expression. (A) IgG2b and IgG2c ANA and anti-RNP-70 serum autoantibody levels of 6 month-old RIIB-/-TLR9-/- (n=24) and RIIB-/-TLR9-/-IFNγR1-/- (n=24) mice were analyzed by ELISA. Symbols represent data from individual mice. Horizontal lines show mean values with standard error of the mean (SEM). (B) Frequencies of $CD138^+$ plasma cells in the spleen of RIIB-/-TLR9-/- (n=9) and RIIB-/-TLR9-/- IFNγR1-/- (n=7) mice were analyzed by flow cytometry. Bar graphs show the mean value with SEM for each group of mice. (C) Kaplan-Meier survival curves for RIIB-/-TLR9-/- (n=21) and IFNγR1 deficient RIIB-/-TLR9-/- (n=13) mice. ns $P>0.05$, * $P\leq0.05$, ** $P<0.01$, ***$P<0.001$

Increased frequencies of Th1, and Th17 cells were associated with exacerbated lupus disease symptoms (Figure 4.3A+B). To clarify whether IFNγR1 deficiency influences T helper cell differentiation and activation flow cytometric analysis of six month-old C57BL/6,

RIIB-/-TLR9-/- and RIIB-/-TLR9-/- IFNγR1-/- was performed. Th1 cell differentiation was significantly decreased in blood, spleen, and lymph nodes of RIIB-/-TLR9-/-IFNγR1-/- compared to RIIB-/-TLR9-/- mice (Figure 4.5A). To determine the activation status of CD4 T cells, cells were stained for CD44 and CD62L. Activated CD4 T cells express high levels of CD44 whereas the expression of CD62L is downregulated. IFNγR1 deficiency in RIIB-/-TLR9-/- diminished the activation of CD4 T cells ($CD4^+CD44^+CD62L^-$) detected in RIIB-/-TLR9-/- (Figure 4.5B). Furthermore, frequencies of Th17 cells ($CD4^+IL\text{-}17^+$) were reduced in RIIB-/-TLR9-/- lacking IFNγR1 (Figure 4.5C) probably due to their impaired activation observed in RIIB-/-TLR9-/- IFNγR1-/- mice.

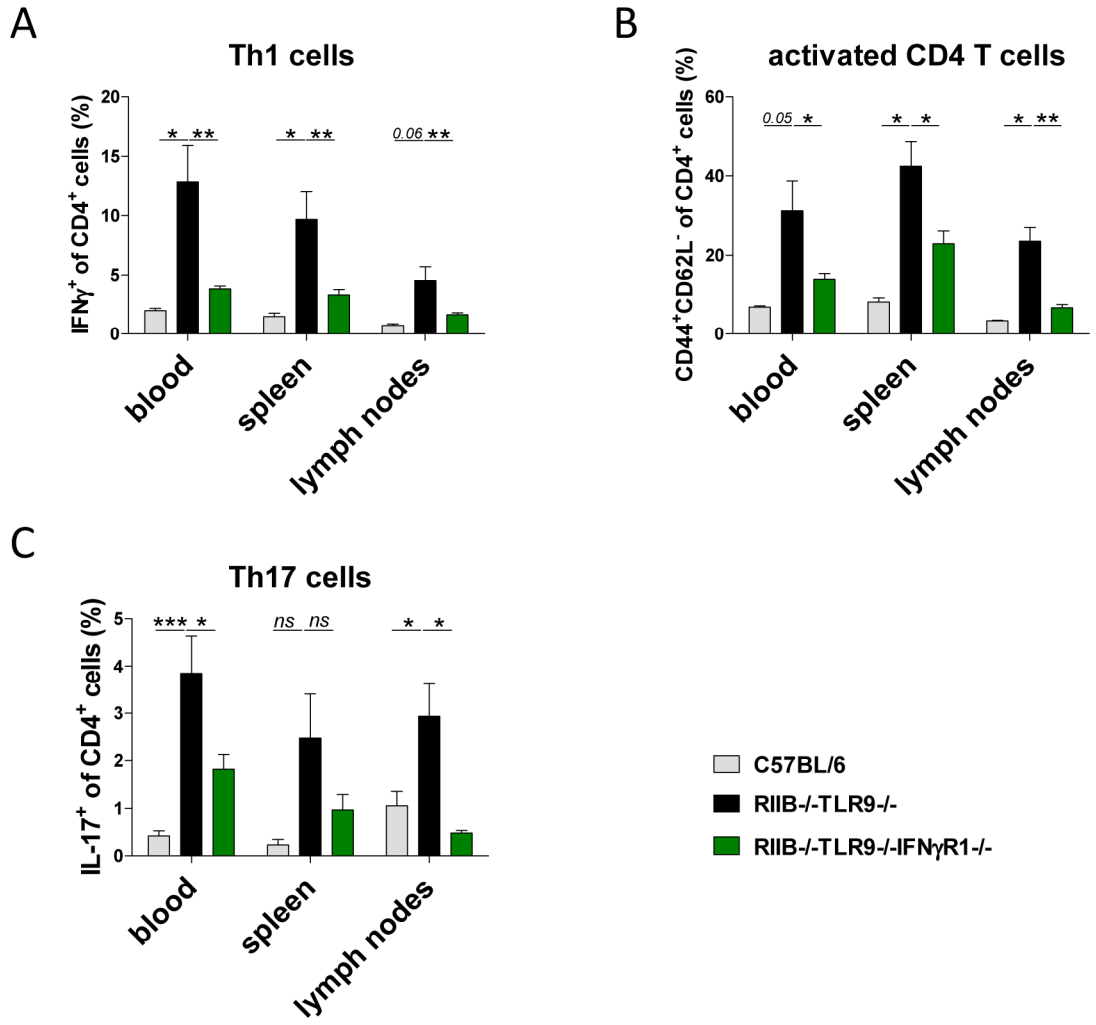


Figure 4.5 Increased expansion of pro-inflammatory Th1 and Th17 cells and activated CD4 T helper cells in RIIB-/-TLR9-/- mice is IFNγR1 dependent. Frequencies of CD4+IFNγ+ Th1 cells (A), activated CD4 T cells (CD4+CD44+CD62L-) (B) and CD4+IL-17+ Th17 cells (C) were determined by flow cytometry in blood, spleen and lymph nodes of 5-6 month-old wild-type C57BL/6 (n=4), RIIB-/-TLR9-/- (n=6) mice and RIIB-/-TLR9-/- IFN▪R1-/- (n=9) mice upon stimulation with PMA/Ionomycin. Bar graphs show the mean value with standard error of the mean (SEM) for each group of mice. ns $P>0.05$, * $P\leq0.05$, ** $P<0.01$, *** $P<0.001$

As I showed before (4.1.2.) interaction between B and T cells is of importance for the development of autoantibodies leading to lupus nephritis. Thus, I analzyed GC formation using flow cytometry. Secondary lymphoid organs were analyzed for GL7 and Fas expression. The frequency of $GL7^{+}Fas^{+}$ in spleen and lymph nodes of RIIB-/-TLR9-/-IFNγR1-/- mice was significantly decreased compared to RIIB-/-TLR9-/- mice (Figure 4.6A).

During development of lupus nephritis macrophages infiltrate the kidney, leading to inflammation and tissue destruction. As the cytokine IFNγ is involved in macrophage activation (Kelchtermans, Billiau et al. 2008), I examined macrophage differentiation in RIIB-/-TLR9-/-IFNγR1-/- mice. Compared to RIIB-/-TLR9-/- mice RIIB-/-TLR9-/- IFNγR1-/- displayed reduced frequencies of $MacI^+$ lymphocytes (Figure 4.6B). Significantly reduced frequencies compared to RIIB-/-TLR9-/- mice were observed in RIIB-/-TLR9-/-IFNγR1-/- for another pro-inflammatory cell type, namely $MacI^+Gr\text{-}1^+$ neutrophils (Figure 4.6C).

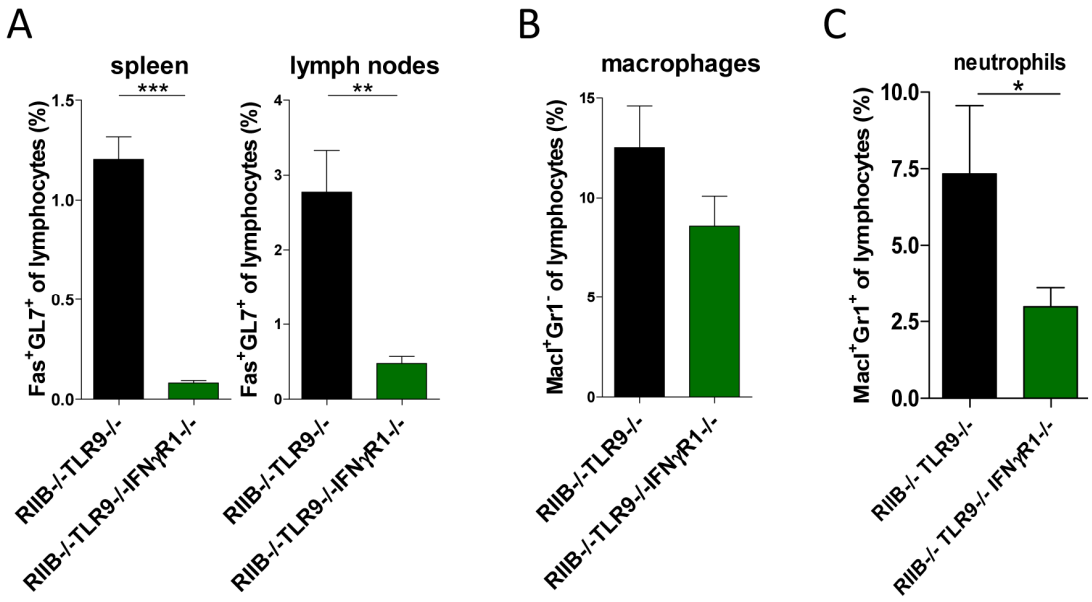


Figure 4.6 IFNγR1 deficiency inhibits GC formation and expansion of pro-inflammatory innate immune cells in the spleen of RIIB-/-TLR9-/- mice. (A) Frequencies of GL7+Fas+ GC cells in the spleen and lymph nodes of 5-6 month-old RIIB-/-TLR9-/- (n=9) and RIIB-/-TLR9-/- IFN▪ R1-/- (n=14) mice. Bar graphs show the mean value with standard error of the mean (SEM) for each group of mice. (B) Frequencies of MacI+ macrophages or (C) MacI+Gr-1+ neutrophils in the spleen of 5-6 month-old RIIB-/-TLR9-/- (n=13) and RIIB-/-TLR9-/- IFN▪ R1-/- (n=6) mice. Data were analyzed using flow cytometry. Bar graphs show the mean value with SEM for each group of mice. ns $P>0.05$, * $P\leq0.05$, ** $P<0.01$, ***$P<0.001$

4.1.4.2 IL-17RA deficiency in RIIB-/-TLR9-/- mice delays disease onset

In the last decade Th17 cells were identified to be involved in autoimmunity (Bettelli, Carrier et al. 2006). I have shown that T cells from lupus-prone mice express high levels of IL-17A and that Th17 cells can be detected in kidneys of mice with active nephritis. Accordingly, I hypothesized that IL-17 is central in the development of lupus nephritis in our mouse model. To determine whether the interaction of IL-17 with its receptor is associated with disease development, I generated lupus-prone RIIB-/-TLR9-/- mice lacking IL-17 receptor A. (IL-17RA). These mice were healthy and fertile and were monitored for nine months for the development of symptoms of lupus. I found that RIIB-/-TLR9-/-IL-17RA-/- mice developed milder proteinurea at the age of six months than age- and sex-matched RIIB-/-TLR9-/- (Figure 4.7A). In accordance, mortality in lupus-prone mice deficient for IL-17RA was significantly delayed compared to RIIB-/-TLR9-/- mice (Figure 4.7B).

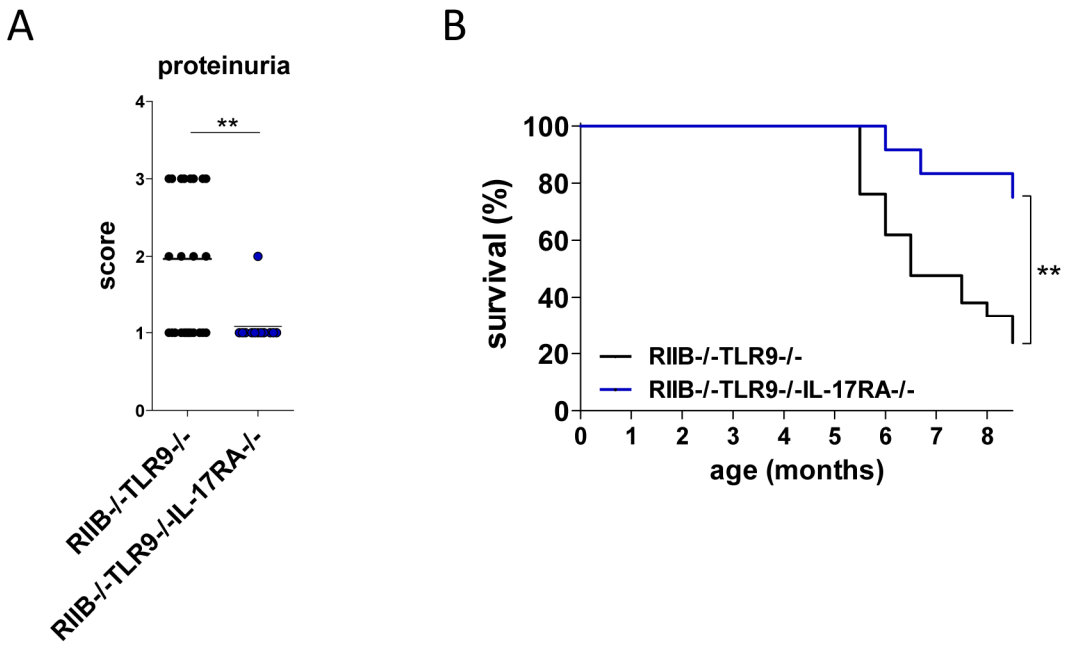


Figure 4.7 IL-17RA deficiency in RIIB-/-TLR9-/- mice delays disease onset. (A) Proteinurea scores (6 month-old mice) and (D) Kaplan-Meier survival curves of RIIB-/-TLR9-/- (n=29) and RIIB-/-TLR9-/-IL-17RA-/- (n=12) mice. Symbols represent data from individual mice. ns P>0.05, * P≤0.05, ** P<0.01, ***P<0.001

To determine whether the IL-17R-IL-17 interaction had an impact on antibody formation and plasma cell differentiation in general, we analyzed serum of six months old RIIB-/-TLR9-/-IL-17RA-/- for total IgG and IgM production. Neither total IgG nor total IgM titers were affected by IL-17RA deficiency suggesting that IL-17RA is not required to maintain the homeostasis of antibodies generated in RIIB-/-TLR9-/- mice (Figure 4.8A). Flow cytometric analysis of $CD138^+$ plasma cells in the spleen revealed no difference between lupus prone RIIB-/-TLR9-/- or RIIB-/-TLR9-/-IL-17RA-/- mice and thus confirmed data obtained by ELISA (Figure 4.8B). Hence, IL-17RA deficiency in our lupus mouse model did not influence plasma cell differentiation and therefore total antibody production.

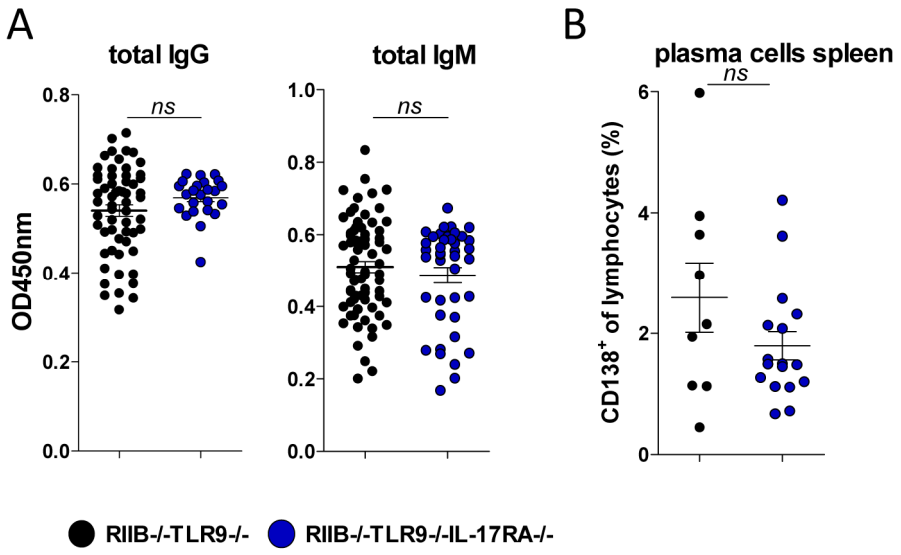


Figure 4.8 Total antibody production and plasma cell differentiation in lupus prone RIIB-/-TLR9-/- mice is independent of IL-17RA expression. (A) Total IgG and IgM antibody titers in the sera of 6 month-old old RIIB-/-TLR9-/- (n=59) and RIIB-/-TLR9-/-IL-17RA-/- (n=24) mice were determined by ELISA. Symbols represent data from individual mice. Horizontal lines show mean values with standard error of the mean (SEM). (B) Frequencies of CD138+ plasma cells in the spleen of RIIB-/-TLR9-/- (n=9) and RIIB-/-TLR9-/- IL-17RA-/- (n=17) mice were analyzed by flow cytometry. Symbols represent data from individual mice. Horizontal lines show mean values with SEM. ns $P>0.05$, * $P\leq0.05$, ** $P<0.01$, ***$P<0.001$

Antibody production against different autoantigens in RIIB-/-TLR9-/-IL-17RA-/- was analyzed by serum ELISA. Unexpectedly, as depicted in Figure 4.9, we detected no significant differences in autoantibody production between IL-17RA deficient RIIB-/-TLR9-/- mice and control RIIB-/-TLR9-/- lupus-prone mice. Autoantibody titers reactive to RNA-

related antigens (RNP-70) were even increased in RIIB-/-TLR9-/-IL-17RA-/- compared to RIIB-/-TLR9-/- mice (Figure 4.9C).

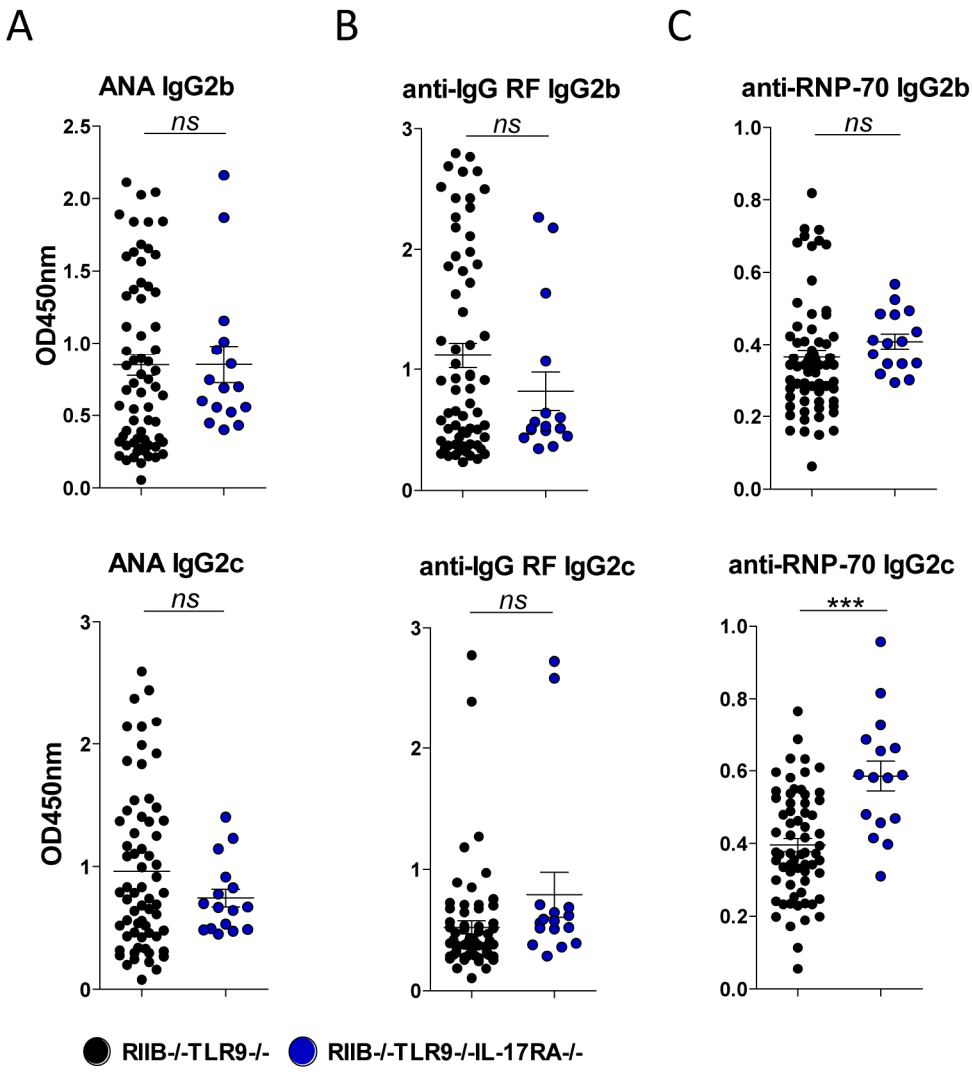


Figure 4.9 Autoantibody production in lupus prone RIIB-/-TLR9-/- mice is independent of IL-17RA expression. IgG2b and IgG2c (A) anti-DNA, (B) ANA, and (C) anti-RNP-70 serum autoantibody levels of 6 month-old RIIB-/-TLR9-/- (n=65) and RIIB-/-TLR9-/- IL-17RA-/- (n=16) mice were analyzed by ELISA. Symbols represent data from individual mice. Horizontal lines show mean values with standard error of the mean (SEM). ns $P>0.05$, * $P\leq0.05$, ** $P<0.01$, ***$P<0.001$

Previous studies in our laboratory have shown that autoantibody production is a prerequisite for lupus disease, but not sufficient to actually induce lupus nephritis (unpublished data by Maria Mertes). IgG2b and IgG2c autoantibodies in RIIB-/-TLR9-/- mice form immune complexes (ICs), which deposit in the kidney. Then macrophages are recruited which induce tissue inflammation and destruction and finally mice die of nephritis. To determine IC

formation and macrophage infiltration in RIIB-/-TLR9-/-IL-17RA-/- mice cyro-sections of kidneys were prepared and stained for ICs (anti-IgG2b/c; red) and macrophages (anti-MacI, anti-F4/80; green). Immunohistological stainings revealed IC formation and their deposition in the kidney in RIIB-/-TLR9-/- and RIIB-/-TLR9-/-IL-17RA-/-. But interestingly, infiltration of macrophages (green) into renal tissues as observed in RIIB-/-TLR9-/- mice could not be detected in RIIB-/-TLR9-/-IL-17RA-/- mice (Figure 4.10). Hence, IL-17RA plays a crucial role in macrophage recruitment in lupus prone mice.

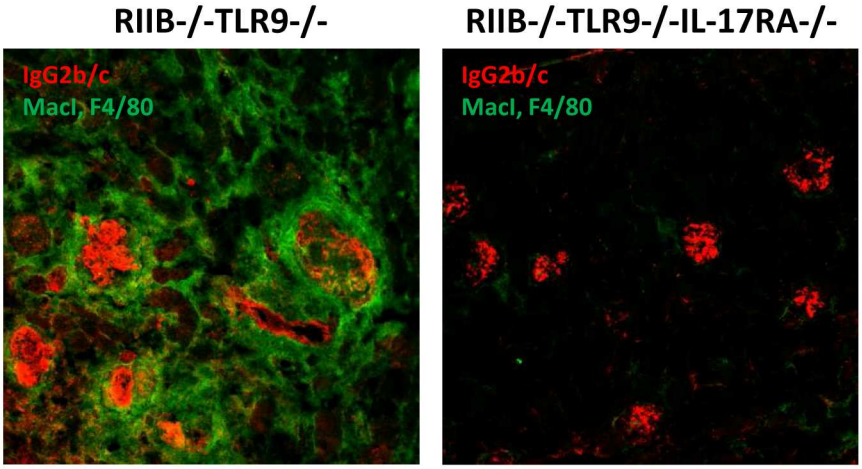


Figure 4.10 IL-17RA deficiency in RIIB-/- TLR9-/- mice inhibits macrophage infiltration into renal tissues. Immunofluorescence staining of frozen sections of kidneys from 6 month-old RIIB-/-TLR9-/-IL-17RA-/- or RIIB-/-TLR9-/- mice stained with anti-MacI/F4/80 (green) and anti-IgG2b/c. Images are representative of 3 different mice per group.

To analyze immunopathology, I sacrificed 6-month-old RIIB-/-TLR9-/- and RIIB-/-TLR9-/-IL-17RA-/- mice. Although, none of the IL-17RA deficient lupus-prone mice exhibited severe disease symptoms like glomerulonephritis, mice showed splenomegaly as observed in RIIB-/-TLR9-/- (data not shown). Similar, no reduction in the cell population of spleen in IL-17RA lupus-prone mice compared to RIIB-/-TLR9-/- mice was found (Figure 4.11A). Only total cell count in the lymph nodes of RIIB-/-TLR9-/-IL-17RA-/- mice was slightly reduced compared to RIIB-/-TLR9-/- lymph nodes (Figure 4.11B). Interestingly, frequencies of IL-17$^+$ CD4 T helper cells in blood and spleen of RIIB-/-TLR9-/-IL-17RA-/- mice were even higher than in RIIB-/-TLR9-/- mice (Figure 4.11C).

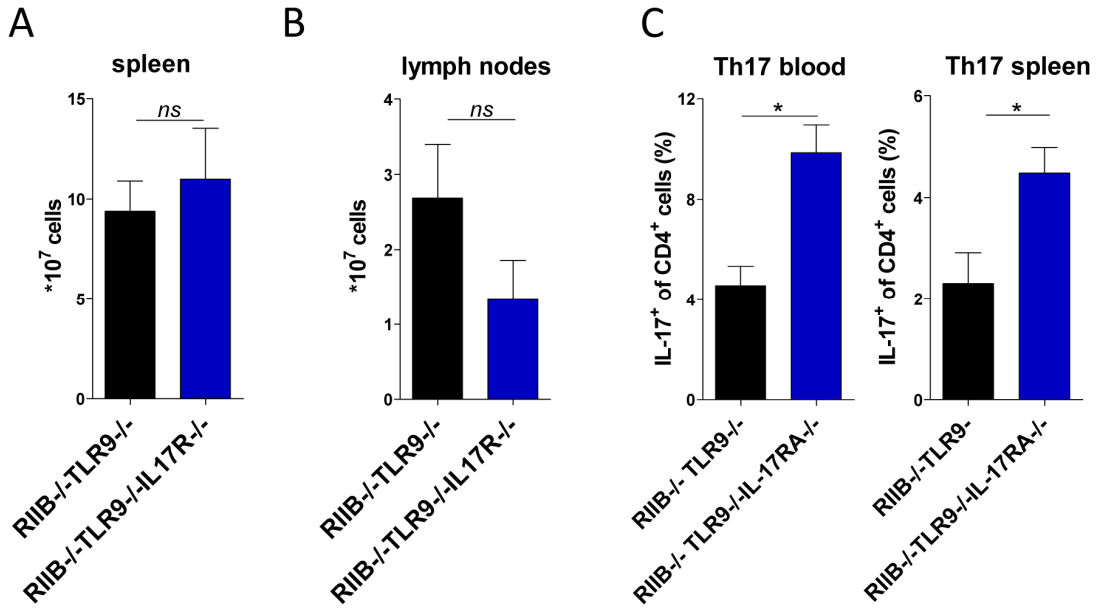


Figure 4.11 IL-17RA deficiency does not inhibit splenomegaly in RIIB-/-TLR9-/- mice. Total cell number of spleen (A) and lymph nodes (B) of RIIB-/-TLR9-/- (n=13) and RIIB-/-TLR9-/- IL-17RA-/- (n=8) mice. Bar graphs show the mean value with standard error of the mean (SEM) for each group of mice. (C) Frequencies of IL-17$^+$CD4$^+$ Th17 cells in blood and spleen of RIIB-/-TLR9-/- (n=6) and RIIB-/-TLR9-/-IL-17RA-/- (n=4) mice. Bar graphs show the mean value with SEM for each group of mice. ns P>0.05, * P≤0.05, ** P<0.01, ***P<0.001

Hsu et al. have reported that IL-17 can promote the formation of GCs in autoimmune mice. In their autoimmune model administration of IL-17 is sufficient to accelerate the development of autoantibody-producing GCs (Hsu, Yang et al. 2008). To determine the effect of IL-17RA on GC formation in our lupus-prone mice, I analyzed spleen and lymph nodes for GC B cells (Fas$^+$GL7$^+$) of RIIB-/-TLR9-/- and RIIB-/-TLR9-/-IL-17RA-/- mice. Despite the high frequencies of Th17 cells in the spleen of RIIB-/-TLR9-/-IL-17RA-/- mice flow cytometric analysis of spleen and lymph nodes revealed about 50% less Fas$^+$GL7$^+$ GC cells in lupus-prone mice deficient for IL-17RA compared to RIIB-/-TLR9-/- (Figure 4.12A), implying the relevance of IL-17A-IL-17RA interaction for GC formation.

In 2008 Hsu et al. reported an effect of IL-17 on the development of marginal zone B cells (Hsu, Yang et al. 2008). Further examinations in our mouse model revealed increased frequencies of CD21$^+$CD23$^{lo/neg}$ marginal zone B cells in the spleen of RIIB-/-TLR9-/-IL-17RA-/- mice compared to RIIB-/-TLR9-/- mice. Whereas recirculating follicular B cells

(CD21^{+}CD23^{high}) are reduced in IL-17RA deficient RIIB-/-TLR9-/- mice, suggesting reduced migration of the B cells from the marginal zone to the follicular region (Figure 4.12B).

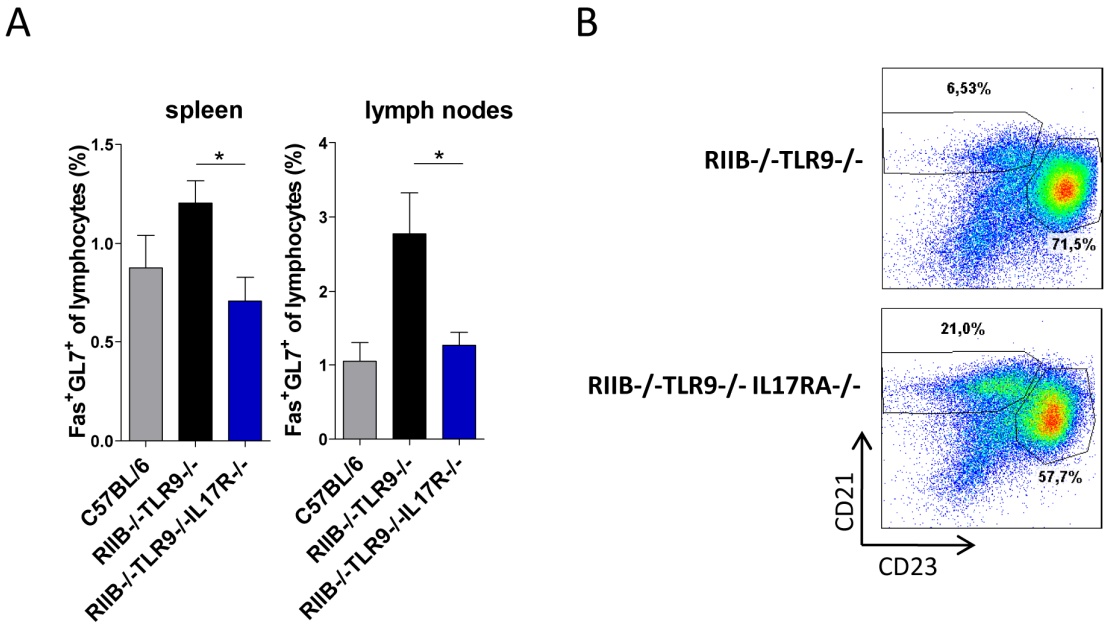


Figure 4.12 IL-17RA deficiency inhibits germinal center formation and induces marginal zone B cells in RIIB-/-TLR9-/- mice. (A) Frequencies of GL7^{+}Fas^{+} GC cells in spleen and lymph nodes (LN) of RIIB-/-TLR9-/- (spleen n=9; LN n=17) and RIIB-/-TLR9-/-IL-17RA-/- (spleen and LN n=17). Bar graphs show the mean value with standard error of the mean (SEM) for each group of mice. (B) Representative FACS staining for CD21^{hi}CD23$^{lo/neg}$ marginal zone and recirculating follicular CD21^{+}CD23^{hi} cells gated within the B220^{+} population of spleens of 6 month-old RIIB-/-TLR9-/- and RIIB-/-TLR9-/-IL-17RA-/- mice. ns P>0.05, * P≤0.05, ** P<0.01, ***P<0.001

Deletion of the IL-17RA reduced GC formation in secondary lymphoid organs and inhibited macrophage infiltration in the kidneys of lupus prone RIIB-/-TLR9-/- mice. Thus, the data obtained from RIIB-/-TLR9-/-IL-17RA-/- mice further support the pro-inflammatory role of Th17 in SLE.

4.2 Function of Th17 cells on disease manifestation in a collagen-induced arthritis mouse model (CIA)

4.2.1 Treatment with anti-IL-23R antibody inhibits arthritis development independently of FcγRIIB

The pro-inflammatory role of Th17 cells is suggested for various autoimmune diseases, e.g. rheumatoid arthritis (RA). There is considerable evidence that IL-17 contributes to the inflammation associated with RA: IL-17 is spontaneously produced by RA synovial membrane cultures and high levels of IL-17 were detected in the synovial fluid of patients with RA (Kotake, Udagawa et al. 1999). In addition, mice deficient for IL-17 or IL-17R were found to be less susceptible for induction of collagen-induced arthritis (CIA) (Nakae, Nambu et al. 2003). Thus, I chose the CIA mouse model, an animal model reminiscent in several aspects to RA, to investigate the pro-inflammatory characteristics of Th17 cells in RIIB-/- mice. The cytokine IL-23 is important for proliferation and maintenance of Th17 cells (Bettelli, Carrier et al. 2006) and to prevent the differentiation of Th17 cells after the induction of CIA I injected an anti-IL-23R antibody.

RIIB-/ mice were immunized subcutaneously (s.c.) on day 0 with chicken collagen type II (CII) in *mycobacteria tuberculosis (M.tb)* enriched complete freund´s adjuvant (enriched CFA). 21 days later mice were boosted s.c. with chicken CII in incomplete freund´s adjuvant (IFA) (Steinman, Hawiger et al.). Additionally, one group of RIIB-/- mice (n=10) received 500µg anti-IL-23R antibody to inhibit Th17 cell proliferation and maintenance; control mice received PBS (n=10). After day 21 mice were monitored and a mean clinical score was assessed. The clinical score was assessed by scoring each limb of a mouse from 0 (healthy) to 3 (severe swelling of digits, paw and ankle). The maximum score per mouse can be 12. The mean clinical score was calculated by including all animals of one group. Arthritis-associated symptoms appeared from day 22 in control RIIB-/- and reached a mean clinical score of 6,9 on day 36. In contrast, anti-IL-23R treated mice developed a less severe form of arthritis with a lower mean clinical score of 3 on day 36 (Figure 4.13A). The incidence of disease in untreated RIIB-/- mice was 70% whereas only 40% of anti-IL-23R treated mice became arthritic (Figure 4.13B).

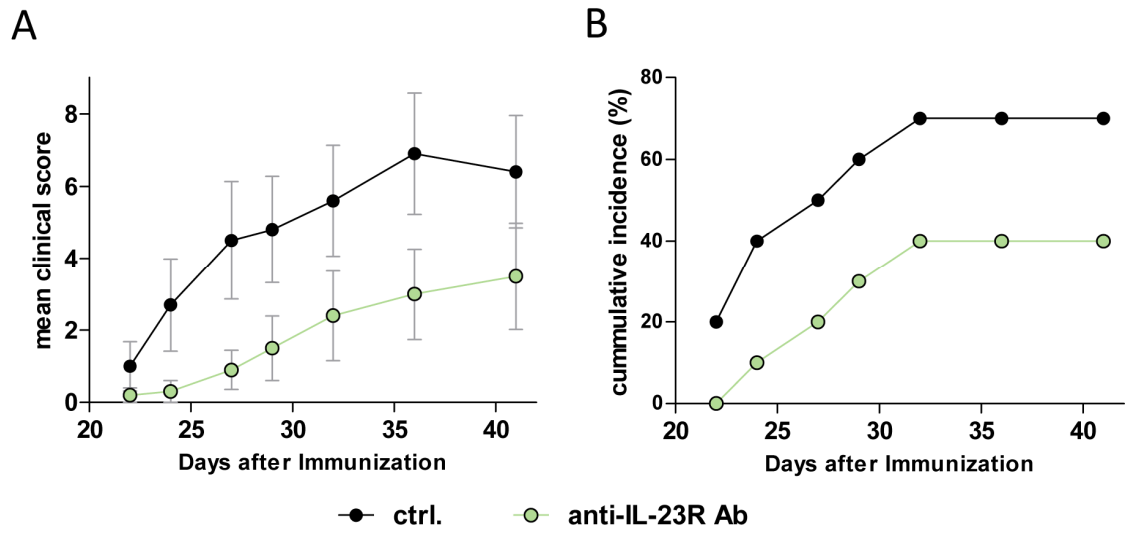


Figure 4.13 Treatment with anti-IL-23R antibody protects from CIA independently of RIIB. CIA was induced in RIIB-/- mice by immunization with chicken CII in M.tb-enriched CFA on day 0 and chicken CII in IFA on day 21. Additionally, mice were injected with PBS (pos. ctrl n=10) or 500µg anti-IL-23R antibody (n=10) once a week; first injection was done on day -1. Mice were assessed over time for development of arthritis. (A) Mean clinical score and (B) cumulative incidence of all mice are shown and plotted against time after secondary immunization with CII/IFA on day 21.

4.2.2 Collagen II-specific humoral immune response is independent of anti-IL23R antibody treatment

Pathogenesis of CIA is generally considered to depend on both humoral and cellular immune responses against CII. Here, I wanted to define whether the protective effect of anti-IL-23R antibody in CIA in RIIB-/- mice results from modulation of the humoral immune response. 42 days after the immunization and therefore after induction of CIA sera from all mice were collected and analyzed for total IgG and total IgM production. RIIB-/- mice treated with anti-IL-23R antibody exhibited significantly reduced levels of total IgG antibodies but increased levels of total IgM antibodies (Figure 4.14A+B). Contrary to IgG antibodies, IgM antibodies do not correlate with disease activity and are known to exhibit protective capacities in autoimmunity (Forger, Matthias et al. 2004).

Sera from both groups were further analyzed for chicken CII-specific antibodies, regarding total chCII IgG, IgG1, IgG2b, IgG2c, and IgM antibodies. Surprisingly, no differences for antigen-specific antibodies could be observed. Neither total CII-specific IgG antibody titer,

nor isotype distribution, nor IgM antibody titers were affected by inhibition of Th17 cell differentiation (Figure 4.14C-G).

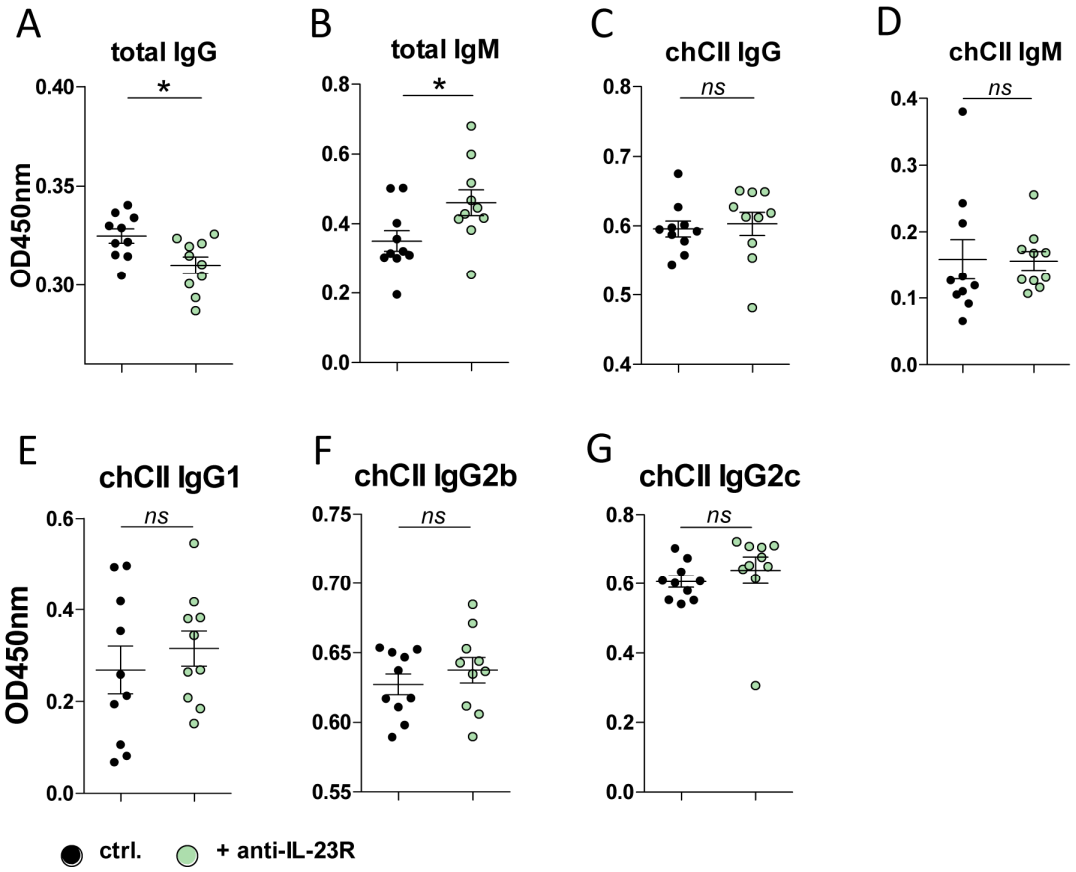


Figure 4.14 Treatment with anti-IL-23R antibody did not influence the CII-specific humoral immune response in CIA. RIIB-/- mice were immunized and injected i.p. with anti-IL-23R (n=10) or PBS (n=10) once a week. Sera of all mice from d42 were analyzed for (A) total IgG, (B) total IgM, (C) anti-CII IgG, (D) anti-CII IgM, (E) anti-CII IgG1, (F) anti-CII IgG2b, and (G) anti-CII IgG2c antibodies. Symbols represent data from individual mice. Horizontal lines show mean values with standard error of the mean (SEM). ns $P>0.05$, * $P\leq0.05$, ** $P<0.01$, ***$P<0.001$

Although the generation of total IgG and total IgM antibodies seemed to be affected by administration of anti-IL-23R antibody during induction of CIA, inhibition of Th17 cell differenation via anti-IL-23R antibody had no impact on antigen-specific antibody generation (Figure 4.15).

4.2.3 Inhibition of arthritis by administration of anti-IL-23R antibody is associated with reduced expansion of innate immune cells

As no significant differences could be detected in the antigen-specific humoral immune response, the influence of IL-23 on CIA induction was additionally examined on the cellular level. Therefore, flow cytometric analysis of secondary lymphoid organs was performed on day 42 after immunization. First, I determined Th17 and Th1 cell differentiation in RIIB-/- mice treated with anti-IL-23R antibody. As anticipated, administration of anti-IL-23R inhibited the accumulation of Th17 cells, but not Th1 cells, in lymph nodes of RIIB-/- mice after induction of CIA (Figure 4.15A+B).

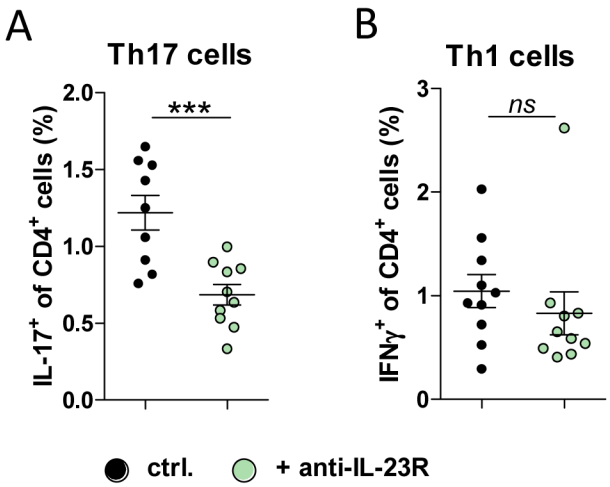


Figure 4.15 Treatment with anti-IL23R antibody effectively inhibits Th17 cell expansion in CIA. RIIB-/- mice were immunized and injected i.p. with anti-IL-23R antibody (n=10) or PBS (n=10) once a week. On day 42, lymph nodes of individual mice were isolated and analyzed by flow cytometry. Frequencies of (A) Th17 (IL-17+CD4+) cells and (B) Th1 (IFN▪ +CD4+) cells in lymph nodes of all mice were analyzed by flow cytometry upon stimulation with PMA/Ionomycin. Symbols represent data from individual mice. Horizontal lines show mean values with standard error of the mean (SEM). ns P>0.05, * P≤0.05, ** P<0.01, ***P<0.001

IL-17 is known to induce the production of pro-inflammatory IL-6, IL-8, RANKL and G-CSF *in vitro* and thus stimulates granulopoiesis *in vivo* (Fossiez, Djossou et al. 1996; Hwang, Kim et al. 2004; Shen and Gaffen 2008). Expansion of granulocytes, particularly of the $MacI^+$ cell population is associated with a more severe form of arthritis (Matthys, Vermeire et al. 1999). This $MacI^+$ population contains mostly immature mononuclear phagocytes and neutrophils that can act as a source of osteoclasts and may thus indirectly account for bone destruction in CIA and contribute to neutrophil mediated inflammation in the joints. Thus,

I analyzed the effect of IL-23 and consequently Th17 cells on the expansion of MacI$^+$ cells. Therefore, spleens and lymph nodes were isolated from anti-IL-23R-treated RIIB-/- mice and non-treated RIIB-/- mice. The total cell number of splenocytes was significantly reduced in anti-IL-23R-treated RIIB-/- compared to non-treated RIIB-/-. Total cell number of lymph nodes was also dimished in anti-IL-23R-treated mice (Figure 4.16A). Figure 4.16B illustrates that reduction of Th17 cells by anti-IL-23R antibody inhibits the expansion of MacI$^+$ cells in the spleen. Moreover, treatment with anti-IL-23R antibody resulted in significantly reduced numbers of MacI$^+$Gr-1$^+$ neutrophils in the spleen (Figure 4.16C). Frequencies of activated CD4 T cells (CD4$^+$CD44$^+$CD62L$^-$) were also slightly reduced in anti-IL-23R-treated RIIB-/- compared to non-treated RIIB-/- (Figure 16D). However, frequencies of CD3$^+$, CD4$^+$, and CD8$^+$ T cells in lymph nodes were comparable in both groups of mice (Figure 4.16F). Only double negative immature T cells (CD3$^+$CD4$^-$CD8$^-$) were significantly decreased in anti-IL-23R-treated mice (Figure 4.16E).

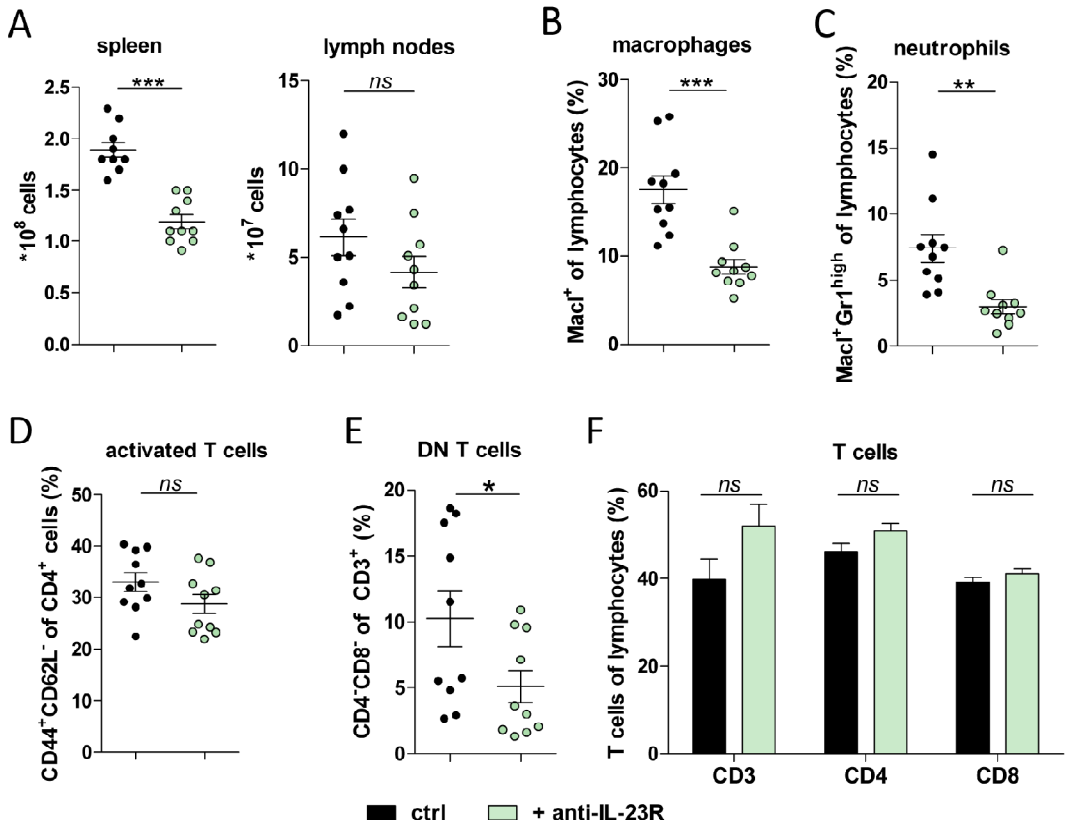


Figure 4.16 Anti-IL-23R antibody treatment reduces splenic MacI$^+$ cell expansion in CIA. RIIB-/- mice were immunized and injected i.p. with anti-IL-23R antibody (n=10) or PBS (n=10) once a week. On day 42, spleen and lymph nodes of individual mice were isolated and total cells were counted (A). Lymphocytes were characterized by flow cytometry and the percentage of (B) MacI+cells in spleen, (C) MacI+Gr-1hi neutrophils in spleen, (D) activated CD4 T cells (CD4+CD44+CD62L-) in lymph nodes, and (E) CD3+, CD4+ and CD8+ T cells in lymph nodes was determined. 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. ns $P>0.05$, * $P\leq0.05$, ** $P<0.01$, ***$P<0.001$

4.3 Application of sialylated antigen-specific antibodies to treat rheumatoid arthritis in a collagen-induced arthritis mouse model (CIA)

In CIA cellular as well as humoral immune mechanisms act in concert to mediate the progression of the disease (Seki, Sudo et al. 1988). A requirement for the generation of collagen II (CII)-specific antibodies in the progression of CIA is well documented (Stuart, Townes et al. 1982; Stuart and Dixon 1983; Holmdahl, Rubin et al. 1986). B cell-deficient mice do not develop type II collagen-induced arthritis (CIA) (Svensson, Jirholt et al. 1998). In fact, transfer of a combination of two CII-specific monoclonal antibodies, CIICI and M2139, can induce arthritis in naive mice (Johansson, Hansson et al. 2001; Nandakumar, Andren et al. 2003; Nandakumar and Holmdahl 2005). In the past it has been shown that antibodies can mediate pro- and anti-inflammatory effector functions and the glycosylation pattern of the IgG Fc fragment was identified to be responsible for the type of immune response (Nimmerjahn and Ravetch 2005; Nimmerjahn and Ravetch 2008). Interestingly, de-glycosylation of Fc glycans correlates with pro-inflammatory responses and increased disease activity in patients with rheumatoid arthritis (Parekh, Dwek et al. 1985; Rook, Steele et al. 1991; Scherer, van der Woude et al. 2010). In accordance, sialylated IgGs in intravenously injected immunoglobulins (IVIG) mediate anti-inflammatory effects during treatment of autoimmunity. However, the specific mechanisms through which sialylated antigen-specific antibodies influence disease progression in CIA remains to be elucidated. Here, I investigated whether sialylated CII-specific IgG antibodies can be used at low amounts to inhibit the onset of arthritis in an antigen-specific manner.

4.3.1 Sialylated antigen-specific antibodies inhibit arthritis development independently of FcγRIIB

The VDJ heavy and VJ light chain sequences of two previously described anti-CII antibodies (M2139 and CII1-5) (Mo and Holmdahl 1996; Ito, Ueda et al. 1997) were cloned into expression vectors containing the murine IgG1 constant region or the murine kappa region sequence, respectively. The two murine IgG1 antibodies produced in human HEK293T

cells and purified with a protein G sepharose column exhibited no Fc sialylation. Thus, antibodies were enzymatically glycosylated *in vitro* after purification and their glycosylation pattern was measured by MALDI-TOF analysis (Figure 4.17A+B). Finally, reactivity to mouse and chicken CII was verified using ELISA. Both, M2139 and CII1-5, were reactive to mouse collagen II, whereas only M2139 bound to chicken collagen II. Most notably, reactivity to collagen was not influenced by their glycosylation pattern (Figure 4.17C).

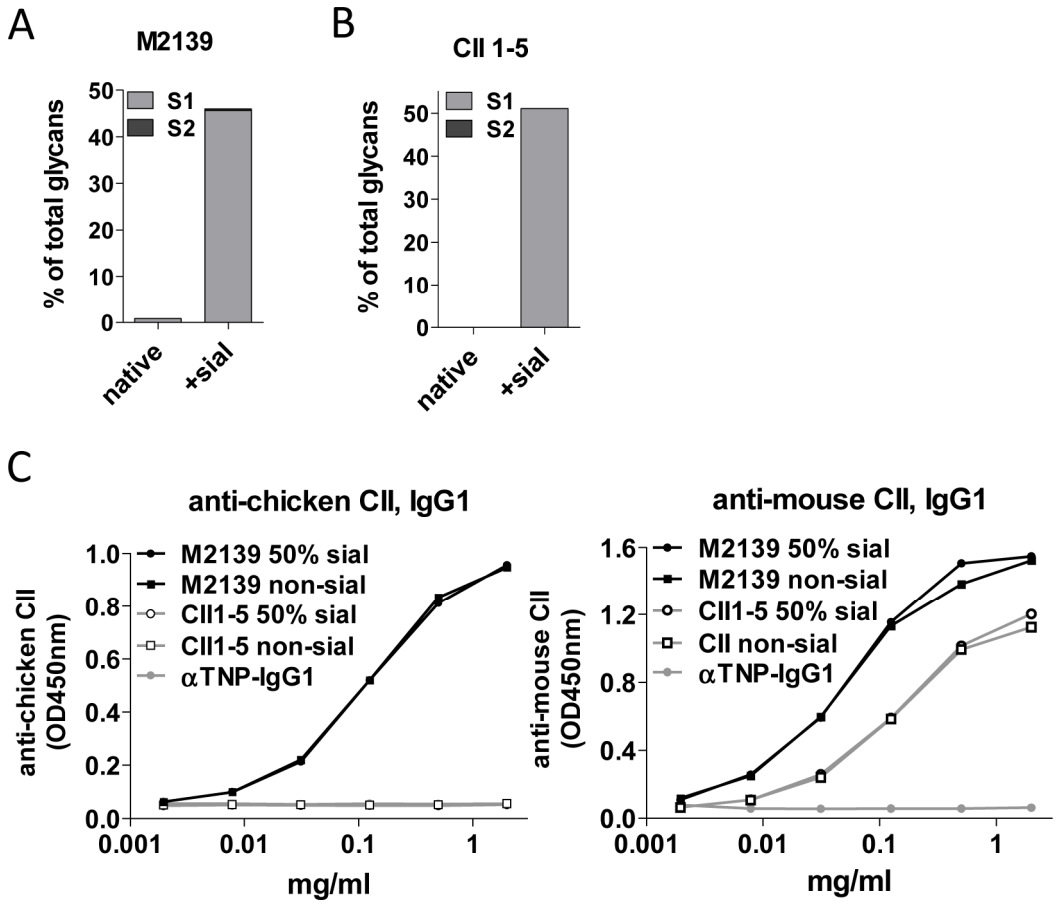


Figure 4.17 Glycan and antigen-binding analysis of sialylated CII-specific murine IgG1 monoclonal antibodies. (A) The percentage of Fc sialylation (S1 and S2) on native and in vitro galactosylated and sialylated (+sial) CII-specific murine M2139 or 1-5 IgG1 antibodies was determined by MALDI-TOF mass spectrometry. (B) Reactivity of non-sialylated (non-sial) and sialylated (+sial) M2139 and 1-5 IgG1 antibodies against chicken and mouse collagen type II and as a negative control an anti-trinitrophenyl-specific (anti-TNP) murine IgG1 antibody (clone H5) was determined by ELISA.

Next, I examined the anti-inflammatory effect of sialylated CII-specific murine IgG1 antibodies in comparison to non-sialylated CII-specific murine IgG1 antibodies and IVIG on

the development of CIA. To exclude any inhibitory effects of RIIB, experiments were performed in mice lacking RIIB-/-. Disease was induced by s.c. injection of chicken CII in *M.tb*-enriched CFA and boosted again on day 21 with chicken CII in IFA. To compare the inhibitory potential of low amounts of antigen-specific sialylated antibodies and different amounts of IVIG in CIA, we injected RIIB -/- mice with low amounts of sialylated or non-sialylated anti-collagen II mouse IgG1 antibodies (5mg/kg M2139 and CII 1-5), 25mg/kg ($IVIG^{low}$) or 2g/kg ($IVIG^{high}$) IVIG, and PBS as a positive control. The total amount of sialylated antigen-unspecific antibodies contained in $IVIG^{low}$ is comparable to the total amount of sialylated antigen-specific antibodies contained in anti-CII IgG1 (M2139 and CII 1-5) antibodies. PBS and all antibodies were injected twice, on day -1 and day 9 (Figure 4.18A). After day 21 mice were monitored and a mean clinical score was assessed.

Only low amounts of *in vitro* sialylated CII-specific antibodies and high amounts of IVIG but neither low amounts of IVIG nor non-sialylated CII-specific antibodies prevented from severe arthritis. The mean clinical score, which resembles disease severity, as well as disease incidence, was reduced in mice treated with sialylated antibodies or IVIG (Figure 4.18B+C). Only 20% of the mice, which received CII-specific sialylated antibodies, became arthritic whereas 50% of the non-sialylated antibody-treated mice showed severe footpad swelling (Figure 4.18C). The mean clinical score was significantly reduced in the CII-specific sialylated antibody-treated group compared to the CII-specific non-sialylated antibody-treated group (1.3±0.6506 to 3.8±0.8794) (Figure 4.18B).

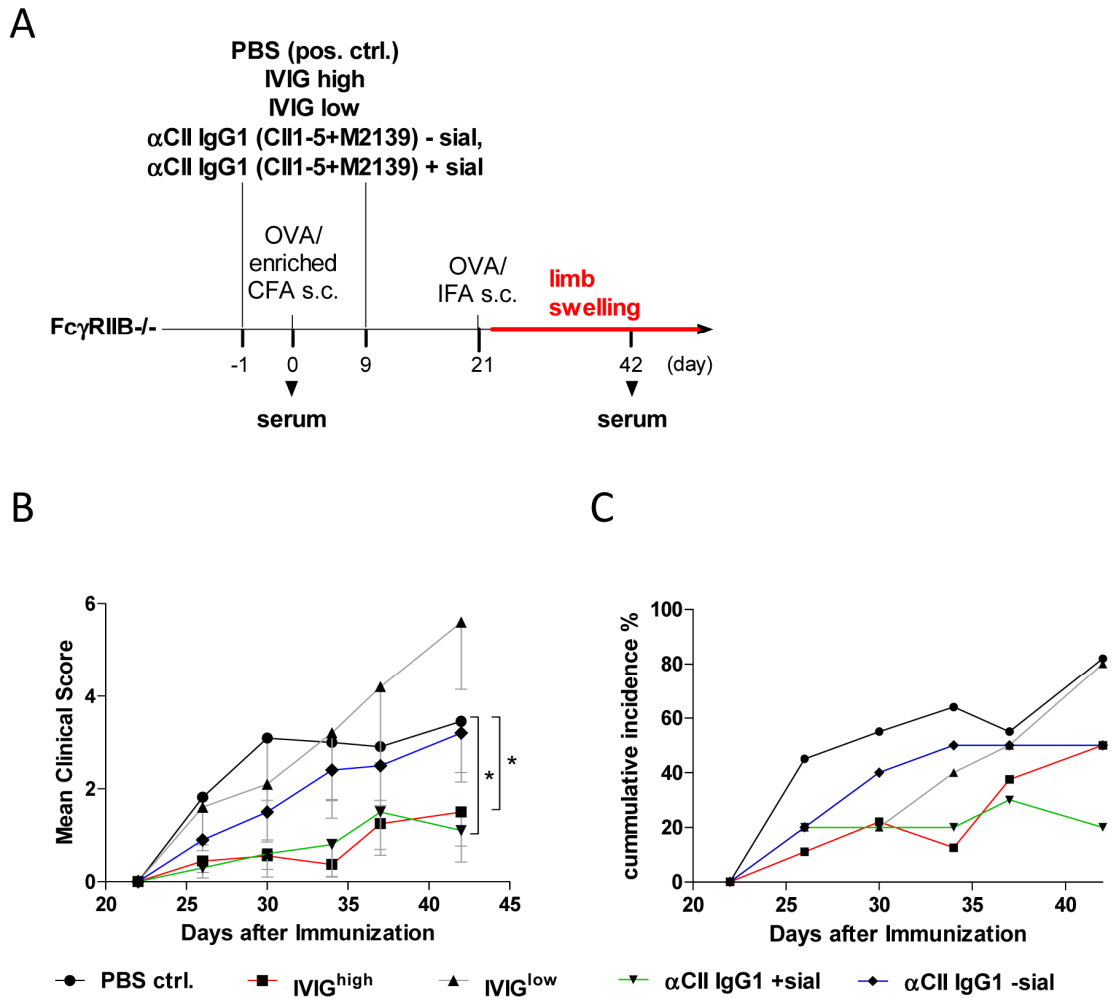


Figure 4.18 Treatment with CII-specific sialylated antibodies prevents from CIA independently of RIIB. (A) Schematic plan of the experimental setup followed in **B-C** and **Figure 4.18-4.20**. CIA was induced in RIIB-/- mice by immunization with chicken CII in *M.tb*-enriched CFA on day 0 and chicken CII in IFA on day 21. Additionally, mice were injected intravenously with PBS (n=11), 2g/kg IVIG (IVIG^{high}; n=9), 25 mg/kg IVIG (IVIG^{low}; n=10) or 5mg/kg sialylated (aCII IgG1 +sial; n=10) or non-sialylated (aCII IgG1 non-sial; n=10) IgG1 antibodies (M2139 and 1-5, 50µg each) on day -1 and again on day 9. Mice were assessed over time for development of arthritis. **(B)** Mean clinical score and **(C)** cumulative incidence of all mice are shown and plotted against time after primary immunization with CII/CFA on day 21. Error bars (mean clinical score) indicate SEM. Data are representative for two independent experiments.

During steady state the synovium that lines the non-cartilaginous surfaces within joints is one or two cell layers deep. In contrast, the synovium during inflammation displays intense infiltrations with multiple layers of cells. For histological examination of the feet, mice were sacrificed on day 47. Figure 4.19 shows representative light microscopy pictures of hematoxylin- and CD3-stained section of representative feet of all groups. As evident from the

data in Figure 4.18 B and C, reduced severity of arthritis in sialylated-antibody-or IVIG ^{high}-treated mice is associated with inhibition of infiltrated cells, hyperplasia and cartilage-bone destruction (Figure 4.19A). Furthermore, sialylated CII-specific antibodies and high amounts of IVIG but neither non-sialylated CII-specific antibodies nor low amounts of IVIG inhibited the infiltration of $CD3^+$ T cells (Figure 4.19B). Thus, histology of the feet reflected disease severity of arthritis and supported the results obtained by assessing a mean clinical score.

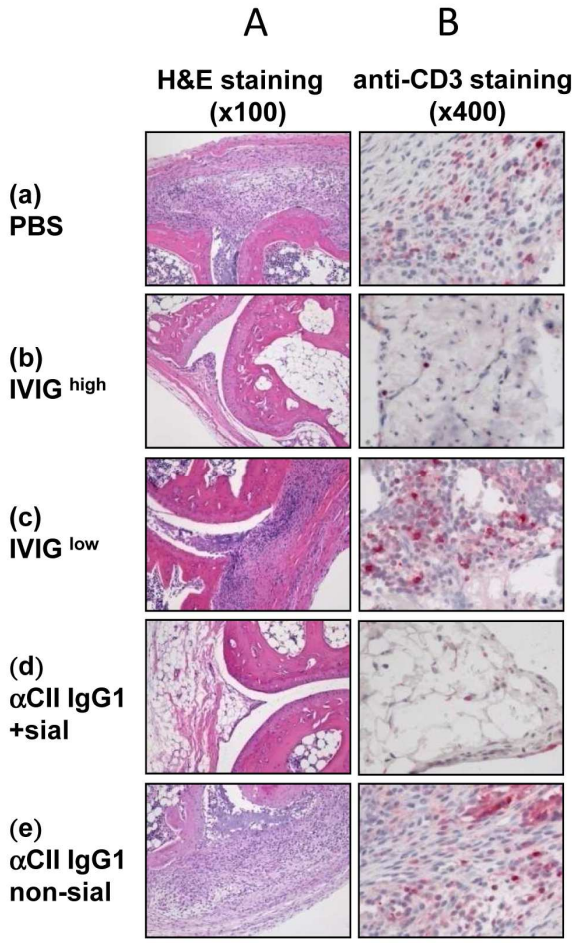


Figure 4.19 Treatment with CII-specific sialylated antibodies inhibits infiltration of cells in the feet of arthritic mice. On day 47 two mice of each group were sacrificed and paraffin sections of the ankles were stained for **(A)** hematoxylin and eosin (H&E) or **(B)** additionally co-stained with an anti-CD3 antibody.

These data clearly show that low amounts (2x5mg/kg) of *in vitro* sialylated CII-specific antibodies, but not non-sialylated CII-specific antibodies, protect from severe arthritis as efficiently as high amounts of IVIG (2x2g/kg) independently of negative signaling by RIIB.

4.3.2 Inhibition of arthritis is associated with reduced expansion of pro-inflammatory Th17 cells and antigen-specific antibodies

To define whether the protection against CIA by sialylated anti-CII antibodies resulted of modulation of the humoral immune response, the accumulation of total IgG and chicken CII-specific IgG2b and IgG2c serum antibodies were determined in the sera of all mice at day 42 by ELISA. Serum levels of CII-specific IgG2b and IgG2c antibodies were significantly reduced in mice treated with sialylated CII-specific, but not in mice treated with non-sialylated CII-specific antibodies comparable to untreated control mice. Comparing antibody titers from CII-specific-treated mice, the data revealed that mice treated with sialylated or non-sialylated CII-specific antibodies showed reduced CII-specific IgG2b and IgG2c serum antibody titers whereas only CII-specific IgG2b antibodies show a significant difference. High amounts of IVIG did not reduce CII-specific serum antibody levels significantly (Figure 4.20B+C). Total IgG titers were not affected by treatment with sialylated antibodies (Figure 4.20A).

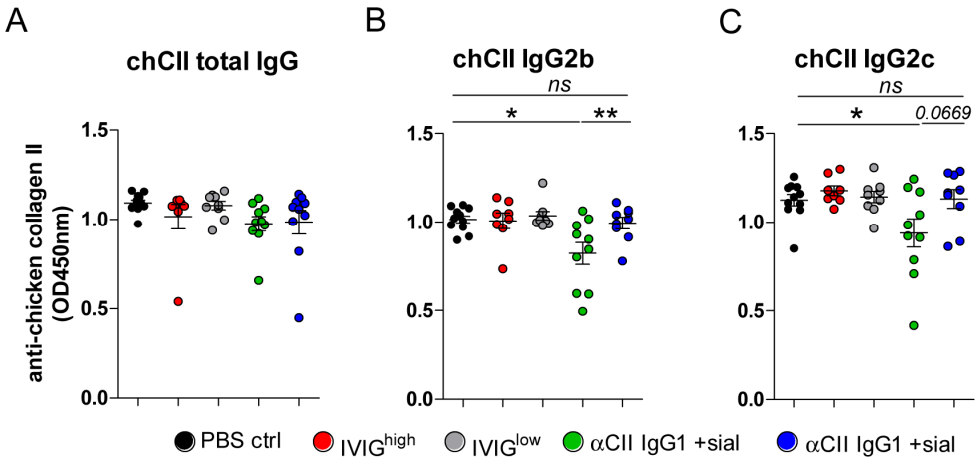


Figure 4.20 Treatment with CII-specific antibodies inhibits production of antigen-specific IgG2b and IgG2c antibodies in CIA. Sera from day 42 of all mice were analyzed for **(A)** anti-CII IgG, **(B)** anti-CII IgG2b, and **(C)** anti-CII IgG2c by ELISA. Symbols represent data from individual mice. Horizontal lines show mean values with standard error of the mean (SEM). ns $P>0.05$, * $P\leq0.05$, ** $P<0.01$, ***$P<0.001$

To investigate whether sialylated and/or non-sialylated CII-specific antibodies influence T cell responses in CIA popliteal and brachial lymph nodes were isolated from all mice at day 47 and analyzed by flow cytometry for activated CD4 T cells and pro-inflammatory Th1 and Th17 cells. The total cell number was higher in mice treated with non-sialylated CII-

specific antibodies, IVIG^{low} or PBS treated mice compared to mice treated with sialylated CII-specific antibody or high amounts of IVIG (Figure 4.21A). Flow cytometric analysis revealed that only low amounts of sialylated antibodies and high amounts of IVIG but neither non-sialylated antibodies nor low amounts of IVIG inhibited the differentiation of activated CD44$^+$CD62L$^-$CD4$^+$T cells and Th17 cells (Figure 4.21B+C). Most notably, frequencies of Th1 cells were comparably reduced but not significantly (Figure 4.21D).

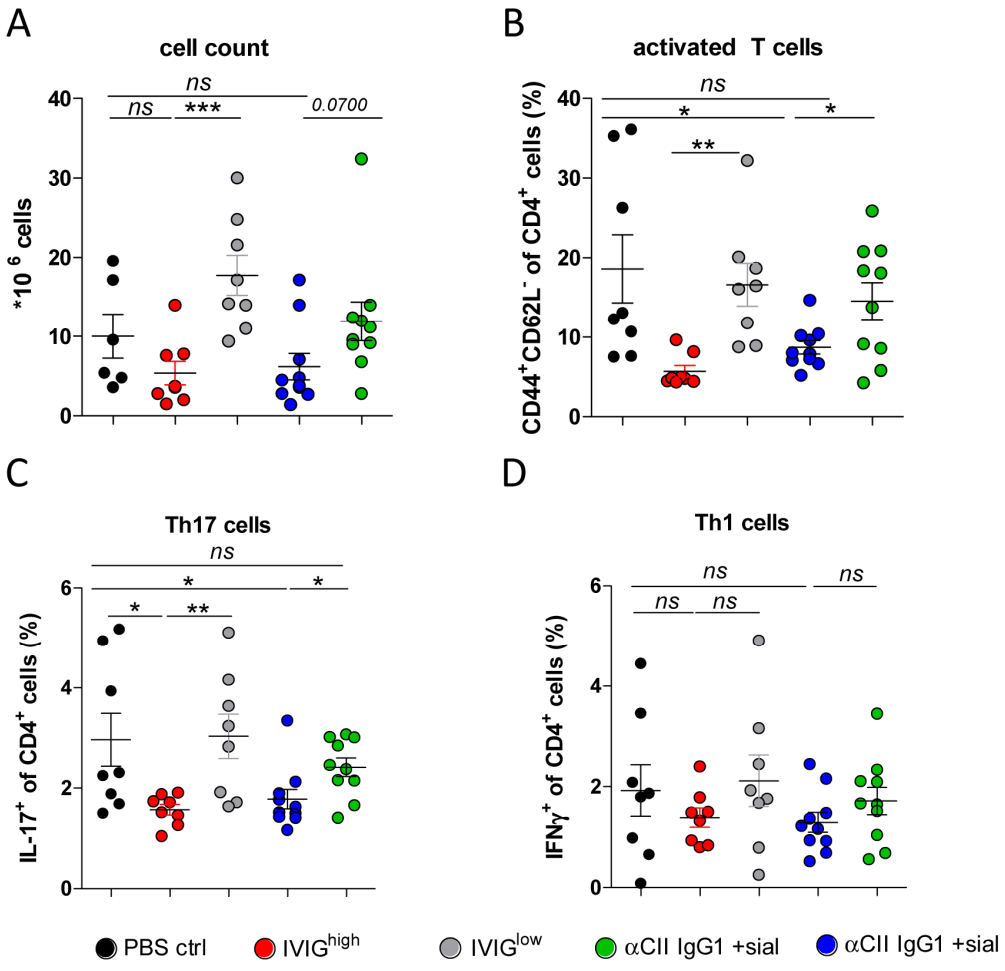


Figure 4.21 Treatment with CII-specific sialylated antibodies inhibits Th17 cell differentiation in CIA. On day 47, popliteal and brachial lymph nodes of all mice were isolated and their total cell number was counted **(A)**. Lymphocytes were characterized by flow cytometry and the percentage of **(B)** activated CD4 T cells (CD4$^+$CD44$^+$CD62L$^-$), **(C)** Th17 cells (IL-17$^+$CD4$^+$) and **(D)** Th1 cells (IFNγ$^+$CD4$^+$) was determined. Symbols represent data from individual mice. Horizontal lines show mean values with standard error of the mean (SEM). ns P>0.05, * P≤0.05, ** P<0.01, ***P<0.001

I conclude that low amounts (2 x 5 mg/kg) of sialylated but not non-sialylated antigen-specific murine IgG1 antibodies inhibit two key players of autoimmunity: pro-inflammatory Th17 cells and antigen-specific IgG2b and IgG2c antibodies.

4.4 Immune complexes containing antigen-specific sialylated antibodies do not induce IL-6 secrection of dendritic cells

Recently, it has been reported that IVIG has no direct effect on T cells, but IVIG can inhibit T cell activation by acting on antigen-presenting cells (Bayry, Lacroix-Desmazes et al. 2003; Aubin, Lemieux et al. 2010). Because antigen-specific sialylated antibodies inhibit the induction of antigen-specific inflammatory T and B cell responses (see 4.3.2), I want to further investigate the impact of immune complexes (ICs) containing sialylated antibodies on cytokine production of dendritic cells (DCs), a prerequisite for the induction of inflammatory T and B cell responses. DCs are one major source for IL-6, which together with TGFβ induces Th17 cell differentiation. Interestingly, I demonstrated that sialylated antibodies had a remarkable effect on Th17 cell differentiation *in vivo* (Figure 4.21C). Thus, the following section aimed to investigate the impact of sialylated antibodies specifically on IL-6 production of DCs *in vitro*.

To compare the influence of sialylated and non-sialylated antibodies on cytokine secretion by bone marrow derived dendritic cells (BMDC), we incubated ICs containing 10μg chicken CII and 40μg non-sialylated or sialylated anti-CII antibodies (clone M2139) 36h with wild-type BMDCs and analyzed their effects on IL-6 production of BMDCs (Figure 4.22A). ICs containing non-sialylated anti-CII antibodies strongly induced IL-6 production, which was inhibited by addition of IVIG. In contrast, sialylated IgG1 ICs were not able to induce IL-6 secretion. Accordingly, only ICs containing sialylated anti-CII antibodies failed to induce IL-6 production in BDMCs of wild-type mice. This effect is dose-dependent, i.e. mixed ICs containing sialylated and non-sialylated anti-CII antibodies with altered amounts of sialylation induced different levels of IL-6 production (Figure 4.22A).

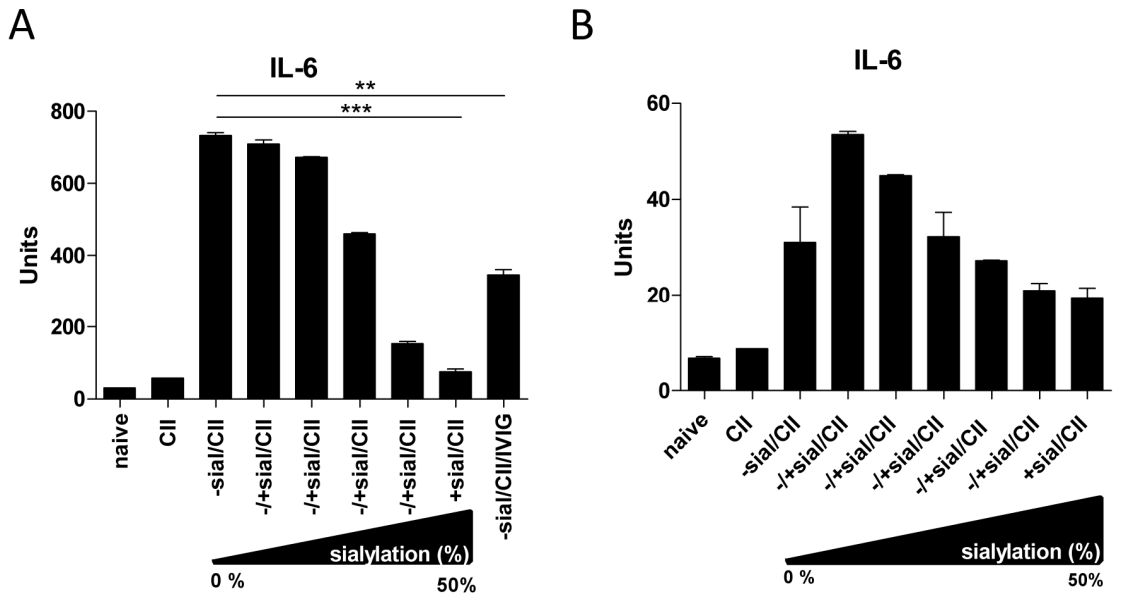


Figure 4.22 Antigen-specific sialylated antibodies inhibit IL-6 secretion of dendritic cells in a dose-dependent manner. DCs were generated from (A) C57BL/6 or (B) RIIB-/- bone marrow for 8 days with GM-CSF and IL-4 in IMDM. ICs containing sialylated or non-sialylated antibodies (40μg/ml M2139) were added with altered amounts of sialylation (from 0% sialylation up to 50% sialylation). After 24h of incubation IL-6 production in the supernatant was determined by ELISA. Bar graphs show the mean value with standard error of the mean (SEM) for each group of mice. Data are representative of four independent experiments. ns $P>0.05$, * $P\leq0.05$, ** $P<0.01$, ***$P<0.001$

Besides triggering activation of innate effector cells, FcγRs function in antigen presentation and immune-complex-mediated maturation of DCs (Smith and Clatworthy 2010). To determine whether FcγRIIB is required for the inhibitory effect of sialylated antibodies observed in wild-type BMDCs, BMDCs from RIIB-/- were incubated with anti-CII antibodies as described before and IL-6 secretion was measured 36h later. The results showed a comparable decrease in IL-6 secretion in both wild-type and RIIB-/- BMDCs in the presence of ICs containing sialylated antibodies. The effect seen in RIIB-/- BMDCs was also dependent on the amount of sialylation in the ICs (Figure 4.22B+C), indicating that the anti-inflammatory effect of sialylated antibodies on BMDC is RIIB independent.

These data point out that ICs containing sialylated antigen-specific antibodies cannot induce cytokine secretion of DC and therefore restrain inflammatory T and B cell responses and most likely contribute to an anti-inflammatory effect in autoimmune diseases. However, additional experiments showing inhibitory effects of sialylated antigen-specific antibodies on activated DCs are necessar.

5 Discussion

5.1 CD4 T helper cells and their interaction with B cells during development of lupus nephritis

T cells contribute to lupus pathogenesis by stimulating the activation and functional differentiation of autoreactive B cells, which secrete high-affinity, isotype-switched autoantibodies (Shlomchik, Craft et al. 2001). Furthermore, T cells can enhance lupus nephritis by the secretion of cytokines that promote recruitment and activation of innate immune cells such as macrophages and neutrophils (Crispin, Oukka et al. 2008).

In this work, the importance of CD4 T helper cells and their interaction with B cells during the development of SLE was investigated in RIIB-/-TLR9-/- mice, a spontaneous mouse model of SLE. Therefore, anti-CD4 or anti-CD154 antibodies were administered to deplete all existing CD4 T cells and to inhibit de novo generation of CD4 T cells or to inhibit CD40-CD154 B-T cell interaction and thereby germinal center formation, respectively. Both experiments clarify that the production of pathogenic IgG2b and IgG2c ANA and RNA-related autoantibodies as well as development of lupus nephritis in RIIB-/-TLR9-/- mice are critically dependent on the presence of CD4 T cells and B-T cell interaction. Furthermore, class switch to high affinity autoantibodies of the pathogenic isotypes IgG2b and IgG2c is highly dependent on CD4 T cell help. Our findings are consistent with the results obtained by Seery et al. demonstrating an essential role for $\alpha\beta$ T cells in the pathogenesis of lupus in another murine model of the disease (Seery, Wang et al. 1999). Moreover, my work among other studies confirmed the importance of CD40-CD154 interaction. Here, treatment with an anti-CD154 antibody resulted in absence of autoantibodies and prevention of lupus nephritis (Early, Zhao et al. 1996; Daikh, Finck et al. 1997; Kalled, Cutler et al. 1998). Thus, manipulating CD40-CD154-interaction has been a promising therapeutic target in SLE since B and T cells overexpress CD154 in patients with SLE (Desai-Mehta, Lu et al. 1996). However, clinical trials using two antibodies against human CD154 resulted in thromboembolic events and are currently in abeyance (Sidiropoulos and Boumpas 2004).

Taken together, reduction of CD4 T cell numbers or inhibition of B and T cell interaction successfully prevents autoantibody generation and therefore disease progression in a mouse model for SLE. However, inhibiting CD4 T cell differentiation or B and T cell interaction

in SLE patients is not recommended as it could lead to a systemic immune suppression, which could result in various co-morbidities, possibly induced by pathogens. Consequently, investigating the individual role of the different T cell subsets on autoantibody generation and activation of other immune cells is necessary to find beneficial treatment for lupus nephritis patients.

5.2 Th1 and Th17 cells in a murine model of systemic lupus erythematosus (SLE)

To date, glomerulonephritis, found in SLE, has been believed to be predominantly Th1 cell mediated. However, the Th1 paradigm has been challenged by the identification of pro-inflammatory Th17 cells (Tipping and Timoshanko 2005). Increasing evidence suggests that Th17 cells are crucial during development of autoimmune diseases in various experimental mouse models (Kyttaris, Zhang et al. ; Bush, Farmer et al. 2002; Nakae, Nambu et al. 2003; Komiyama, Nakae et al. 2006; Hsu, Yang et al. 2008; Kelchtermans, Schurgers et al. 2009). Aim of this thesis was to clarify the impact of Th1 as well as Th17 cells on the development of pathogenic autoantibodies and lupus nephritis in RIIB-/-TLR9-/- mice.

Analysis of the blood and secondary lymphoid organs of 6-month-old C57BL/6 and RIIB-/-TLR9-/- mice for IFNγ and IL-17A-producing $CD4^+$ T cells showed highly increased frequencies of Th1 and Th17 cells, respectively. These results led to the proposal that Th1 as well as Th17 cells are involved in either development of autoantibodies or development of nephritis or both. To identify the specific role of Th1 and Th17 cells in lupus nephritis, I crossed IFNγR1 or IL-17RA knock out mice with lupus prone RIIB-/-TLR9-/- mice. IFNγR1 encodes the ligand-binding α-chain of the heterodimeric gamma interferon receptor. IFNγ-IFNγR1 signaling is crucial to promote and maintain Th1 cell differentiation properly, for full expression of MHC II on APCs, proper B cell help and leukocyte recruitment (O'Garra 1998; Timoshanko, Holdsworth et al. 2002). IL-17RA binds four different types of IL-17, in particular IL-17A, which has been associated with autoimmunity (Yang, Chang et al. 2008; Ishigame, Kakuta et al. 2009).

Mice overexpressing IFNγ in the epidermis develop T cell-dependent lupus-like symptoms with ANA autoantibodies and kidney deposits of ICs (Seery, Wang et al. 1999). Furthermore, concentrations of IFNγ and IL-12 are increased in the serum of lupus patients, par-

ticularly those in the active stages of the disease (al-Janadi, al-Balla et al. 1993; Tokano, Morimoto et al. 1999). Analysis of RIIB-/-TLR9-/- mice deficient for IFNγR1 revealed no production of pathogenic autoantibodies of the IgG2c isotype and significantly reduced IgG2b autoantibodies for various lupus-associated antigens. Abrogation of autoantibody production was associated with inhibited plasma cell differentiation in the spleen of RIIB-/-TLR9-/-IFNγR1-/- mice. The cytokine IFNγ regulates B cell differentiation and provokes class-switch to IgG2c antibodies. The known impact of IFNγ-producing Th1 cells on B cell differentiation and IgG antibody generation explains data obtained from RIIB-/-TLR9-/-IFNγR1-/- mice (Snapper and Paul 1987). The significant decrease of IgG2b autoantibodies observed in RIIB-/-TLR9-/-IFNγR1-/- mice might depend on reduced numbers of TGFβ-producing CD4 T cells that provoke class-switch to IgG2b (Stavnezer 1996; Stavnezer 1996). Inhibition of pathogenic IgG2b and IgG2c autoantibody development prevents IC formation and their deposition in the kidney, which is a prerequisite for lupus nephritis. Indeed, none of the RIIB-/-TLR9-/-IFNγR1-/- mice developed lupus nephritis and died until the age of nine months. Thus, elevated autoantibody formation in RIIB-/-TLR9-/- compared to wild-type mice and RIIB single knock out mice can be explained by increased Th1 cell differentiation. The importance of IFNγ or IFNγR has been confirmed in several other mouse models for lupus nephritis (Jacob, van der Meide et al. 1987; Peng, Moslehi et al. 1997; Haas, Ryffel et al. 1998; Schwarting, Wada et al. 1998).

Besides its effect on B cells, Th1 cells secrete cytokines, which influence the immune response. IL-2, for example, produced by Th1 cells, induces T cell proliferation and additional secretion of cytokines. IFNγ activates macrophages, inhibits Th2 cell differentiation and activates MHC molecules on APCs (Tipping and Holdsworth 2006). GM-CSF secreted by Th1 cells induces macrophage differentiation in the bone marrow. Furthermore, Th1 cells secrete chemokines such as MCP-1 (monocyte chemotactic protein-1), which recruits macrophages to the site of infection (Timoshanko, Holdsworth et al. 2002; Timoshanko, Kitching et al. 2005). In an experimental mouse model for inducible nephritis, IFNγ production by intrinsic renal cells and bone marrow-derived cells are required for full development of glomerulonephritis. Timoshanko et al. suggest that IFNγ from BM-derived cells in secondary lymphoid tissue is important for development of the Th1 response to nephritogenic antigens, but that intrinsic renal cells are an important source of IFNγ for the effec-

tor response in the kidney (Timoshanko, Holdsworth et al. 2002). Similarly, RIIB-/-TLR9-/-IFNγR1-/- mice displayed decreased frequencies of macrophages and neutrophils in the spleen indicating less pro-inflammatory potential.

Interestingly, not only Th1 cell but also Th17 cell frequencies are increased in RIIB-/-TLR9-/- mice compared to RIIB-/- and wild-type mice. The role of Th17 cells and their cytokines during autoimmunity has been widely discussed and there is growing evidence for the participation of IL-17 in human SLE: (1) IL-17A levels are elevated in the sera of patients with lupus and IL-17 positive $CD4^+$ T cells are present in SLE patients (Wong, Ho et al. 2000; Wong, Lit et al. 2008; Cheng, Guo et al. 2009); (2) IL-23 is upregulated in the plasma and in peripheral blood mononuclear cell (PBMC) mRNA of SLE patients (Huang, Hua et al. 2007; Cheng, Guo et al. 2009); (3) stimulation with IL-17A resulted in higher production of anti-ds-DNA autoantibodies and IL-6 in PBMCs from lupus nephritis patients (Dong, Ye et al. 2003) and (4) there are increased numbers of double negative ($CD4^-$ $CD8^-$) T cells producing IL-17A that infiltrate the kidneys of patients with lupus nephritis (Crispin, Oukka et al. 2008). However, while some studies confirm elevation of IL-17A in SLE patients (Wong, Lit et al. 2008), other studies fail to correlate IL-17A increase with disease activity (Zhao, Pan et al. ; Cheng, Guo et al. 2009).

Studies in lupus prone autoimmune mice provide additional evidence for the participation of the Th17 pathway in the pathogenesis of SLE. Splenocytes from SNF1 mice show enhanced IL-17A production *ex vivo* and IL-17A-producing T cells were demonstrated to infiltrate the kidneys of these mice (Kang, Ecklund et al. 2009). Furthermore, Zhang et al. reported increased IL-17 expressing $CD3^+CD4^-CD8^-$ T cells, their presence in the kidneys of lupus-prone mice with active nephritis and the ability of IL-23-treated lymphocytes from lupus prone mice to induce nephritis in Rag-1-/- mice (Zhang, Kyttaris et al. 2009).

In RIIB-/-TLR9-/- mice I detected increased Th17 cell frequencies in the blood, secondary lymphoid organs and in renal tissues compared to wild-type mice. Cryosections of kidneys of wild-type mice display no IC deposition and therefore no macrophage or T cell infiltration. In contrast, RIIB-/-TLR9-/- mice exhibit IC deposition resulting in macrophages recruitment and tissue destruction. In addition, a high number of $IL\text{-}17A^+$ CD4 T cells were present in kidneys of RIIB-/-TLR9-/- mice with active nephritis. These observations provide a link between IL-17A and immunopathology in this murine lupus model. Th17 cells trigger pro-inflammatory immune responses by secreting pro-inflammatory cy-

tokines, such as IL-6 and IL-22. *Ex vivo* cultured splenocytes from RIIB-/-TLR9-/- mice produced increased titers of IL-6 and IL-22 compared to wild-type splenocytes. IL-6 induces differentiation of Th17 cells together with TGFβ and therefore promotes and maintains Th17 cell differentiation. Additionally, Th17 cells themselves produce IL-6 and consequently promote additional pro-inflammatory responses by recruitment of other leukocytes (Romano, Sironi et al. 1997). IL-22 is produced mainly by T helper cells and its receptor is expressed only on tissue-resident cells, including fibroblasts or epithelial cells. Together with IL-17, IL-22 may promote tissue inflammation and tissue destruction (Bettelli, Oukka et al. 2007). However, these data support but do not prove a role for IL-17A in renal lupus.

To clarify whether the interaction between IL-17A and its receptor IL-17RA plays a role in the development of lupus nephritis, I crossed RIIB-/-TLR9-/- lupus prone mice with IL-17RA knock out mice. Because of the importance of autoantibodies for the development of lupus nephritis I first analyzed autoantibody generation in RIIB-/-TLR9-/-IL-17RA-/-. IL-17RA deficiency in RIIB-/-TLR9-/- mice did not significantly influence total IgG and IgM production or autoantibody generation for various lupus-associated antigens. In accordance, the number of ICs stained in kidney sections of RIIB-/-TLR9-/-IL-17RA-/- mice was similar to RIIB-/-TLR9-/- mice. However, these findings were surprising as Hsu et al. showed significant lower amounts of IgGs specific for DNA or histones in IL-17RA deficient BXD2 autoimmune mice compared to control BXD2 mice (Hsu, Yang et al. 2008). Furthermore, Kyttaris et al. reported recently that IL-23R deficiency in lupus prone B6.MRL-Faslpr mice resulted in reduced Th17 cells numbers and therefore reduced production of inflammatory anti-DNA autoantibodies and less IC depostition in the kidneys (Kyttaris, Zhang et al. 2010). Although I did not see an effect of IL-17RA deficiency on autoantibody production in RIIB-/-TLR9-/- mice, significantly less RIIB-/-TLR9-/-IL-17RA-/- mice developed proteinurea or died of nephritis. Thus, IL-17A-IL-17RA signaling is clearly important during development of lupus nephritis. Flow cytometric analysis indicated that there were significantly lower percentages of GC cells in the spleen and lymph nodes of 6-month-old RIIB-/-TLR9-/-IL-17RA-/- than in age-matched RIIB-/-TLR9-/- mice. By contrast, the percentage of marginal zone B cells ($CD21^{hi}CD23^{lo/neg}B200^{+}$) was higher in RIIB-/-TLR9-/-IL-17RA-/- than in age-matched RIIB-/-TLR9-/- mice, suggesting reduced migration from the marginal zone to the follicular region. These results demonstrate that IL-17 signaling is essential during the formation of GCs. IL-17-dependent GC

formation was also reported in BXD2 autoimmune mice (Hsu, Yang et al. 2008). Hsu et al. showed that (1) IL-17 can induce GC development in vivo, (2) is required to stabilize developed GCs and (3) observed reduced GC formation in BXD2 IL-17RA-/- mice. They propose that B cells and $CD4^+$ T cells exposed to IL-17 have a diminished chemotactic response to both CXCL12 and CXCL13, ligands for CXCR4 and CXCR5, respectively. This is thought to be due to higher expression of Rgs13 and Rgs16, known inhibitors of CXCR4 and CXCR5. Ordinarily, B cells migrate between the dark and light zone of the GC under the direction of CXCL12 and CXCL13. Somatic hypermutation (SHM) and proliferation are concentrated in the dark zone. In the light zone, B cells are subjected to selection resulting in apoptotic death or return to the dark zone for additional rounds of mutation or differentiate into antibody-secreting cells that emigrate from the GC, possibly under the direction of the CXCL12-CXCR4. B cells in the GCs of BXD2 mice, however, are now proposed to have diminished migration capacities because of their exposure to IL-17 (Hsu, Yang et al. 2008). This may result in less emigration and more rounds of mutation, indicated by the greater number of mutations per V gene observed in the BXD2 mouse model. Plasma cells eventually emigrate from the BXD2 GCs and are enriched for self-reactive cells (Tarlinton 2008). Thus, although the level of autoantibodies produced in RIIB-/-TLR9-/-IL-17RA-/- mice is similar in age-matched RIIB-/-TLR9-/- mice the quality of these antibodies is most likely different due to reduced GC formation in these mice. A higher mutation rate and therefore development of higher affinity antibodies in RIIB-/-TLR9-/- compared to antibodies from RIIB-/-TLR9-/-IL-17RA-/- mice could lead to higher pathogenicity of these autoantibodies.

The role of IL-17 on B cells has been investigated lately. Doreau et al. reported the influence of IL-17 together with BAFF on B cell biology and the pathophysiology of SLE showing (1) IL-17 and BAFF drive human B cell survival, (2) IL-17 and BAFF can substitute for TLR signaling and allow B cell proliferation and differentiation and (3) serum from SLE patients with high IL-17 and BAFF concentrations promote survival, proliferation and differentiation of peripheral B cells (Doreau, Belot et al. 2009). In addition, BAFF-transgenic mice display a lupus-like phenotype characterized by deregulated B cell activation and maturation (Batten, Groom et al. 2000). Another group recently demonstrated that Th17 cells can induce B cell proliferation and isotype class switching *in vitro* and *in vivo* (Mitsdoerffer, Lee et al. 2010). My data provide evidence that IL-17 signaling is involved in the pathogenesis of lupus nephritis in RIIB-/-TLR9-/- mice. However, no direct influence

on autoantibody titers can be detected. Probably, the importance of IL-17RA during GC formation influences the pro-inflammatory potential of the autoantibodies in RIIB-/-TLR9-/- mice.

Furthermore, IL-17RA deficiency abolished macrophage infiltration in the kidneys of RIIB-/-TLR9-/- mice thereby inhibiting a pro-inflammatory response in the kidneys of RIIB-/-TLR9-/-IL-17RA-/- mice which results in lower proteinurea incidence and a decreased mortality rate. IL-17 induces granulopoiesis both *in vitro* and *in vivo* (Fossiez, Djossou et al. 1996; Schwarzenberger, La Russa et al. 1998). Moreover, IL-17R signaling is able to induce the production of TNFα by macrophages and is critical for CXC chemokine generation and induction of G-CSF (Jovanovic, Di Battista et al. 1998; Ye, Rodriguez et al. 2001). Generally it is known that G-CSF is required for ongoing neutrophil production in response to infections (Dale, Liles et al. 1995). Thus, deleting IL-17RA in RIIB-/-TLR9-/- mice most likely prevents renal tissue destruction by inhibiting macrophage infiltration into the kidney.

Deleting IFNγR1 or IL-17RA in RIIB-/-TLR9-/- mice made clear that both CD4 T cell subsets are involved in the pathogenesis of lupus nephritis. IFNγR1 deletion in lupus prone mice prevents disease onset by complete inhibition of autoantibody development. Th17 cells most likely influence GC formation and thereby promote affinity maturation of autoantibodies. Additionally, deletion of IL-17RA inhibited macrophage infiltration in renal tissues (see Figure 5.1). Hence, a reagent that targets both Th1 and Th17 cells might actually have advantages over reagents that target only one T cell subset. One such reagent can be an antibody binding p40, a subunit shared by IL-12 and IL-23. IL-12 induces IFNγ in T cells and IL-23 maintains Th17 cell differentiation. Moreover, blockade of a proper Th17 cell differentiation not only reduces IL-17 expression but also additional pro-inflammatory cytokines produced by Th17 cells which promote autoantibody production (e.g. IL-21) or tissue destruction (e.g. IL-22).

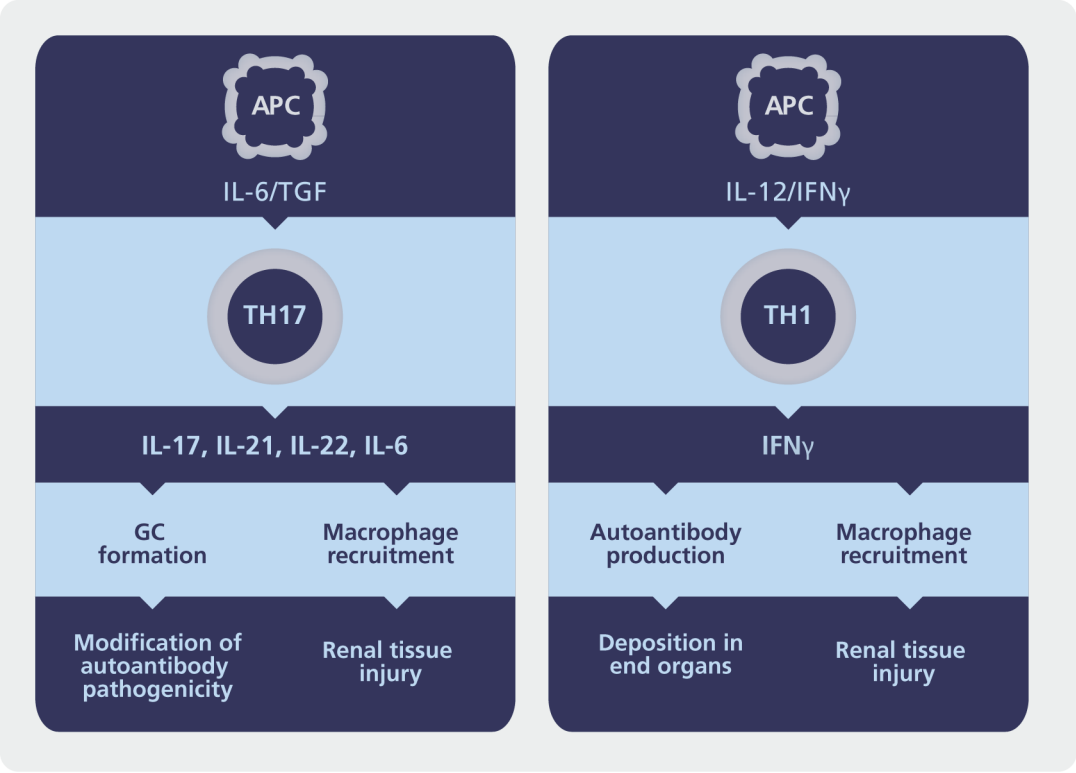


Figure 5.1 Increased frequencies of Th17 as well as Th1 cells promote lupus nephritis in RIIB-/-TLR9-/- mice. Upon IL-6 and TGFβ or IL-12 and IFNγ stimulation by antigen-presenting cells (APCs) Th17 or Th1 cells, respectively, can differentiate. Th17 cells produce various cytokines, which promote germinal center (GC) formation thereby changing the pathogenicity of autoantibodies and macrophage recruitment, which leads to renal tissue destruction. IFNγ produced by Th1 cells enhances pathogenic autoantibody production, which form immune complexes that deposit in the kidney. In addition, IFNγ can also promote macrophage recruitment and therefore renal tissue injury. TGFβ-Transforming growth factor β, IFNγ-Interferon γ

5.3 Th17 cells in a murine model for rheumatoid arthritis (RA)

IL-17 promotes inflammation by enhancing IL-1β, TNFα, IL-6, receptor activator of nuclear factor-κB ligand (RANKL), as well as chemokines such as macrophage inflammatory protein (MIP)-2 and IL-8 (Fossiez, Djossou et al. 1996; Laan, Cui et al. 1999; Hwang, Kim et al. 2004). Increased Th17 cell differentiation during inflammation in RA could therefore induce bone destruction and infiltration of neutrophils or macrophages into the synovium. There is considerable evidence that IL-17 contributes to the inflammation associated with RA: (1) IL-17 is produced in RA synovial membrane cultures (Kotake, Udagawa et al. 1999), (2) IL-17 titers in the synovial fluid of patients with RA are increased (Chabaud,

Durand et al. 1999), (3) in a murine mouse model for RA, namely CIA, an IL-17 neutralizing antibody reduced symptoms of arthritis (Lubberts, Koenders et al. 2004) and (4) mice deficient for IL-17 or IL-17R were found to be less susceptible for induction of CIA (Nakae, Nambu et al. 2003). Although Th17 cells and IL-17 have been studied extensively in previous years, much of their effector functions remain unknown. To determine the relative role of Th17 cells in RIIB-/- mice regarding the establishment and maintenance of autoimmune inflammation, we have analyzed the cellular and humoral immune response after the induction of CIA.

The cytokine IL-23 promotes proliferation and maintenance of Th17 cells and is therefore essential for a full Th17 cell response (Langrish, Chen et al. 2005). I used an IL-23R blocking antibody to effectively and specifically diminish Th17 cell proliferation during induction of CIA. For this experiment RIIB-/- mice were used because of increased Th17 cell differentiation compared to C57BL/6 (data not shown) and enhanced susceptibility to CIA (Yuasa, Kubo et al. 1999). Preventive treatment with anti-IL-23R antibody (starting from the day of the immunization) reduced disease severity as well as disease incidence. CIA is thought to depend in part on collagen-specific humoral immunity and as mentioned before Th17 cells are most likely involved in B cell biology. Treatment of RIIB-/- mice with an anti-IL-23R antibody during CIA, however, resulted in equal CII-specific IgG autoantibody titers as found in untreated RIIB-/- mice. Only total IgG and IgM titers were affected by anti-IL23R blocking, whereas IgG antibody titer is reduced and IgM antibody titer is increased in IL-23R-treated RIIB-/- mice compared to untreated RIIB-/- mice. Contrary to IgG antibodies, IgM antibodies do not correlate with disease activity but are known to exhibit protective capacities in autoimmunity (Forger, Matthias et al. 2004).

Increased disease severity of CIA can be associated with an increased CFA-induced expansion of immature $MacI^+$ macrophages and $MacI^+Gr\text{-}1^+$ neutrophils (Matthys, Vermeire et al. 1999). Here, prevention of a proper Th17 cell differentiation by IL-23R blocking antibodies during induction of CIA inhibits the expansion of $MacI^+$ macrophages and $MacI^+Gr\text{-}1^+$ neutrophils in the spleen. The lower numbers of neutrophils present in the spleen may provide an explanation for the reduced disease severity because of less neutrophil infiltration in the joints. The reduced granulopoiesis observed in IL-23R-treated RIIB-/- mice could result from reduced production of hematopoietic cytokines, such as GM-CSF, IL-6 and IL-12 (Kelchtermans, Schurgers et al. 2009). Analysis of CD3, CD4 and CD8 T cells in

the lymph nodes revealed no difference between IL-23R-treated and untreated RIIB-/- mice after induction of CIA. Only activated CD4 T cells ($CD4^+CD44^+CD62L^-$) were slightly decreased in IL-23R-treated RIIB-/- mice compared to untreated RIIB-/- mice.

During RA, activation of CD4 T cells is often linked to pathological bone resorption and Th17 cells most likely promote this process. Osteoclasts are multinucleated cells of the monocyte/macrophage lineage that are responsible for bone resorption. Together with osteoblasts they control the balance between bone formation and bone resorption to remodel the skeleton. Several signals are important to keep this system in balance whereas RANKL is the key osteoclastogenic cytokine expressed by mesenchymal cells and T cells, indicating that RANKL bridges the skeletal and immune system. During RA, osteoclastogenesis promotes bone resorption. Osteoclast activity is mediated by RANKL, and IL-17 is known to be a potent stimulator of osteoclastogenesis through induction of RANKL (Kotake, Udagawa et al. 1999; Lubberts, van den Bersselaar et al. 2003; Sato, Suematsu et al. 2006). In contrast, Th1 cells inhibit osteoclastogensis via IFNγ. I could show that RIIB-/- mice with diminished Th17 cell differentiation, but regular Th1 cell differentiation, exhibit ameliorated disease symptoms and less arthritic incidents. In line, it is known that IFNγR -/- mice display exacerbated CIA (Manoury-Schwartz, Chiocchia et al. 1997; Vermeire, Heremans et al. 1997). Based on these observations, the IL-23–IL-17 axis inducing Th17 cells, rather than the IL-12–IFN-γ axis inducing Th1 cells, is critical for the development of autoimmune arthritis. Thus, my data together with previous studies by others suggest that Th17 facilitate local inflammation by recruiting and activating immune cells (Sato, Suematsu et al. 2006; Kelchtermans, Schurgers et al. 2009). Th17 cells themselves or the activated immune cells can enhance RANKL expression, which promotes ostecolastogenesis.

Hence, Th17 cells most likely are not only important during the onset of RA, but also during the destruction phase of arthritis characterized by the T cell-mediated activation of osteoclastogenesis. As a result, I hypothesize Th17 cells as a powerful therapeutic target in rheumatoid arthritis. Antibodies directed against IL-17 or IL-23 or their receptors most likely ameliorate the pathogenesis of RA.

5.4 Sialylated antigen-specific IgG antibodies prevent from severe arthritis

Recent publications report that IgG antibodies trigger pro- or anti-inflammatory immune responses depending on their glycosylation pattern (Kaneko, Nimmerjahn et al. 2006; Kaneko, Nimmerjahn et al. 2006; Anthony, Wermeling et al. 2008; Nimmerjahn and Ravetch 2008). The anti-inflammatory effect of intravenously injected immunoglobulins (IVIG) is based on these findings. As mentioned before, IVIG is successfully used to treat a variety of acute and chronic autoimmune disorders (Sullivan, Kopecky et al. 1990; Olopoenia, Young et al. 1997; Negi, Elluru et al. 2007; Siberil, Elluru et al. 2007). Nevertheless, IVIG is gained from pooled serum of thousands of donors bearing the risk of increased susceptibility to pathogens and even the transfer of allergies. Furthermore, due to the small effector-mediating fraction of sialylated IgGs high doses of IVIG must be transferred into patients (Nimmerjahn and Ravetch 2008). Consequently, the unspecific systemic immunosuppression can cause side effects, such as headache and fatigue. Despite beneficial effects of IVIG in various autoimmune diseases, there is still a wide range of chronic autoimmune disorders that exclude the usage of IVIG therapy. In our laboratory we were able to show that *in vivo* generated antigen-specific sialylated IgG antibodies protect from subsequent inflammatory T cell dependent immune responses. De-sialylation of these *in vivo* generated antigen-specific sialylated IgG antibodies abrogates their protective potential. Furthermore, the total amounts of antigen-specific antibodies needed to reduce pro-inflammatory effects were drastically reduced compared to the high doses of IVIG required to treat autoimmune diseases (PhD thesis of Constanze Heß). Based on these findings I aimed to investigate the effect of sialylated antigen-specific IgG antibodies in a preclinical autoimmune mouse model for RA.

CIA is an autoimmune mouse model for RA where collagen type II (CII) is the defined arthritic phenotype causing self-antigen. To investigate the anti-inflammatory potential of antigen-specific sialylated IgG antibodies, two CII-specific IgG antibodies were cloned and *in vitro* sialylated. The V-region sequences of these CII-specific antibodies are published and a specific mixture of different anti-CII antibodies is known to efficiently induce arthritis (Iribe, Kabashima et al. 1988; Mo and Holmdahl 1996; Ito, Ueda et al. 1997). The glycosylation profile of antibodies detected in patients with RA defines their pro-inflammatory effector properties due to more than 2/3 of the Fc-glycans being in the G0 glycoform

(Parekh, Dwek et al. 1985; Parekh, Dwek et al. 1988; Huhn, Selman et al. 2009; Scherer, van der Woude et al. 2010). The cloned murine IgG1 antibodies produced in HEK293T cells did not contain any sialylated Fc-glycans. To compare the inflammatory potential of sialylated and non-sialylated CII-specific antibodies in CIA part of these CII-specific antibodies were *in vitro* sialylated and finally contained about 50% sialylated Fc-glycans after enzymatic glycosylation, which did not affect antigen-reactivity.

To evaluate the impact of IVIG, non-sialylated CII-specific or sialylated CII-specific IgG antibodies on the development of CIA, IVIG or antigen-specific antibodies were administered before and after immunization with chicken CII in *M.th.*-enriched CFA. IVIG was administered in two different doses: $IVIG^{high}$ or $IVIG^{low}$. Total amounts of sialylated antibodies contained in $IVIG^{low}$ or sialylated CII-specific IgGs was comparable. A mixture of CII-specific antibodies was used to enhance the possibility of IC formation with CII *in vivo*. The transfer of sialylated, but not non-sialylated IgG antibodies, prevents from antigen-specific induction of CIA. I could point out that enzymatic glycosylation of antigen-specific antibodies is crucial to switch their effector function to trigger anti-inflammatory effects and thereby protect from inflammatory immune responses. Furthermore, I made clear that antigen-specific IgG antibodies could substitute the immunosuppressive effect of IVIG, but in an antigen-specific manner. By contrast, low dose treatment with IVIG does not prevent from development of arthritis supporting the observations that IVIG can only exert anti-inflammatory activity when administered in high doses. Inhibition of CIA in mice treated with sialylated CII-specific IgG antibodies or $IVIG^{high}$ was associated with reduced cell infiltration, bone destruction, pro-inflammatory Th17 cell response and antigen-specific IgG2b and IgG2c antibody responses. In contrast, Th1 cell differentiation was not affected by treatment with sialylated CII-specific IgG antibodies or $IVIG^{high}$. Increased bone destruction and expansion of Th17 cells but not Th1 cells in arthritic mice is in line with the knowledge that Th17 cells promote osteoclastogenesis, whereas Th1 cells inhibit osteoclastogenesis. These data clearly show a beneficial effect of antigen-specific sialylated IgG antibodies with up to 100-fold less amount of sialylated IgGs compared to total amount of sialylated antibodies in $IVIG^{high}$ in treating arthritis. Treatment with $IVIG^{low}$ containing comparable amounts of sialylated unspecific IgGs did not show reduced disease severity, cell infiltration or reduced Th17 cell differentiation indicating an antigen-specific anti-inflammatory effect of the CII-specific sialylated IgGs responsible for disease reduction. However, a clear proof that this protective effect is dependent on antigen-specificity

remains to be done and thus mice have to be treated with antigen-unspecific monoclonal sialylated IgG1 antibodies during CIA as a control in the future.

In summary, my data are the first to show that low dose of antigen-specific sialylated IgG1 antibodies are sufficient to inhibit the development of CIA via inhibiting both arms of the adaptive immune system independently of FcγRIIB: autoantibody production and Th17 cell differentiation. Existing treatments of arthritis inhibit the immune system systemically and have several side effects such as the increased susceptibility to infections. Our findings may provide the basis for the development of more specific strategies to abolish autoimmune diseases with known autoantigens by inhibiting the induction of the pro-inflammatory adaptive immune system with sialylated antigen-specific IgG antibodies.

5.5 Immune complexes containing sialylated IgG antibodies mediate antigen-specific tolerance

In the previous section I discussed the effect of sialylated antigen-specific IgG antibodies on the development of arthritis. Reduced activated CD4 T cells, Th17 cells and CII-specific IgG2b and IgG2c antibodies led to the question whether ICs containing sialylated antigen-specific IgG antibodies have a direct or indirect effect on B and T cells. Central to the pathogenic loop that maintains the ongoing autoantibody production in autoimmune diseases are APCs such as DCs. APCs phagocytose self-antigens and present them to CD4 T cells, which in turn provide the necessary help to sustain autoantibody production. Recently, it has been published that high amounts of IVIG can inhibit maturation of APCs (DCs) by a yet unknown mechanism independently of RIIB (Bayry, Lacroix-Desmazes et al. 2003; Bayry, Lacroix-Desmazes et al. 2003; Aubin, Lemieux et al. 2010) indicating that IVIG indirectly influences the development of pro-inflammatory B and T cells. Low amounts of ICs containing sialylated antigen-specific IgGs might be even more efficient in their ability to cross-link unknown inhibitory surface receptors on DCs compared to monomeric sialylated IgGs contained in IVIG.

To investigate the tolerogenic effect of CII-specific IgG antibodies I established an *in vitro* culture system using bone marrow derived DCs (BMDCs) and measured their IL-6 cytokine production as indicator of DC activation. Both wild-type C57BL/6 and RIIB-/- BMDCs were able to produce IL-6 in high amounts upon incubation with ICs containing

non-sialylated IgG antibodies while incubation with ICs containing sialylated IgG antibodies failed to induce IL-6 production of BMDCs. Moreover, this effect was dose dependent, i.e. the higher the amount of sialylated antibodies within the ICs the lower IL-6 cytokine production. DCs are an important source for the pro-inflammatory cytokine IL-6, which promotes Th17 cell differentiation and is crucial for the induction of B lymphocyte maturation into plasma cells and augmentation of antibody secretion (Bettelli, Carrier et al. 2006; Rabe, Chalaris et al. 2008). Furthermore, IL-6 has been described to promote the pathogenesis of autoimmune diseases as the deletion of IL-6 in lupus prone mice reduced renal macrophage infiltration and autoantibody production, thereby delaying the onset of lupus nephritis (Cash, Relle et al. 2010). My findings indicate that sialylated IgG antibodies could indirectly inhibit T and B cell responses by preventing IL-6 secretion of APCs. (Figure 5.2) This is in line with reduced Th17 cell differentiation and IgG autoantibody development observed in CIA mice treated with antigen-specific sialylated IgG antibodies. These *in vitro* experiments could explain the anti-inflammatory effect of *in vivo* administered antigen-specific sialylated IgG antibodies before onset of arthritis.

However, sialylated ICs might also directly influence effector immune cells, like macrophages, B and T cells, by a yet unknown receptor. One possible candidate is CD22, an inhibitory co-receptor of the BCR, which plays a crucial role in modulating B cell signaling. It is a member of the sialic acid-binding immunoglobulin-like lectin (Siglec) family of adhesion molecules (Sgroi, Varki et al. 1993) and specifically binds to sialic acid attached in α-2,6-linkage to galactose residues on glycoproteins (Powell, Sgroi et al. 1993; Powell, Jain et al. 1995; Walker and Smith 2008). Besides its potential to provide positive signals to the B cell, CD22-deficient mice have been demonstrated that CD22 functions predominantly as an inhibitory receptor *in vivo* (O'Keefe, Williams et al. 1996; Otipoby, Andersson et al. 1996; Nitschke, Carsetti et al. 1997). Furthermore, it has been shown that defects in CD22 might predispose to autoimmunity, likely in a similar fashion to RIIB (Pritchard and Smith 2003). Although the role of CD22 is still being discussed, there are implications for CD22 playing a key part in establishing the BCR threshold and the generation of B cell hyper- and hypoactivity. Thus, investigating the role of CD22 in mediating anti-inflammatory effects by antigen-specific sialylated IgG antibodies might be a challenge in the future (Figure 5.2).

It is well recognized that the inhibitory RIIB is an important mediator of the anti-inflammatory effect of IVIG (Anthony, Wermeling et al. 2008). However, in the present

work, the effect of sialylated CII-specific IgGs is similar between BMDCs derived from wild-type mice or RIIB-/- mice, indicating that RIIB might not play an important role in the monoclonal sialylated antibody-mediated inhibition of antigen-presentation. A yet unknown receptor on APCs could be responsible for the RIIB-independent effects observed in this work. However the exact RIIB-independent mechanism behind the inhibitory effect of sialylated antigen-specific antibodies remains to be elucidated.

In brief, the data obtained in CIA mice treated with sialylated or non-sialylated antigen-specific IgG antibodies *in vivo* and the data obtained from BMDCs *in vitro* suggest sialylated antigen-specific IgG antibodies as promising therapeutic tools to substitute IVIG during treatment of autoimmune diseases with known autoantigens.

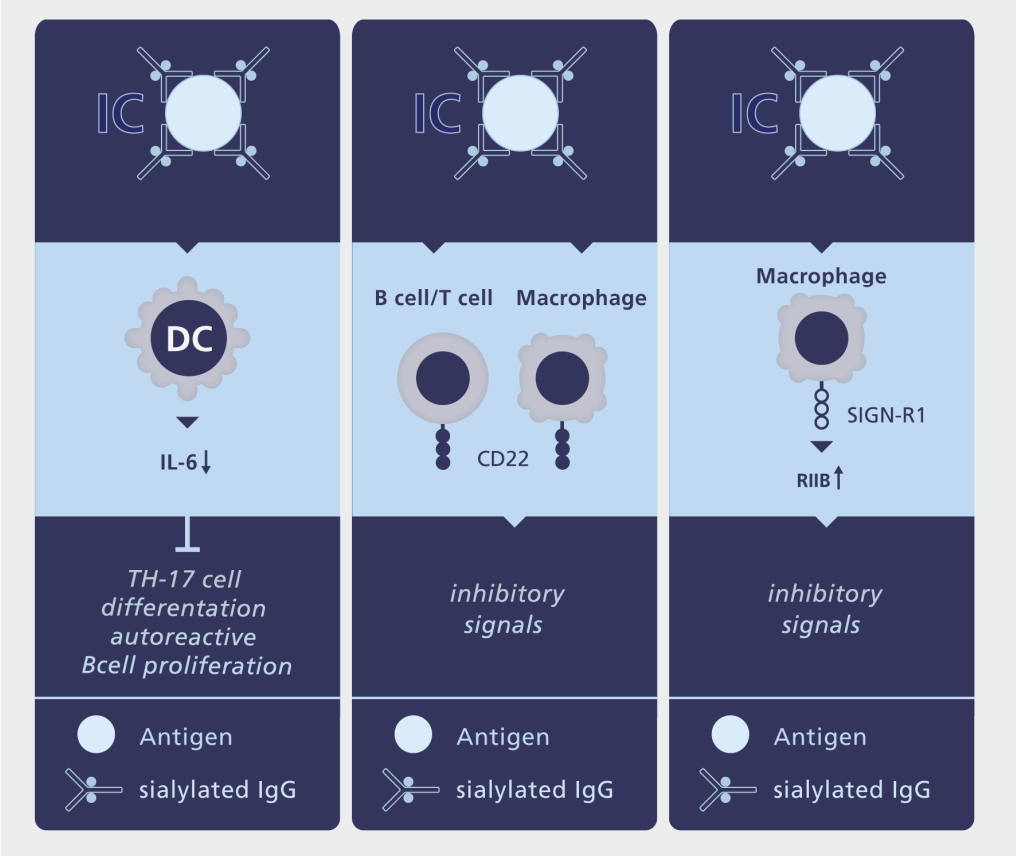


Figure 5.2 Antigen-specific sialylated IgG antibodies most likely mediate anti-inflammatory immune responses through different mechanisms. Immune complexes (ICs) containing sialylated antibodies bind to dendritic cells (DCs) through a yet unknown receptor and thereby inhibiting IL-6 production. Reduced IL-6 secretion prevents Th17 cells differentiation and B cell proliferation thereby possibly preventing inflammatory responses (left). A second possible pathway is shown in the middle row: ICs bind to the CD22 receptor, expressed on different cell types and thereby inducing inhibitory signals. Finally, ICs bind to SIGN-R1 on splenic macrophages, which leads to an upregulation of FcγRIIB (RIIB) that promotes inhibiting signals (right).

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Abbreviation

AgeI	restriction enzyme
ANA	anti nuclear antigens
APC	antigen presenting cell
Asn	Asparagine
BCR	B cell receptor
BSA	Bovine serum albumin
BsIWI	restriciton enzyme
C57BL/6	wild type mouse model
CCL	chemokine ligand, inducing effector cell chemotaxis
CCR	chemokine receptor
CD	cluster of differentiation
CD	cluster of differentiation
CFA	complete freund´s adjuvant
CSR	class switch recombination
CTL	cytotoxic T lymphocyte
CXCR	chemokine receptor
Cy5	carboxymethylindocyanin-5
Cy5	carboxymethylindocyanin-5
CII	collagen type II
DMEM	Dulbecco/Vogt modified Eagle's minimal essential medium
DNA	desoxyribonucleic acid
ds	double strand
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EndoS	Endoglycosidase
EtBr	ethidium bromide
Fab	antibody variable region; antigen binding fragment
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

FITC . . . . . fluoresceinisothiocyanat
FSC . . . . . forward scatter
GC . . . . . germinal center
GlcNac . . . . . N-acetylglucosamine
Hek293T . . . . . human embryonic kidney cells with T-antigen
HLA . . . . . human leukocyte antigen
HRP . . . . . horse radish peroxidase
HSC . . . . . hematopoetic stem cell
IC . . . . . immune complex
IFA . . . . . incomplete freund´s adjuvant
IFNγ . . . . . interferone γ
Ig . . . . . immunglobulin
IgH . . . . . BCR heavy chain
IgL . . . . . BCR light chain
IL . . . . . interleukin
i.p. . . . . intra peritoneal
IMDM . . . . . Iscove's Modified Dulbecco's Medium
ITAM . . . . . immunoreceptor tyrosine-based activation motif
ITIM . . . . . immunoreceptor tyrosine-based inhibitory motif
i.v. . . . . intra venous
IVIG . . . . . intravenous immuno globulin
La . . . . . protein binding to RNA
LN . . . . . lymph node
lpr . . . . . lymphoproliferation gene
LPS . . . . . lipopolysaccharide
MacI . . . . . surface marker
MALDI-TOF . . . . . Matrix-assisted laser desorption/ionization
MHC . . . . . major histocompatibility complex
MIP . . . . . macrophage inflammatory protein
MRL . . . . . mouse model
MZ . . . . . marginal zone
NF-κB . . . . . nuclear factor κ B
nuc . . . . . nucleosomes
NK . . . . . natural killer
N-linked . . . . . chemical bound via nitrogen
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 . . . . . riboncleic complex
RPMI . . . . . Roswell Park Memorial Institute medium
SalI . . . . . restriction enzyme
s.c. . . . . subcoutaneous
SEM . . . . . standard error of the mean
SDS . . . . . sodium lauryl sulfate
SIGN-R1 . . . . . Specific ICAM-3 grabbin non-integrin related 1
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

TGF-β	transformal growth factor β
Th	T helper
TE	Tris/EDTA
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
Treg	regulatory T cell

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