TLR/MyD88 signaling in B cells suppresses T cell-mediated CNS autoimmunity

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der Technischen Universität Berlin
zur Erlangung des akademischen Grades
Doktorin der Naturwissenschaften
Dr. rer. nat.

genehmigte Dissertation

Promotionsausschuss:

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Tag der wissenschaftlichen Aussprache: 28.10.2011

Berlin 2012,

D83

In fond memory of Dr Alevizos

who sparked my interest in research

“ Twice two equals four: ´tis true,

But too empty and too trite.

What I look for is a clue

To some matters not so light. ”

Wilhelm Busch
(*1832-1908*)

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides Statt, dass die vorliegende Dissertation in allen Teilen von mir selbstständig und ohne Hilfe Dritter angefertigt wurde. Alle benutzten Hilfsmittel sind vollständig angegeben worden.

Die Dissertation wurde bisher nicht für eine Prüfung oder Promotion oder für einen ähnlichen Zweck zur Beurteilung eingereicht. Ich versichere, dass ich die vorstehenden Angaben nach bestem Wissen vollständig und der Wahrheit entsprechend gemacht habe. Weiter erkläre ich, dass ich nicht schon anderweitig einmal die Promotionsabsicht angemeldet oder ein Promotionseröffnungsverfahren beantragt habe.

Abstract

Toll like receptors (TLR) mediate recognition of microbial structures. Engagement of TLR has been shown to initiate an inflammatory cascade in innate immune cells that in turn orchestrate and instruct effector adaptive immune responses. B cells hold a particular position in the immune system displaying characteristics of both adaptive and innate immune cells. On one hand they elicit antigen-specific responses and produce high affinity antibodies, whereas by virtue of their expression of pattern recognition receptors, including TLR, they are equipped to sense and rapidly respond to microbes. Here we show that MyD88, a major signaling adaptor downstream most TLR, can have also anti-inflammatory functions through B cells. We found that TLR-activated B cells, but not dendritic cells (DC), produced high amounts of the anti-inflammatory cytokine IL-10 that suppressed effector functions of TLR-activated DC and consequently, limited T cell activation *in vitro*. Our *in vivo* studies revealed that mice lacking MyD88 selectively in B cells suffered a more severe chronic form of experimental autoimmune encephalomyelitis (EAE) - a mouse model of multiple sclerosis. In contrast, mice lacking MyD88 in all cells were resistant to disease. The exacerbated disease observed in mice with MyD88-deficient B cells correlated with increased autoreactive effector $CD4^+$T cells, suggesting that MyD88 in B cells promoted disease resolution by limiting inflammatory T cell responses. The suppressive function of B cells required TLR2/4 but not TLR9, indicating that distinct TLR differentially contribute to disease development and resolution. Absence of MyD88 in B cells was dispensable for germinal centre formation and antigen-induced antibody production, arguing against a requirement for MyD88 in B cell activation. However it resulted in strikingly reduced titers of natural IgM. Using mice deficient for secreted IgM, we found that IgM had a protective function in EAE that interfered with the development of autoreactive $CD4^+$T cell responses and controlled disease onset and severity. Altogether, these findings demonstrated a dual role for MyD88 in immune responses: MyD88 signaling in cells other than B cells, presumably DC and macrophages, is critical for the initiation of inflammation and T cell responses, whereas MyD88-signalling in B cells antagonizes this effect, thereby controlling overt inflammatory responses. This work identified a previously unappreciated role for TLR/MyD88 pathway in B cells linking recognition of microbial products to regulation of T cell-mediated autoimmunity.

Zusammenfassung

Toll-like Rezeptoren (TLR) dienen der Erkennung mikrobieller Strukturen. Die Aktivierung von TLRs löst Entzündungskaskaden in Zellen des angeborenen Immunsystems aus, welche wiederum adaptive Immunantworten steuern. B Zellen nehmen eine besondere Stellung im Immunsystem ein, da sie sowohl Charakteristika von Zellen des adaptiven als auch solche des angeborenen Immunsystems aufweisen. Einerseits lösen sie Antigen-spezifische Immunantworten aus und produzieren hochaffine Antikörper, andererseits können sie durch die Expression von so genannten Pattern-Recognition Rezeptoren, einschliesslich der TLRs, mikrobielle Strukturen erkennen und gegen diese reagieren.

In der vorliegenden Arbeit zeigen wir, dass MyD88, ein wichtiges Adaptorprotein im Signalweg der meisten TLR, in B Zellen auch anti-inflammatorische Funktionen erfüllen kann. Im Gegensatz zu dendritischen Zellen (DZ) produzieren B Zellen, die über TLR aktiviert wurden, grosse Mengen des anti-inflammatorischen Zytokins IL-10, welches seinerseits die Effektorfunktionen von TLR-aktivierten DZ supprimiert und darüber die T-Zell-Aktivierung *in vitro* inhibiert. Unsere *in vivo* Studien zeigen, dass Mäuse, die eine B-Zell-spezifische Defizienz für MyD88 aufweisen, einen schweren, chronischen Verlauf der experimentellen Autoimmunenzephalomyelitis (EAE), einem Mausmodell der Multiplen Sklerose, zeigen. Im Gegensatz dazu sind Mäuse mit genereller MyD88 Defizienz resistent gegen die Erkrankung. Der schwerere Krankheitsverlauf in Mäusen mit B-Zell-spezifischer MyD88 Defizienz korreliert mit einer erhöhten Zahl an autoreaktiven CD4 positiven Effektor-T-Zellen. Dies legt die Vermutung nahe, dass MyD88 in B Zellen zur Unterdrückung entzündlicher T-Zell-Antworten beiträgt. Die suppressive Funktion der B Zellen ist von TLR2/4 jedoch nicht von TLR9 abhängig, was darauf hinweist, dass verschiedene TLR auf unterschiedliche Art und Weise zur Krankheitsentstehung und Heilung beitragen. Eine MyD88 Defizienz wirkt sich nicht auf die Bildung von Keimzentren und die Antigen-abhängige Antikörperproduktion aus, was gegen eine Funktion in der B-Zell-Aktivierung spricht. Es werden jedoch deutlich reduzierte Titer an natürlichem IgM beobachtet. Wie unsere Studien in Mäusen mit einer Defizienz für sekretiertes IgM zeigen, kommt IgM eine protektive Funktion während der EAE, sowohl in der Unterdrückung von autoreaktiven CD4 positiven T-Zell-Antworten als auch in der Kontrolle von Krankheitsausbruch und Schwere der Erkrankung, zu. Zusammen genommen zeigen diese Befunde zwei gegensätzliche Funktionen von MyD88 während einer Immunantwort. In Nicht-B-Zellen, vornehmlich in DZ und Makrophagen, ist der MyD88 Signalweg essentiell für die Ausbildung von Entzündung und von T-Zell-Antworten. In B Zellen hingegen wirkt MyD88

diesen Effekten entgegen und hilft übermässige Entzündungsreaktionen zu inhibieren. Die vorliegende Arbeit beschreibt eine zuvor unbekannte Funktion des TLR-MyD88 Signalweges in B Zellen, und bringt darüber die Erkennung mikrobieller Strukturen mit der Regulation von T-Zell-vermittelter Autoimmunität in Verbindung.

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1 Introduction

1.1 Basic features of the immune system and its response

The mammalian immune system serves as the host´s guard against infections and thereby promotes its adaptation to the environment and survival through evolution. Primary and acquired immunodeficiencies in humans result in recurrent infections and often, fatal pathology [1-2]. There are numerous kinds of pathogens (intracellular and extracellular bacteria, viruses, fungi and parasites), with diverse macromolecular structures, tissue tropism and routes of infection. Organized in an extensive lymphatic system that drains almost every tissue and organ in the body, and that is in connection with the circulatory system via the thoracic duct, cells of the immune system continuously scan their environment for foreign invaders and upon pathogenic encounters readily acquire diverse effector functions collectively referred to as the immune response. The ability to sense a wide variety of microbial structures (antigens), yet to mount appropriate antigen-specific effector responses, and to generate long-term immunity to re-infection, termed as memory, are hallmarks of a successful immune response.

Innate and adaptive immune system: Diverse cells with distinct functions shape the immune response, and are broadly grouped into two categories: those comprising the innate immune system, including dendritic cells and macrophages, and those belonging to adaptive immune system, namely T cells and B cells (Fig.1.1). The innate immune system provides the first line of immune defense. It efficiently senses pathogen associated molecular patterns (PAMPs), becomes activated and initiates inflammatory cascades and anti-microbial defenses that serve to neutralize pathogens, and recruits phagocytic cells to locally contain infection. At the same time, it presents pathogen-derived antigens to cells of the adaptive immune system and orchestrates their activation. Adaptive immune responses then ensue directed against specific antigenic determinants and facilitate pathogen clearance and elimination of infected cells. A few antigen specific cells of the adaptive immune response, including CD4 and CD8 T cells, B cells and plasma cells, survive as memory cells [3-4] to rapidly and robustly defend the host upon subsequent exposure to a microbe. Memory is solely conferred by T and B cells, although memory features have been recently suggested for a cell subset of the innate immune system

[5]. Cytokines and chemokines are proteins mediating the activation and shaping of immune responses, migration and trafficking of immune cells during steady state and disease. Many of these mediators are pleitropic, with some of them being critical for the development and fine compartmentalization of secondary lymphoid organs.

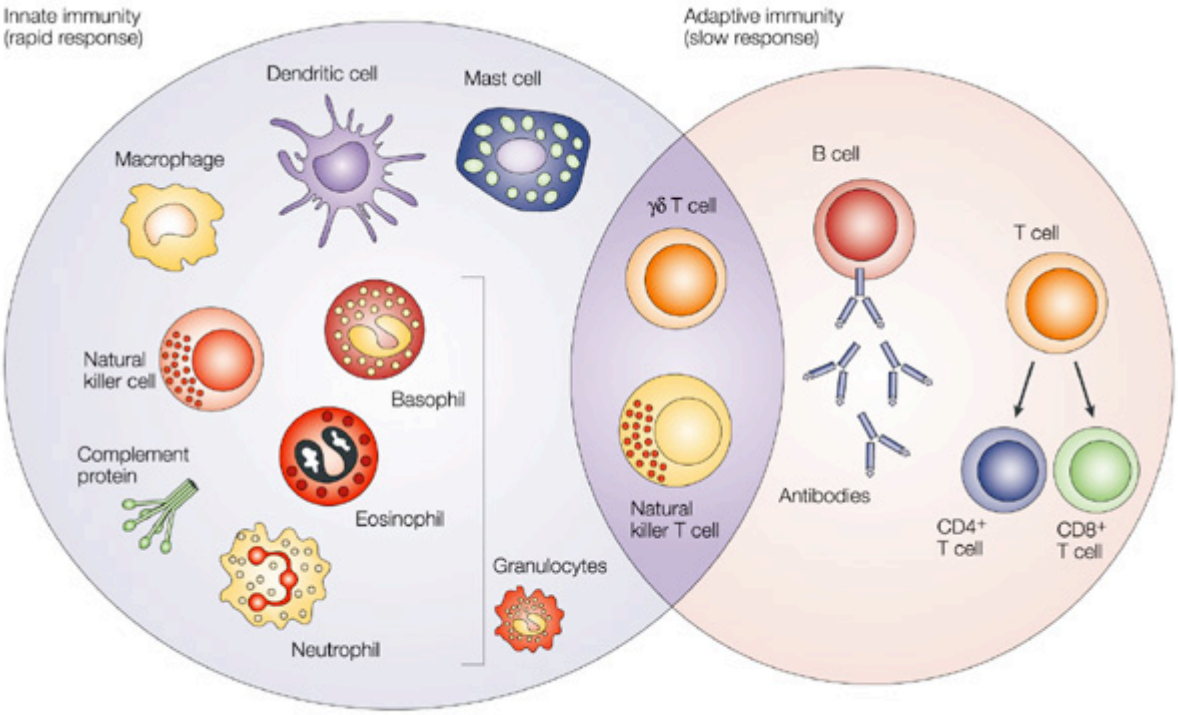

Figure 1.1 Cells of the innate and adaptive immune system.
Innate and adaptive immune resposnses are mediated by distinct cell types of the immune systems, schematically shown in the left and right circle, respectively. Cells like gd T cells and NK T cells display features of innate and adaptive immune cells, and can participate in early immnate immunity and adaptive immune responses. They are thus represented part of of both innate and adaptive immune cells. Adapted from [6]

Antigen recognition: A notable difference between the innate and adaptive immune system is the nature of the receptors each utilizes for antigen recognition. The former use germ-line encoded receptors, called pattern recognition receptors (PRRs) that recognize invariant pathogen-associated molecular patterns (PAMPs), conserved structures usually not present on

healthy host cells. As proposed by Janeway [7], these receptors contribute to the ability of the immune system to discriminate between self (host) and non-self (foreign) antigens, thereby mounting responses to microbes while sparing host integrity. Indeed, via PRRs the innate immune system is able to determine the specific nature of the invading pathogen and mount appropriate responses. Of note, accumulating evidence suggests that PRRs can also recognize host-derived macromolecules expressed upon tissue damage during infections or under sterile conditions, often referred to as danger-associated molecular patterns (DAMPs), as originally suggested by Matzinger [8-9]. In contrast, T cells and B cells recognize antigens via surface receptors that are generated *de novo* in each organism, the T cell receptor (TCR) and B cell receptor (BCR), respectively. The generation of BCR and TCR repertoires relies on random somatic recombination of several gene segments, resulting in various specificities to numerous foreign antigens. Thus, adaptive immune responses are highly specific and this is one of the features likely accounting for the rapidity and effectiveness of memory responses. Yet, adaptive B and T cell responses cannot discriminate the nature of infectious agents solely as result of antigen recognition through the BCR and TCR, respectively. Therefore, these different modes of antigen recognition by the innate and adaptive immune response complement each other in optimizing pathogen detection and elimination.

Activation and amplification of immune responses: The immune system generates diverse responses tailored to each class of pathogens. Dendritic cells (DC) are equipped with a diverse array of PRRs and are known to be the main antigen-presenting cells (APC) to activate naïve CD4 T cells in most circumstances [10-12]. They are thus, considered as critical sentinels of the immune system that link recognition of pathogens to the generation of appropriate types of adaptive immune response. Upon capturing microbial antigen at the periphery, resting DC acquire an activated phenotype through a process known as maturation [13-14]. Maturation allows them to leave the site of infection and migrate to the nearest lymphoid tissue, usually a draining lymph node (LN), where they encounter naïve T lymphocyte in the T cell zones [11, 15-16]. T cells scan the surface of the “incoming” DC and those bearing an antigen specific TCR become activated and primed via DC-delivered signals to differentiate into appropriate effectors. Broadly, antigen-specific CD4 T cells can differentiate into T helper cell 1 (Th), Th2, Th17 cells, though more Th subsets may exist [14, 17]. It should be noted that, apart from DC [12], Th2 priming may require signals from other cells, including basophils, mast cells and other recently discovered innate immune cells [18-21]. Each of these Th subsets produce characteristic set of

cytokines and accessory surface receptors, necessary for the clearance of distinct pathogens. Generally, Th1 cells are induced in response to intracellular bacteria and viruses, Th2 in response to helminths and Th17 in response to extracellular bacteria and fungi [22]. In addition, these Th subsets may differentially mediate immune responses to self-structures during autoimmune disorders (described in later sections). CD4 T cells together with DC are required for the activation of naïve antigen-specific CD8 T cells in a CD40-CD40L dependent manner in certain infections [23-25], but not others [26-28]. Upon activation CD8 T cells produce cytokines (mainly IFN-γ) and acquire cytotoxic functions, which mediate killing of cells infected by viruses or intracellular bacteria, and transformed cells. Another main function of Th subsets is to provide "help" signals that promote high affinity antigen-specific B cell responses, including specific antibody classes, memory B cells and long-lived plasma cells (described in later sections). Of note, T cell-independent B cell responses also occur, which as recently suggested may also give rise to memory B cells [29]. Highly specific antibodies are essential mediators of adaptive host defense, as they can directly neutralize pathogens or opsonize them for phagocytosis by innate phagocytic cells. Activated B cells on the other hand also present antigen to CD4 T cells via MHC II, can produce cytokines, and thereby can further influence Th responses (see section1.3.4). Notably, adaptive immune responses further enhance effector functions of innate immune cells. Cytokines produced by Th cells and CD8 T cells can enhance the phagocytic and other anti-microbial activities of innate immune cells. Similar effects can be mediated by B cell-derived cytokines and antibodies (see sections 1.3.2 and 1.3.5). Thus, upon pathogen invasion the innate and adaptive system engage into a mutual exchange of signals to generate and amplify appropriate immune responses.

Regulation: Strong immune responses are critical for efficient elimination of harmful microbes yet, if uncontrolled they can result in host pathology. Conceivably, a major limiting factor is antigen availability, as following pathogen clearance immune responses normally subside, a phase commonly referred to as contraction. Nevertheless, starting from innate recognition of antigen onwards, the magnitude and duration of innate and adaptive immune responses are also actively regulated. Several layers of regulation exist that act in a cell autonomous manner, via the expression of activation-induced inhibitory molecules, or at an intercellular level, whereby congenitally dedicated or activation-induced regulatory cell populations suppress inflammatory cells [30-32]. Examples, of cell-autonomous regulation include activation-induced suppressor molecules of PRR signaling [33-34], T cell and B cell inhibitory receptors [35-37],

inhibitory antibody receptors on B cells and other APC (see section 1.3.3). Regarding regulatory cell subsets, it should be noted that their inhibitory functions often rely on their secretion of anti-inflammatory cytokines, including IL-10 and TGF-b. Abrogation of these regulatory mechanisms can be deleterious to the host, resulting for instance, in toxic shock –due to excessive secretion of PRR-induced inflammatory cytokines [38] or fatal lymphoproliferative syndrome [35]. Conversely, nucleotide polymorphisms leading to increased expression of negative regulators may foster chronic infections [39-42]. Therefore, optimal regulation of immune responses is critical for the maintenance of functional homeostasis of the immune system.

Tolerance to self: While mounting responses to harmful pathogens, the immune system should spare responses to host-tissues to avoid autoimmunity. The latter is characterized by aberrant immune responses to self-molecules expressed systemically or in specific organs. Although somatic recombination ensures a broad range of TCR and BCR specificities, it can also generate potentially harmful self- reactive B and T cell clones. Overtly self-reactive cells (T and B cells) are purged from the mature repertoires via the process of central tolerance [43-46], as originally suggested by experiments of Burnet and Medawar [47]. During their development, immature T and B cell clones are tested for their ability to react against a broad range of self-antigens ectopically presented in thymus and bone marrow, respectively. Upon strong binding, they are eliminated by clonal deletion [48-49]. Genetic mutations interfering with central tolerance, can lead to autoimmune disorders. For example, mutations in the gene encoding for the transcription factor AIRE, which regulates ectopic expression of peripheral self-antigens in the thymus, abrogates central tolerance to certain self antigens, resulting in the so called APECED syndrome (autoimmune polyendocrinopathy, candidiasis and ectodermal dystrophy) [50-52]. Despite their efficiency, central tolerance mechanisms are insufficient to eliminate all self-reactive cells, as cells bearing low-avidity receptors for self-molecules are found in the periphery of healthy individuals. There they are kept unresponsive to antigen (anergic) or quiescent by a series of molecular and cellular mechanisms collectively referred to as peripheral tolerance [49, 53-58]. An important mechanism among the latter is the suppression of self-reactive T cells by a specialized naturally occurring T cell subset called regulatory T cells (Treg). Mutations in the X chromosome-linked gene encoding for FoxP3, a transcription factor required for the generation of Treg cells, are fatal in females early on in life, whereas males suffer from the also fatal disorder called IPEX (immune dysregulation, polyendocrinopathy, enteropathy and X-linked inheritance; [59]). Lastly, besides recurrent infections, patients with primary

immunodeficiencies often develop autoimmune disorders, that have been recently linked to defective central and peripheral tolerance [60], further exemplifying the importance of the immune system in protecting the host both from infections and self-attack. It should be noted here that the immune system is in constant interaction with the environment, and this interaction likely contributes to the disruption of tolerance, as indicated by discordant development of several autoimmune disorders in monozygotic twins and relevant epidemiological studies (Multiple sclerosis is presented as an example in section 1.4)

1.2 Antigen recognition via toll-like receptors and their role in activation of immune responses

As mentioned earlier, cells of the innate immune system sense pathogens by recognizing PAMPs. Recognition is mediated by a diverse array of receptors called pattern recognition receptors (PRRs), which often act in concert to discriminate among distinct types of pathogens. PRRs are expressed by many cell types including macrophages, dendritic cells (DCs), neutrophils, natural killer cells and epithelial cells, and they mediate the early detection of pathogens directly at the site of infection. There are several classes of PRRs that according to their cellular localization can be broadly grouped into secreted, transmembrane and intracellular ones. Among the secreted PRRs, often referred to as part of humoral innate immunity, are pentraxins (e.g. C-reactive protein), ficolins and collectins (eg.mannose-binding lectin), which collectively promote early pathogen neutralization either through direct binding, or by activating the complement cascades and opsonising pathogens for phagocytosis via cell-surface receptors [61-63]. Cytosolic receptors include the nucleotide binding domain and leucine-rich repeats-containing receptors (NLRs), and the retinoic acid-inducible gene I (RIG I)-like receptors (RLRs). Some NLRs can detect microbial signals, degradation products of peptidoglycans [64-65], whereas others-those forming the so-called inflammasome structures, can also sense nonmicrobial stress [66]. RLRs are expressed by most cell types and recognize ssRNA and some dsRNA viruses [67-68]. Transmembrane receptors include members of the C-type lectin family receptors (CLRs; eg. Dectin-1, scavenger and mannose receptors) and the Toll-like receptors, (TLR) although some of the latter recognize pathogens in intracellular compartments as well (described below). Surface CLRs recognize mannose, fucose or glucan carbohydrate structures on viruses, fungi, mycobacteria, certain bacteria and helminth parasites [69]. Signaling through most PRRs induces the expression of several genes leading to activation of

innate immune cells, their production of anti-microbial defenses, cytokines, chemokines and chemokine receptors that orchestrate activation and recruitment of other innate and adaptive immune cells.

1.2.1 Antigen recognition by Toll-like receptors (TLR)

Toll-like receptors were first discovered in Drosophila and shown to be required for its development and for anti-fungal defenses [70]. Since the identification of their human homologues [71] in mid-1990s, TLR have emerged as crucial sensors of PAMPs and have been the focus of extensive research. Their significance in evolutionary terms is exemplified by the presence of the vast repertoire of 222 TLR recently predicted to be encoded in the genome of sea urchin [72-73], as well as their presence in teleost fish [74]. The TLR family consists of at least 10 functional members in human and 12 in mice. TLR can be categorized into two groups based on their cellular localization. Surface TLRs include TLR1, 2, 5, 6 and 11 and recognize mostly lipids, lipoproteins and proteins present on the surface of pathogens (Fig. 1.2). Among the intracellular TLRs are TLR3, 7, 8 and 9 (Fig. 1.3). These reside in endoplasmic reticulum (ER), endosomes/phagosomes, lysosomes and endolysomes, and recognize nucleic acids derived from the genome of bacteria and viruses. TLR4, the founding member of the TLR family, which responds to LPS (lipopolysaccharide) present on the cell wall of gram negative bacteria, is so far the unique TLR described to signal both at the cell surface and from intracellular location. Occurring as homo- or hetero- dimers, TLRs recognize a wide variety of microbial structures and types of pathogens, including polysaccharides, proteins present on bacteria, viruses and parasites [75]. Upon ligand regognition, TLR signaling is initiated resulting in specific gene transcription in sentinel cells, like dendritic cells, and activate innate immune responses.

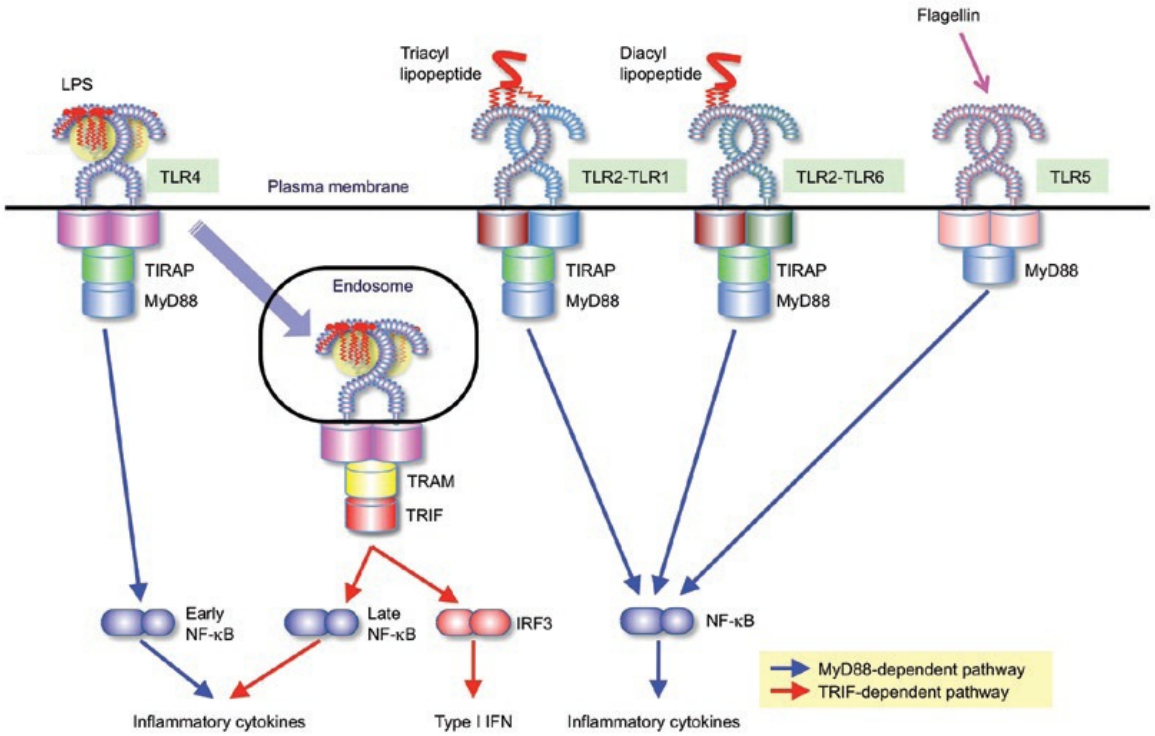

Figure 1.2 Cell surface Toll-like receptors and downstream signaling pathways.
Surcafe TLR can mediate recognition of gram-negative derived (LPS; flagellin) or gram-positive-derived (lipopeptides) products. TLR4 is the only TLR known thus far to trigger signaling cascades both at the cell membrane and intracellularly. Upon TLR4-LPS interaction the adaptor TIRAP recruits MyD88 to the membrane to induce a first wave of NF-kB activation and inflammatory cytokines (MyD88-dependent pathway). Another adaptor TRAM mediates the translocation of the TLR4-LPS complex into endosomes, where it recruits TRIF. This MyD88-independent pathway leads to late NF-kB activation and subsequent inflammatory cytokines as well as to IRF3-mediated induction of type I IFN. The rest of the surface TLR shown can form heterodimers (TLR2-TLR1 and TLR2-TLR6) and trigger MyD88-depedent signaling cascades to induce inflammatory cytokines. IRF: interferon regulatory factor. Adapted from [76]

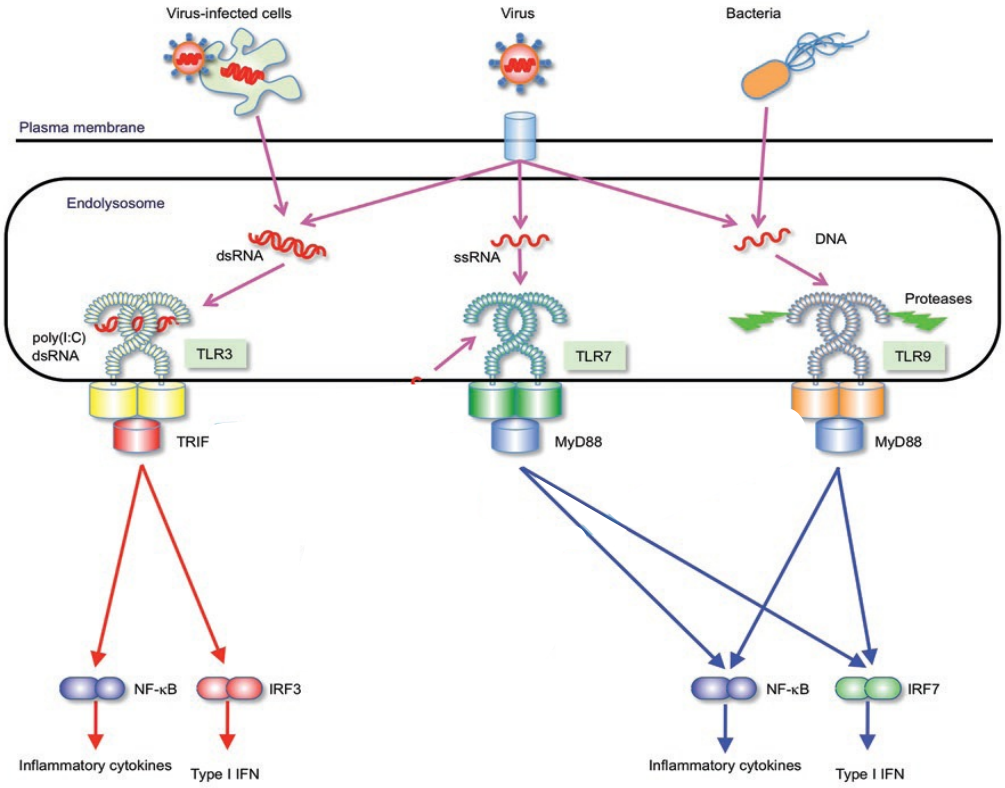

Figure 1.3 Intracellular Toll-like receptors and downstream signaling adaptors.
Intracellular TLR reside in endosomes and mediate recognition of nucleic acid structures (RNA or DNA) from viruses, virus-infected cells or bacteria once these are endocytosed. TLR7 and TLR9 propagate signals via the MyD88-dependent pathway leading to NF-kB-induced inflammatory cytokines and IRF7-mediated induction of type I IFN. TLR3 signals exclusively the TRIF-mediated MyD88-independent pathway to induce, respectively, NF-kB- mediated and IRF-mediated inflammatory cytokines and type I IFN. Adapted from [76]

Signaling through individual TLR elicits specific cellular responses that contribute to their ability to discriminate among different classes of pathogen. For example, TLR3 and TLR4 can generate both inflammatory cytokines like IL-6, IL-12, TNF, and type I interferon (IFN) responses, whereas TLR2-TLR1, TLR2-TLR6 and TLR5 induce only the former (Fig. 1.2a and b). In addition, certain TLR may participate in the recognition of different classes of microbes. These features can be attributed to several mechanisms that operate at multiple levels upon TLR-TLR ligand binding, as described below.

TLR signaling: TLR are type I transmembrane receptors and their cytoplasmic tail contains the Toll/interleukin 1 (IL-1) receptor (TIR) domain which is unique to this class of PRRs. Through

this domain, TLR associate with several signaling adaptors, including MyD88 (myeloid differentiation fator 88), Mal (MyD88-adaptor like; also called TIRAP), TRIF (TIR-domain containing adaptor protein inducing IFNβ) and TRAM (TRIF-related adaptor molecule) (Fig.1.2). A fifth adaptor has also been recently described, called SARM (sterile a- and armadillo-motif-containing protein), that interacts with TRIF [77]. MyD88 is required for signaling downstream all TLR except TLR3, which exclusively recruits TRIF. TLR4 is unique among TLR, in that it signals both via a MyD88-dependent and a MyD88-independent, TRIF-dependent pathways (Fig.1.2a). MyD88 and TRIF do not directly interact with the TIR domain of TLR, but via the adaptors Mal and TRAM, respectively. Whereas TRAM is required to mediate binding of TRIF to TLR4 and TLR3, Mal-mediated coupling of MyD88 is necessary for signaling through TLR2 and TLR4, but not TLR 9 and 5 [78-79] (Fig.1.2). In the case of TLR 4, the MyD88 pathway is activated first at the plasma membrane, where Mal recruits MyD88 to TLR4 and leads to initial activation of NF-kB and MAPK [80]. Subsequently, TLR4 is endocytosed and trafficked to the endosomes, where it now binds TRAM that couples it to TRIF, inducing activation of IRFs and a late-phase activation of NF-KB and MAPK [80]. Differential recruitment of signaling components among individual TLR also occurs downstream of MyD88 and TRIF, including members of the IRAK (IL-1 Receptor-associated kinases), TRAF (Tumor necrosis factor and IRF families [81]. MyD88-dependent activation of NF-kB and MAPK kinases, results in the production of inflammatory cytokines while the TRIF-mediated pathway stimulates type I IFNs via activation of IRF molecules. Notably though, inflammatory cytokine production in response to TLR4 requires both MyD88 and TRIF. Therefore, engagement of individual TLR results in the formation of distinct signaling complexes that mediate the induction of distinct cellular responses.

Cooperation and antagonism among TLR and other PRRs: A given TLR may mediate the recognition of distinct classes of pathogens. This often relies on the cooperation between different TLR. For instance, distinct conformational features of TLR2-TLR1 and TLR2-TLR6 dimers allow the discrimination between diacylated and triacylated lipopeptides, respectively, from Gram-positive and Gram-negative bacteria and mycoplasma ([82-83]. Considering that many pathogens carry agonists for multiple TLR [84], it is likely that the ensuing cellular response relies on the cooperation of signaling through several TLR. Indeed, stimulation of myeloid cells with a combination of TLR agonists had synergistic effects in the production of inflammatory cytokines, compared to individual agonists [85-87]. Conversely, negative regulation may also occur upon TLR cooperation. Simultaneous engagement of certain TLR

resulted in synergistic downregulation of some genes that was not observed after activation with a single TLR [88]. Moreover, TLR9 can antagonize signaling through TLR7, while agonists of TLR8 have been reported to antagonize signaling via TLR7 and TLR9 [89]. In the case of certain TLR, further specificity is conferred by their ability to act in concert with other PRRs or co-receptors to mediate PAMPs recognition. A common example is the co-receptor CD14, which can facilitate discrimination of different forms of LPS (namely smooth and rough LPS) by TLR4 [90]. Furthermore, the adhesion molecule CD36 and the CLR dectin-1 co-operate with TLR2-TLR6 dimers in the recognition of respectively, bacterial and fungal molecules [91-92]. Certain CLRs can also influence both the duration of TLR signaling and the resulting cytokine profile [69].

Cell type specific TLR responses: Specific immune responses to different pathogens can also be mediated by preferential or altered TLR triggering in a cell specific manner. This notion is indicated by several findings. First, TLR are differentially expressed in various cells of immune and non-immune origin [93]. With regard to the immune system, the TLR repertoire of plasmacytoid DC (pDC) is restricted to intracellular nucleic acid sensing TLR, like TLR7 and TLR9 [94]. pDCs are a specialized DC subset with distinct phagocytic and other functional properties compared to conventional DC [95] and their preferential activation via TLR9 and TLR7 promotes the activation of type I IFN responses. Interestingly, in pDCs TLR7- and TLR9-induced type I IFN requires recruitment of MyD88, in contrast to other myeloid cells in which TLR9 activation induces MyD88-dependent inflammatory cytokines but not type I IFNs [94, 96-97]. Along this line, Barbalat et al, recently showed that TLR2 on monocytes (a subset of phagocytic cells), but not dendritic cells or macrophages, can respond to viral but not bacterial structures [98]. TLR signaling in non immune cells may also shape host responses to pathogens. Emerging evidence suggests that TLR signaling in these cells maybe different from that in cells of the immune system. An epithelial cell line were found to express only intracellular TLR4 and displayed altered cytokine responses to LPS, though the physiological significance of this finding during infection remains to be determined [99]. Altogether, cell type-specific expression, cellular compartmentalization, distinct signaling pathways, of individual TLR as well as TLR cooperation facilitate optimal microbe detection and distinct biological responses,

1.2.2 TLRs as instructors of innate and adaptive immune responses

The importance of TLR in host defense has been demonstrated by numerous *in vivo* studies in experimental animals and a growing body of evidence in humans. Mice deficient in MyD88 are susceptible to infections caused by several pathogens, including bacteria, parasites, and viruses [81]. Similar phenotype has been observed in Mal or TRIF-deficient mice, though fewer pathogens have been tested in these cases [81]. Humans with functional deficiency in MyD88, due to deletions or missense mutations are susceptible to pyogenic infections, including *Staphylococcus aureus*, and *Pseudomonas aeruginosa*, which are life threatening within the first 10 years of life [100]. Resembling these patients are individuals deficient in IRAK-4 a protein recruited to TLR- or to IL-1R (receptor) by MyD88 [101]. Furthermore, mutations resulting in TLR3-deficiency [102], defective TLR3 responsiveness [103] or in impaired transport of the intracellular TLRs 3, 7 and 9 to endolysosomes [104-105] in humans, have been linked to susceptibility to herpes simplex virus – induced encephalitis.

Engagement of TLR by microbial products in various immune and non-immune cells leads to the activation of host defenses with direct anti-microbial activity, including anti-microbial peptides like defensins, nitric oxide-mediated killing. Apart from these pathogen-combating mechanisms, the contribution of TLR to host defense further encompasses both innate and adaptive immunity. TLR activation enhances antigen uptake by DC [106], induces upregulation of MHC II molecules and via endocytosis, TLR bound to microbial products can influence the selection of antigen presented on MHC II [107]. TLR agonists also alter the migration pattern of DC by inducing the expression of chemokine receptors, like CCR7 allowing them to migrate from the periphery to the T cell zones in draining lymph nodes [108]. Signaling via TLR also results in up-regulation of co-stimulatory molecules, including CD40, CD80, CD86 and members of the B7h family [108]. Thus, TLR stimulation equips DC with a potent capacity to provide two signals critical for T cell activation; signal 1, through antigen presentation to TCR and signal 2, co-stimulation. Perhaps equally important, TLR signaling in DC leads to their secretion of several cytokines, which act as a third signal guiding the differentiation of CD4 T cells into Th subsets. These cytokines include IL-12 and IL-1, IL-6, which promote differentiation of Th1 [109] and Th17 cells [110-112], respectively. The role of TLR in Th2 priming is not well documented. Of note, although cytokines alone can similarly up-regulate MHC and co-stimulatory molecules on DC [113], in the absence of TLR stimuli they are insufficient to allow DC to induce CD4 T cell differentiation into effector Th subsets [114]. Consistently, MyD88-deficient mice display delayed and impaired Th1 responses to several pathogens [113]. TLR can also influence the activation

of CD8 T cells. A recent study showed that TLR3 and 9 agonists may promote CD4 T cell-independent CD8 T cell activation by acting on DC [115], while DAMPs acting via TLR4 stimulated increased autoreactive CD8 T cells reopnses in a mouse model of systemic autoimmunity [116]

1.3 B cells

B (bursal or bone marrow-derived) lymphocytes constitute together with T cells the adaptive immune system. B cells express clonally diverse cell surface immunoglobulin (Ig) receptors, called B cell receptor (BCR), which is composed of the heavy (H) and light (L) chain, recognizing specific antigenic epitopes. Functional BCR results from the random combinatorial rearrangement of gene segments V (variable), D (diverse) and J (joining) in the H locus, and V and J in the L chain loci, during B cell development [117]. This is an random process that generates a diverse BCR repertoire that binds a variety of molecular structures, including proteins, lipids and nucleic acids. The most prominent function of B cells is antibody production, the host´s unique cell type able to produce antibodies. Antibodies can be produced naturally, that is, without prior exposure to exogenous microorganisms, and in response to foreign antigens. Natural antibodies are generally polyreactive and of low affinity for antigens. They are part of the first layer of host defense against invading pathogens serving to early contain infection. Later, once adaptive immune responses ensue, B cells with highly specific BCR clonally expand and give rise to plasma cells secreting antigen specific antibodies that via a variety of mechanisms help to eliminate infection in a pathogen-specific manner. Subsequently, a proportion of these plasma cells survive as long-lived cells, maintaining antibody serum levels for years that contribute to rapid and robust protective immunity upon pathogen re-encounter [118]. Exemplifying their importance in host defense, antibodies are transferred to the fetus through the placenta and to neonates through lactation, presumably serving as a front-line response to invading pathogens.

In addition to antibody production, B cells can present antigen and produce cytokines. and thus, can influence immune responses in a multifaceted manner. Indeed, the diverse functions

of B cells and their role in immune responses have received renewed interest, especially in the context of autoimmunity. Recent clinical trials of a B cell-depleting antibody, Rituximab, have demonstrated a previously unappreciated pathogenic role of B cells in autoimmune disorders, like multiple sclerosis, rheumatoid arthritis and diabetes [119]. Yet, targeting of B cells has not always been beneficial [119], and several studies in mice and humans point to a dual role of B cells, acting both as drivers and regulators of autoimmunity. Consequently, there is great interest in identifying the pathogenic and protective contributions of B cells as well as the signals stimulating these effects. The following sections describe main features of B cell biology and functions, and provide examples of how they can shape immune homeostasis and responses during disease.

1.3.1 B cell subsets

The murine B cell compartment is heterogeneous and comprises several B cells subpopulations that differ in their ontogeny, anatomical location and function. B cells can firstly be divided into the B1 and B2 types. B1 cells originate mostly in fetal liver and partly in adult BM. They reside predominantly in the peritoneal and pleural cavities and constitute about 5-10% of splenic B cells. Two subsets within B1 cell can be phenotypically distinguished: B1a and B1b B cells (Table 1.1). Although both subsets mount responses to antigens without T cell help, and provide protection against bacterial and viral infections, B1a cells are the main sources of natural antibodies (predominantly IgM), whereas B1b cells can generate long-term adaptive ntibody responses, and have higher capacity to undergo somatic hypermutation. Upon activation, B1 cells can migrate from the peritoneal cavity to the gut where they contribute to generation of T cell-independent of mucosal IgA [120]. B2 cells on the other hand constitute the B cell compartment in spleen, peripheral lymph nodes and mucosa-associated lymphoid structures,

Table 1.1 B cells subsets defined by expression of surface makers and their tissue distribution.

B cell subset		phenotype	location
B1	B1a	$IgM^{high}IgD^{low}CD43^{+}CD23^{-}CD21^{-}$ $CD11b^{+}CD5^{+}$	Peritoneal and pleural cavity
	B1b	$IgM^{high}IgD^{low}CD43^{+}CD23^{-}CD21^{-}$ $CD11b^{+}CD5^{-}$	Peritoneal and pleural cavity
	B1	$IgM^{high}IgD^{low}CD43^{+}CD23^{-}CD21^{-}$ $CD11b^{-}CD5^{+}$	spleen
B2	T1	$IgM^{high}IgD^{-}HSA^{+}CD93^{+}CD21^{-}CD23^{-}$ $CD11b^{-}$	T cell area of spleen
	T2	$IgM^{high}IgD^{-}HSA^{+}$ $CD93^{+}CD21^{high}CD23^{high}CD11b^{-}$ $CD43^{-}CD1d^{high}$	B cell follicle of spleen
	Marginal zone	$IgM^{high}IgD^{-}HSA^{-}CD93^{-}$ $CD21^{high}CD23^{-}CD11b^{-}CD43^{-}$ $CD1d^{high}$	Marginal zone of spleen
	Follicular	$IgM^{high}IgD^{-}HSA^{-}CD93^{-}$ $CD21^{int}CD23^{+}CD11b^{-}CD43^{-}$ $CD1d^{int}$	Follicles of spleen, and all lymph nodes.

like Payer´s patches (PP). They are generated in the bone marrow and enter the spleen as immature cells termed then as transitional B cells (T1 and T2 B cells; Table 1), comprising about 10% of splenic B cells. Among mature B2 cells two subsets can be phenotypically identified: the marginal zone B cells [121] and follicular B cells (FO) (Table 1), that, respectively, localize in the marginal zone and the B cell follicles of the splenic white pulp. Of note, the MZ subset is almost exclusively found in spleen. The expression of CD1d, a lipid-antigen presenting molecule can be used to grossly divide B cells into $CD1d^{lo}$ and $CD1d^{hi}$ cells. To the former belong virtually all B cells (splenic, LN) whereas the $CD1d^{hi}$ subset is restricted to the spleen, where they constitute about 5-10% of splenic B cells during the steady state [117]. Although the various B cell subsets can be phenotypically distinguished during the resting state based on the expression of certain surface markers, the latter can be altered upon activation of the cells, so that subsets become phenotypically inseparable. For example, upon LPS administration *in vivo* B1 cells can migrate to the spleen or gut where they lose expression of CD11b [122]. Conversely, certain inflammatory conditions may induce upregulation of CD1d expression LN-derived B cells [123].

Due to this activation-induced phenotypic plasticity, tracking B cell subsets *in vivo* during immune responses remains a daunting task.

1.3.2 B cell activation and fates

A critical prerequisite for B cell activation and antibody responses to occur is for B cells to encounter antigen. Although the exact mechanisms through which B cells acquire antigen are still being deciphered, several modes have been proposed. B cells may directly bind soluble antigen diffusing in the B cell follicles [124], “pick-up” particulate antigen or antigen in the form of immune complexes (IC, antigen bound to antibody) displayed on DC [125-126], follicular dendritic cells (FDC; [127]) and subcapsular macrophages in the LN [128-129]. After acquiring antigen, B cells may become an antigen-transport vehicle themselves, transferring and depositing Ag to the follicles via complement receptors [128, 130] and/or antigen-IgM complexes [131]. B cells may also encounter antigen outside the LN and subsequently transport it there [132]. Notably, most of these studies did not provide data on the activation fate of the B cells subsequent to their antigen capture. Considering that a) antigen trapping and transport occur within a few hours after immunization, and b) the low frequency of B cells carrying a BCR specific to a given antigen in naive mice, it is unlikely that that the former is mediated by antigen-specific B cells. Indeed, capture of particulate antigen from subcapsular macrophages required complement receptor expression on the B cells, whereas their BCR specificity was not addressed. Early studies demonstrated that B cells capture antigen most efficiently when they carry a cognate BCR [133]. Therefore, B cells may meet their cognate antigen, once this is transported in their vicinity by non-antigen specific B cells.

Antigen engagement to cognate BCR initiates B cell activation and antigen internalization. Subsequently, antigen is processed, loaded onto MHC molecules and presented on the B cell surface [134-135]. Antigen-loaded B cells migrate to the border between the B cell follicle and the T cell zone where they engage into cognate interaction with $CD4^+$ Th cells [136-137], which in turn provide the T cell help signals to complete B cell activation. B cells also interact with antigen specific Th that are found within the B cell follicle, and hence, called follicular helper T cells (Tfh) [138]. Upon B-T cell interaction, B cells may either clonally expand and differentiate into short-lived plasma cells outside the follicle, a response referred to as extrafollicular [139], or form germinal centers [72] within the follicle. In GC, B cells extensively proliferate and undergo three major processes: somatic hypermutation (SHM), affinitiy

maturation and class switch recombination (CSR). SHM introduces specific site-directed mutations in the Ig variable region-genes, in an attempt to improve the BCR specificity for antigen [140]. During afiinity maturation, hypermutated BCR are then tested for their affinity for antigen displayed on local FDC, and B cells bearing BCR of increased affinitiy receive survival signals and exit the GC [140]. Class switch recombination is the process whereby the heavy chain class of an antibody produced by activated B cells changes from IgM to either IgG (1,2c,2b, 3), IgA, IgE. A recent study demonstrated that IgM to IgD class switch can also occur [141]. Isotype switch alters the effector function of the antibody mediated by the Fc part (later section) leaving the re-arranged VDJ region of the antibody unchanged. Both SHM and CSR require the activity of the enzyme activation-induced cytidine deaminase [142]. Although SHM and CSR are hallmarks of GC reaction, they can occur at extrafollicular sites as well [143-144]. After exiting the GC, highly specific B cells differentiate into short-lived effector plasma cells that act during ongoing immune response, long-lived plasma cells or memory B cells that provide long term protective immunity upon antigen re-encounter. The GC reaction and the ensuing B cell fates requires engagement of CD40 on B cells with CD40 ligand (CD40L;CD154) expressed on follicular T cells, because genetic deficiency in CD40 [145] or antibody-mediated blockade of CD40L [146] resulted in absence of GCs, class switched antibodies, and memory B cells. Consistently, engagement of CD40 on B cells *in vitro* (via an agonistic antibody to CD40) stimulates vigorous proliferation and promotes their survival.

B cells can receive T cell help after they have been activated through BCR-specific binding of protein antigens, referred to as Thymus-dependent (TD)-antigens. However, B cells may proliferate, and differentiate into plasma cells in response to certain antigens in the absence of T cells, which are thus called T-independent antigens (TI) and usually derive from pathogens. These fall into two classes: TI-1, that directly induce B cell division (mitogenic) regardless of their BCR specificity, and TI-2 that lack mitogenic activity, and instead activate B cells through extensive cross-linking of their BCR [147].

Role of TLR in B cell activation. An example of TI-1 antigen is LPS that induces B cell proliferation and antibody production by triggering TLR4 on B cells. Mouse B cells indeed express most TLR reported so far making them responsive to various TLR agonists and hence invading microbes. Human B cells also express TLR, although at naive state they do not

respond to TLR4 agonists, like LPS [148-149]. Therefore, by virtue of their expression of PRRs and BCR, B cells can be viewed not merely as part of adaptive immune system, but as cells at the interface of innate and adaptive immunity. TLR can influence the behavior and diverse functions of B cells during immune responses to both foreign and self-antigens. Although the T cell-independent B cell activation in response to TLR stimuli is widely admitted, considerable controversy exists over the role of TLR signaling in B cell humoral immunity to TD-antigens. As previously mentioned, MyD88-deficient mice mount impaired B cell responses and have dramatic defect in the accumulation of long-lived plasma cells and antibody maintenance upon infection, with polyoma virus [150] or vesicular stomatitis virus [151]. This defect was initially attributed to defective T cell help, as Th responses are compromised in these mice. Recently, Pasare and Medzhitov [152] challenged this view, by suggesting that optimal antibody responses to protein antigen-LPS conjugate require B cell-intrinsic TLR signaling [152]. A year later Nemazee and colleagues opposed that study, by showing that antibody responses to haptenated antigens mixed in various adjuvants were largely normal in MyD88.Trif-double deficient mice [153]. The experimental systems used in these studies had certain caveats. The former performed B cell transfers into B cell-deficient mice that may induce host-versus-graft reaction and ultimately lead to elimination of the transferred B cells, whereas the latter study used adjuvants whose ability to trigger specific TLR is not clearly documented. Other studies have indicated that signaling through TLR9 could be selectively required for the isotype switching to IgG2a/c. Of note, a drawback shared by most of the aforementioned studies is that the MyD88/TLR deficiency was not restricted solely to B cells. Thus, their conclusions regarding the B cell-intrinsic requirement of this pathway for B cell responses should be interpreted with caution. In humans, TLR have been proposed as the third signal, in addition to BCR and T cell help, required for the complete activation of naive B cells [154]. Bypassing T cell help, certain TLR could augment activation of autoreactive B cells and autoantibody production. This notion was raised by two studies, which showed that IC of antibodies and nucleic acid-containing self antigens, like chromatin, efficiently activated autoreactive B cells in a BCR- and TLR9-dependent fashion [155-156]. TLR7 and 9, which recognize RNA and DNA structures, respectively, were later reported to support autoantibody production in experimental lupus [157].

1.3.3 Functions of antibodies

A consequence of B cell activation is antibody production. The first antibody class to be produced during an immune response is IgM, followed by switched classes of IgG1,2,3, IgE or IgA isotypes. IgG isotypes are commonly produced in response to bacterial and viral infection, whereas IgE and IgA isotypes are important for host defense against parasitic infections and pathogens invading at mucosal sites, respectively. Although IgG and E antibodies function exclusively as monomers, IgA exists at mucosal surfaces as a secretory dimer, whereas IgM forms a pentameric structure. The effector functions of antibodies rely on both their antigen-binding fragment (Fab') and their constant Fc part (Fragment crystallizable). Through their Fab fragment antibodies bind circulating antigen or whole microbes forming IC and directly neutralize them or opsonize them for destruction by complement, phagocytosis and antibody-dependent cell-mediated cytotoxicity (ADCC) by accessory cells, the latter two processes being mediated by the Fc part of the antibody. The Fc part can activate complement directly [158], whereas phagocytosis and ADCC are mediated by binding of Fc to Fc receptors (FcR) expressed, respectively, on phagocytic cells, like macrophages [159], and cells with cytotoxic activity, like natural killer [160] cells. While beneficial to host defense against pathogens, in autoimmune diseases autoantibodies (self-reactive antibodies)–self-antigen IC can be detrimental, as in the case of lupus nephritis whereby IC deposit in and damage the renal tissue. Apart from direct tissue destruction, IC may affect immune responses by modulating the function of FcR-bearing accessory cells. Depending on the type of signaling motifs present in their cytoplasmic tails the FcR for IgG antibodies are grouped into activatory (FcγRI, III, IV) and inhibitory (FcγRIIb). Engagement of activatory FcγR can trigger oxidative bursts, cytokine release, mast cell degranulation, promote APC function, and B cell proliferation [161-162]. Consistently, antibodies, especially IgG isotypes, have been attributed a pathogenic role in several human autoimmune disorders and their experimental models including SLE, rheumatoid arthritis (RA), multiple sclerosis (MS), and idiopathic thrombocytopenic purpura (ITP) [163-164]. Evidence also supports a contribution of antibodies to tumor growth [162, 165] and to transplant rejection [166].

Conversely, ligation of inhibitory FcγR can dampen immune responses, and their importance is exemplified in humans by the observation that loss-of-function mutations in FcγRIIb gene correlate with increased susceptibility to autoimmune diseases, particularly systemic lupus erythematosus (SLE), and with resistance to certain infections [167]. The inhibitory FcγRIIB is expressed on many cells, including DC, B cells, and mast cells, and their

engagement generally results in effects opposite to those mediated by activatory FcγR [168]. Interestingly, Ivashkiv and colleagues, showed that extensive ligation of activatory FcγR may also propagate suppressive signals that dampen TLR-induced activation of innate immune cells during experimental arthritis [169]. This observation indicated a complex role for IgG antibodies during disease, and highlighted the cross talk between different signaling pathways as a means for regulating overt immune responses [169]. Consistent with a regulatory function of antibodies, intravenous injection of whole serum Ig, termed IVIg, from healthy individuals has been approved for the treatment of autoimmune diseases, like ITP, Guillain-Barren and Kawasaki´s syndrome [170]. The inhibitory action of IVIg involves multiple mechanisms, including suppressive effects via the inhibitory FcγRIIB, targeting of inflammatory cytokines, and attenuation of complement activation [171].

Role of secretory IgM in immune responses. Secretory IgM (sIgM) can be divided into natural, due to its presence in germ-free mice or foreign antigen-free, and immune IgM, produced upon exposure to exogenous antigen [172]. The main cellular sources of natural IgM are thought to be B1a cells and MZ B cells, whereas all B cell subsets can produce antigen-induced IgM [173]. Most of the functions of IgM are attributed to its two basic properties: its pentameric structure and polyreactivity. Owing to its pentameric structure sIgM is the most effective isotype at activating complement, with approximately 1000-fold greater binding capacity compared to IgG [173]. Another accompanying structural feature of sIgM is its multivalent antigen-binding capacity, which enables high avidity binding of antigens of low affinity. The repertoire of natural IgM contains reactivities to viral and bacterial pathogens, such as vesicular stomatitis viruses, vaccinia virus, listeria monocytogenes, lymphocytic choriomeningitis virus (LCMV) [174] and is essential to host defense against *Streptococcus pneumoniae* [175]. Natural and immune sIgM provide protection against a infections by a variety of pathogens, including influenza virus [176-177], LCMV [178], west nile virus [179], bacterial peritonitis [180], *Toxoplasma gondii* [181], and the fungus *Cryptococcus neoformans* [182].

Besides their antimicrobial actions, IgM IC via complement promote the initiation of B cell responses, by aiding capture, transport and deposition of antigen to FDC in the spleen [131]. Indeed, mice lacking secretory IgM ($\mu S^{-/-}$) display impaired antigen trapping on FDC, delayed GC development and antibody responses upon immunization with haptenated TD-antigens

[183-184]. sIgM also facilitates the initiation of a delayed-type hypersensitivity reaction, by mediating via complement local recruitment of antigen-specific T cells that are necessary for elicitation of this response [185-186].

Interestingly, inherent to the natural IgM repertoire is also self-reactivity. Several studies have reported that natural IgM can bind self antigens such as phospholipids, like phosphorylcholine (PC) and lysophospolipids [187], cardiolipin (a lipid constituent of mitochondrial membrane), as well as nuclear antigens and single stranded DNA [188]. A common feature of most of these antigens is that they become exposed on the surface of apoptotic cells. By virtue of these reactivities sIgM has been demonstrated to facilitate clearance of apoptotic primary cells by phagocytic cells, namely macrophages and dendritic cells [187-190]. In humans, natural IgM reactive to Fas, (an apoptosis-inducing receptor) isolated from healthy individuals was also shown to induce apoptosis of human lymphoid cell lines and peripheral blood mononuclear cells, suggesting a protective role of sIgM in lymphoproliferative disorders [191]. In addition, IgM can bind antigens present on atherosclerotic plaques [192], which may facilitate their clearance by phagocytic cells in atherosclerosis-susceptible mouse strains [193]. Another potential function of IgM associated to its self-reactive repertoire is tissue repair, which was indicated from studies on virus-induced demyelination. When chronically infected mice were immunized with spinal cord homogenate they displayed enhanced remyelination and sera from these mice could „transfer“ this effect in unimmunised recipients [194]. Immune sera contained IgM clones that were able to bind antigens on the surface of oligodendrocytes and promote remyelination. Of note, administration of these clones did not affect the viral load, highlighting their specificity in mediating tissue repair [194-195].

The generation of $\mu S^{-/-}$ mice revealed a role for sIgM the development of B cell subsets, as absence of sIgM resulted in increased numbers of B1 and MZ B cells and reduced numbers of FO B cells [183, 196]. Notably, repeated administration of exogenous polyclonal, but not monoclonal, sIgM from naive mice restored this defect, indicating that the polyreactivity of IgM is important in the fate decision of developing B cells [196]. The authors proposed that polyreactive IgM boung to self antigen enhances the signal strength of BCR on developing B cells, which in turn favors the formation of FO over MZ B cells [197]. Although this is an attractive hypothesis, its formal proof as well as how IgM regulates B1 cell development are yet to be shown.

Due to its inherent self-reactivity and its enhancing effects in immunity, IgM would have been expected to greatly contribute to autoimmune pathogenesis. Studies with sIgM-deficient mice revealed the contrary. On lupus-prone background, lack of sIgM resulted in appearance of higher titers of anti-nuclear and histone-reactive IgG antibodies compared to wild-type mice, correlating with accelerated and worse nephritis pathology [198]. Consistently Neuberger and colleagues reported that in the absence of IgM, lupus-resistant naive C57BL/6 mice harbored higher titers of anti-DNA IgG and even displayed, albeit to a small extent, IC deposition in renal tissue [199]. Potentiating the significance of these studies, anti-DNA IgM levels inversely correlated with lupus nephitis in a subgroup of SLE patients [200]. Furthermore, transfer of monoclonal anti-DNA IgM prevented nephritis in lupus-prone mice [201], though not always [198]. These observations demostrated a protective role of IgM in lupus disease, and raised the question whether this is a general property of IgM in other autoimmune disorders, which is yet to be explored.

1.3.4 B cells as antigen presenting cells

It is becoming evident that B cells can influence immune responses through antibody-independent functions. One way by which B cells can modulate immune responses independently of antibody production is through antigen presentation. Similar to DC, activated B cells express high levels of MHC II and co-stimulatory molecules and can thereby provide CD4 T cells with signals essential for their activation. DC have been demonstrated as the most efficient and sufficient APC to activated and prime T cells [11], a notion also supported by *in vivo* imaging studies showing that they are the first APC to interact with T cells in LN upon antigen inoculation [202]. Whether the APC function of B cells is of equal importance in T cell responses has been the subject of several studies, yet conflicting results have been generated. In some studies congenital absence of B cells (B cell-deficient mice) led to impaired T cell responses to model antigens or infections [203-207], whereas in others B cells were found dispensable for T cell priming and expansion [208-210]. A few reports also using B cell deficient mice indicated that B cells could be critical amplifiers of T cell responses to protein, but not peptide antigens [211-212], which are rather more efficiently captured by DC [211]. It should be noted that B cell-deficient mice is not an ideal tool to selectively study the direct APC function of B cells, as B cells can also promote antigen presentation by DC and macrophages through antibodies/IC, which are obviously absent in these mice. In addition, congenital absence of B

cells results in disrupted splenic architecture and defects in gut-associated lymphoid structures (see next section), abnormal frequency of T cells in spleen, altered homeostasis of DC subsets, loss of FDC and certain splenic stromal cells. Conceivably, these abnormalities can affect T cell responses and confound studies that address the role of B cell APC function upon oral antigen-inoculation or oral infections [204].

Avoiding the caveats of B cell-deficient mice, a group of studies assessed the APC function of B cells using BCR-transgenic (tg) B cells, that recognize a known protein antigen. They suggested that antigen-specific B cells could present antigen to T cells as early as a 6 hours after antigen encounter [213-214]. Although these findings are in agreement with the notion that antigen-specific B cells can be efficient APC for T cells [133], they are hampered by the fact that the frequency of antigen-specific B cells in naive wild-type mice is, in contrast to BCR-tg mice, very low. In a physiological setting, Germain and colleagues using an antibody against antigen-MHC II complexes could visualise *in vivo* wild-type antigen-loaded B cells also within few hours after immunization. However, their ability to stimulate T cells *in vivo* was not addressed [215].

Altogether, due to their low frequency in normal naive mice, antigen-specific B cells are unlikely to serve as the primary APC driving the initial activation of cognate naive CD4 T cells. Indeed, even if reduced, CD4 T cell responses were not completely abrogated in B cell-deficient mice. Once they accumulate following activation, antigen-specific B cells may infulence the priming and expansion of T cells via antigen presentation. In agreement with this, bone marrow chimeric mice lacking MHC II selectively in B cells diplayed impaired antigen-specific T cell responses [216]. Presumably, within the context of antigen presentation, B cells may influence CD4 T cell responses also by provision cytokines (see next section) and co-stimulatory signals. For example, expression OX40L by B cells was required for optimal expansion and cytokine production by Th2 cells [217], whereas their expression of CD86 was critical for their ability to suppress Th1 T cell responses [218].

Antigen presentation is considered as a critical antibody-independent function of B cells contributing to pathogenesis in autoimmunity. This is supported by the observation that the effects of B cell depletion in mice and patients suffering from certain autoimmune diseases, do not always correlate with significant changes in antibody titers, despite the pathogenic role of antibodies in these diseases [119]. Schlomchik and colleagues [219] were the first to elegantly demonstrate *in vivo* the antibody-independent role of B cells in experimental lupus, by generating lupus-prone mice that lack the ability to secrete antibodies. Similar to wild-type, but

not to disease-resistant B cell-deficient mice [220], these mice still developed characteristic lupus pathologies, including nephritis, and intact autoantigen-specific T cell responses. Thus, the authors showed that the autoreactive B cells promoted autoreactive T cells in the absence of antibodies, most likely through antigen presentation and/ or cytokine production (see next session), though the exact mechanism was not investigated. This study was in agreement with earlier data proposing that B cells may constitute an important autoantigen-specific APC in augmenting autoreactive T cell activation under immunogenic conditions [221-223]. The contribution of B cells as important APC for CD4 T cell responses has been implicated in pathology of several other autoimmune diseases, including models of RA [224-225], diabetes [226-228] and MS (described in later section). Notably, in some of these diseases, this function maybe preferentially carried out by certain B cell subsets. For example, in mice, diabetes onset and activation of autoreactive T cells coincide with a dramatic expansion of MZ B cells, that express high levels of MHC II and costimulatory molecules and can efficiently present autoantigen to diabetogenic CD4 T cells *in vitro* [229-230]. Why MZ B cells preferentially expand is not clear. Nevertheless, this is consistent with the reported capacity of this B cell subset to directly activate T cells [231]. Besides promoting CD4 T cell responses to a particular autoantigen, antigen-presentation by B cells may also facilitate diversification of self-epitopes under immune attack [232], a process known as epitope spreading. An involvement of B cells in epitope spreading has been suggested for diseases, such as diabetes [233-234], autoimmune thyroiditis [234] and lupus [235].

The role of B cell in CD8 T cell responses has been mostly studied using B cell deficient mice and is not thoroughly explored. These studies revealed that B cells are not required for optimal primary and memory CD8 T cell responses to certain bacteria and viruses, despite that antigen-specific CD8 T profoundly contracted in the absence of B cells [236-238]. In contrast, B cells may influence CD8 T cells during chronic infections [239]. Similarly, absence of B cells in non obese diabetic mice, resulted increased CD8 T cell death in pancreatic islets [240]. In a model of colitis, B cells and CD8 T cells were both required for suppression of colitogenic CD4 T cells, however, whether protection involved B-CD8 T cell contact was not determined [241]. B cells have been reported to efficiently cross-present protein antigens-conjugated to microbial products [242-243], though the *in vivo* relevance of these findings await further demonstration.

Lastly, apart from classical MHC I molecules, B cells express non-classical or class Ib ones, including CD1d, MR1, and Qa-1, of which CD1d the most studied so far. Through CD1d B cells can uptake lipid antigens in BCR-mediated manner, and present them to invariant NKT cells,

that in turn promote extrafollicular B cell proliferation, and antibody production [244]. As discussed below, in certain autoimmune diseases, high expression of CD1d on a subset of B cells coincides with their increased to secrete the anti-inflammatory cytokine IL-10, although whether these two features are functionally linked remains unclear.

1.3.5 B cells as cytokine-producing cells

The ability of primary mouse and human B cells to produce cytokines has been initially documented in the late 1980s [245-249], yet its significance in immune responses has only begun to be elucidated during the last decade. One of the first demonstrated functions mediated by cytokine-producing B cells is the organization secondary lymphoid tissues. B cell-derived TNF and lymphotoxin (LT) are required for development and maturation of splenic FDC networks [121, 250-251]; the organization of B cell follicles in the spleen [251] and Peyer´s patches, a gut-associated lymphoid structure [252]; the development of stromal cells important for the formation of the splenic T cell zone [253]. Lastly, via expression of LT, B cells also regulate homeostasis of splenic DC subsets [253-254]. Defects in these cytokine-dependent functions of B cells may contribute to the immune defects observed in B cell deficient mice and human (for example lack of TD B cell responses like GC, possibly arising from impaired FDC networks).

Additional cytokine expression profiles have been described for B cells. In vitro co-culture of mouse B cells with cognate Th1-polarized cells led to the generation of B cells secreting Th1-related cytokines, like IFNγ and IL-12, whereas in the presence of Th2 cells, B cells produced IL-4, IL-6 and IL-10 [255]. In addition, both co-cultures induced IL-2 secretion by B cells [255]. B cells able to produce IFN-γ or IL-4 have also been isolated from mice infected with Th1-inducing [255-256] or Th2-inducing [255, 257] pathogens, respectively, although their *in vivo* role was not determined. Conversely, B cell-derived cytokines may affect the differentiation and expansion of Th cells. When stimulated *in vitro* with TLR agonists and T cell help signals (CD40 ligation) mouse [258] and human [259-260] B cells made IL-12 and thereby, promoted IFN-γ secretion by Th1 cells. It should be noted that under these stimulation conditions B cells also secreted cytokines, such as IL-6 and TNF cells which may also affect Th cell responses. Indeed, another study suggested that via provision of TNF, B cells could amplify IFNγ production by CD4 T cells during infection with *Toxoplasma gondii* [261]. IL-6 is required for differentiation of Th17 cells

[117]. It is thus plausible that IL-6 from B cells could amplify Th17 responses, for example during Th17-associated autoimmune diseases, such as EAE, or fungal infections, yet its role *in vivo* has not been investigated thus far. B cell-derived cytokines may also aid host defense to Th2-inducing pathogens. Lund and colleagues showed that during infection with the intestinal parasite *H.polygyrus*, TNF from B cells facilitated the maintenance of humoral responses, while B cell-derived IL-2 promoted the expansion of parasite-specific Th2 cells [262]. Recently, LT from B cells was suggested to contribute to metastasis of prostate cancer in mice [263], implicating B cell cytokine production in regulating tumor responses.

B cells are likely active cytokine producers in autoimmune diseases. Tonsils from Sjögren's syndrome patients contain B cell populations with distinct cytokine signatures, producing Th1- or Th2- related cytokines [264], similar to mouse B cells mentioned earlier. Interestingly, these populations resided in different locations, indicating that they may regulate distinct local responses [264]. Cytokine-producing B cells have been also described in MS patients. B cells isolated from peripheral blood of these patients produced *ex-vivo* increased amount of proinflammatory cytokines, including TNF, LT, compared to healthy donors [265]. Although the precise role of these cytokine-producing B cells needs to be defined, these studies indicate that the contribution of B cells in autoimmune disease encompasses not only antibodies, but cytokine secretion as well.

Apart from inflammatory cytokines, B cells can produce cytokines with anti-inflammatory properties, such as TGFβ [266] and IL-10, endowing them with the ability to dampen immune responses. TGF expression by B cells and TGFβ-IgG complexes were shown to suppress host response to *Staphylococcus aureus in vivo* by inhibiting neutrophil functions [267]. Another study indicated that via surface expression of TGFβ, B cells could induce anergic CD8 T cells [268]. Despite these early reports, the role of TGF-producing B cells in immune responses has remained largely unexplored.

Production of IL-10 by B cells is most frequently documented. Although originally identified as B cell-growth factor and mast cell stimulator, IL-10 exerts potent anti-inflammatory effects, and is the signature cytokine for several regulatory T cell subsets, such as natural and type 1 T regulatory cells [117]. Mizoguchi *et al* first described a suppressive role for IL-10 producing B cells in a Th2-driven intestinal inflammation that spontaneously develops in TCRα-deficient mice [123]. The authors had previously observed that B cells suppress inflammation in this model, because their absence results in accelerated and more severe pathology [117]. They

subsequently found that inflammation induces IL-10 mRNA expression in B cells from mesenteric LN, and remarkably, transfer of wild-type B cells ameliorated full-blown disease in recipient mice [123]. B cell-deficiency in mice also leads in exacerbated and chronic CNS autoimmunity, in EAE [269], in contrast to B cell-sufficient mice that spontaneously recover. By generating mice lacking IL-10 selectively in B cells, Fillatreau *et al*, elegantly demonstrated that B cell-derived IL-10 controlled recovery from disease, by limiting autoreactive Th1 responses [270]. Furthermore, B cells from recovered mice displayed recalled IL-10 production *ex vivo*, and ameliorated disease symptoms in recipient mice upon adoptive transfer. A suppressive role of IL-10-producing B cells has also been demonstrated in collagen-induced arthritis (CIA), an experimental model of RA. Stimulation of B cells from arthritogenic, but not naïve, mice with anti-CD40 induced a population of IL-10-secreting B cells, that were able to suppress Th1 responses and prevent arthritis development in recipient hosts [271]. Consistent with a regulation of Th1 responses by B cells, DC from B cell-deficient mice expressed increased amounts of IL-12 compared to those from wild-type mice [272], and patients with X-linked agammaglobulinemia, who lack circulating B cells, show a preferential Th1 response *ex vivo* [273]. Considering that B cells are also critical drivers of pathology RA models (as APC and antibody producers), the finding that they can also exert inhibitory effects, provided a first hint that B cells can act both as perpetuators and regulators of autoimmune responses. Tedder and colleagues recently demonstrated that this notion also applies to EAE by performing B cell depletion experiments. They showed that whereas early B cell depletion exacerbated disease, depletion at later stages ameliorated clinical symptoms, indicating a previously unappreciated pathogenic contribution of B cells in this disease model [274].

Interestingly, IL-10 producing B cells may also exert suppressive functions in autoimmunity in humans. B cells isolated from MS patients made less IL-10 upon stimulation, compared to healthy controls, implicating IL-10-producing B cells in protection from disease [275-276]. Moreover, B cell depletion in an UC patient led to a concomitant loss of intestinal IL-10, correlating with exacerbated pathology [277]. B cells from SLE patients able to produce IL-10 have also been described [278], however their protective role is unclear, consistent with a pathogenic role of IL-10 in this disorder [279].

1.4 Multiple Sclerosis and its experimental model

Multiple sclerosis: Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS; brain and spinal cord) leading to progressive disability. It affects more than one million people worldwide, women twice as frequently as men [280]. Clinical onset of MS usually occurs within 20 to 40 years of age, and it is the most common neurologic disease affecting young adults [281-282]. Following disruption of the brain blood barrier (BBB; [283], inflammation is thought to cause acute lesions and to contribute to gradually-appearing multiple areas of scarring (sclerosis) in the white matter throughout the brain and spinal cord [284-285], and hence the name of the disease. Sclerotic plaques are the “end product” of several processes occurring at affected regions, including destruction of the myelin sheath surrounding the neuronal axons (demyelination), incomplete re-myelination, oligodendrocyte loss, astrocytosis, axonal damage and eventual neuronal loss [286]. Clinical manifestation and disability occurs following damage of the sensory, motor, visual and autonomic systems, and often impairment of neurocognitive functions [281, 286].

The clinical course of disease varies depending on the type of disability progression (Fig. 1.3). A vast majority of approximately 80% of patients initially present with a relapsing- remitting form (RR-MS) [281, 286], whereby episodes of demyelination attacks in different areas of the CNS often resulting in permanent deficits (relapses) alternate with subsequent spontaneous recovery periods (remission) over a period of about 25 years [286]. The incidence of new episodes is unpredictable, but the relapse rate can generally reach a maximum of 1.5 per year [286]. With time recovery becomes incomplete and about 65% of RR-MS patients enter a secondary progressive phase characterized by steady increase of symptoms and disability [286]. The other 10-15 % of MS patients present with an insidious disease onset and directly follow a steady progression course, referred to as primary progressive (PP)-MS [286], which is the most aggressive form of MS. PP-MS occurs in older individuals compared to RR-MS, and with similar frequency in women and men [285]. Noteworthy, PP-MS differs from RR-MS also in that CNS pathology is characterized by little inflammation [286]. The factors and mechanisms underlying these different forms of MS remain enigmatic. Additional heterogeneity exists among patients of a particular MS type, for example among RR-MS patients, with respect to morphological features of pathology, the CNS areas

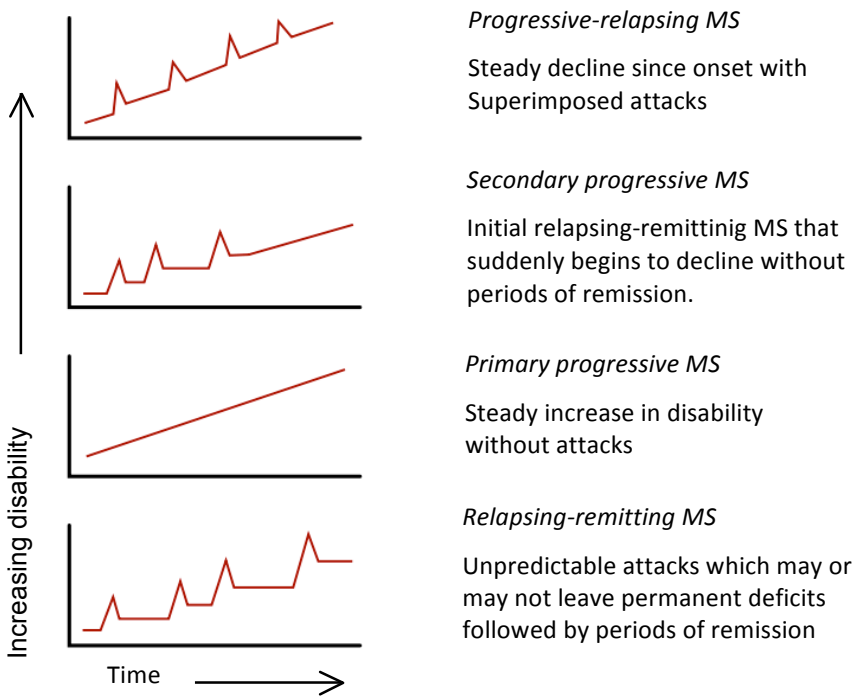

Figure 1.4 Different forms of multiple sclerosis according to the progression of disability. Adapted from [287]

affected and responsiveness to treatment. Furthermore, it is not clear whether inflammation continuously drives all pathogenic processes throughout the disease course or whether a later part of pathogenesis depends on other factors [286]. It is currently believed for example that susceptibility to neurodegeneration rendering neurons vulnerable to accumulating injury, and ability for tissue repair, are inherent factors in MS patients that also determine the degree of disease severity after onset of inflammation [281, 286]. There is no curative therapy for MS, and most of the treatments available, though beneficial in some patients, reduce the frequency of relapses only by 30%, resulting in a modest delay of disease progression in RR-MS and secondary progressive MS [288-289].

The etiology of MS involves both environmental and genetic factors. A role for environmental exposure is supported by the geographical pattern of disease prevalence around the world. In particular, the prevalence of MS varies between 60–200/100,000 individuals in Northern Europe and North America and 6–20/100,000 in low risk areas such as Japan [290]. A series of studies have indicated that MS might be triggered by viral infections, with Epstein-Barr virus being a suspected candidate [291-292]. Such infections would lead to CNS autoimmunity

presumably via molecular mimicry and/or bystander activation [293-294]. This notion led to the development of beta interferons (IFNβ -1a and -1b) as an antiviral therapy for MS, which is one of the current treatments for RR-MS [295]. However, despite the relative efficacy of this treatment, definitive proof for a viral agent triggering disease is missing [296-297]. The contribution of environmental stimuli is further suggested by the relatively low concordance rate of MS in twins, which is below 50% [289]. Nevertheless, according to epidemiological studies in certain areas, concordance rate is higher in monozygotic than dizygotic ones (25% and 5%, respectively), and family history confers higher disease risk [286], indicating a role for hereditable susceptibility. Indeed, genome-wide screens have identified susceptibility alleles encoding for certain HLA class II (MHC II) molecules, as well as susceptibility-conferring single nucleotide polymorphisms in the α chains of IL-2 receptor and IL-7 receptor (*IL2RA* and *IL7RA*) genes [282, 298-300].

MS as an autoimmune disease: Multiple sclerosis is considered an autoimmune disease. Evidence for the involvement of the immune system in mediating pathogenesis derives from several findings. First, CD4 and CD8 T cells, B cells and plasma cells, and activated macrophages are found within CNS lesions [301-304], they form perivascular cuffs and/or migrate to the surrounding parenchyma [305]. In addition, plasma cells and antibodies are increased in the cerebrospinal fluid of MS patients, and, in fact, the presence of intrathecal oligoclonal IgM bands is a diagnostic criterion for MS [286]. The expression of several inflammatory cytokines that are produced by cells of the immune system, though not exclusively, is another consistent finding in CNS lesions MS patients [280]. Advocating a CD4 T cell –mediated pathogenesis is also the strong association of HLA II haplotypes with genetic risk, presumably through preferential presentation of autoantigens [283]. Autoreactive CD4 T cells specific for myelin antigens are found in both healthy individuals (and experimental animals) and MS patients. The over-representation of certain MHC II molecules in MS patients may account for the transient expansion of myelin-reactive CD4 T cells in patients with higher during disease activity [306]. Alternatively, these HLA II molecules may poorly bind myelin antigens in the thymus, thereby promoting the selection of autoreactive CD4 T cells with higher avidity TCR compared to healthy individuals [283]. Finally, current treatments of MS, including IFNβ, glatiramer acetate [307], natalizumab [308-309], mitoxantrone [310], and the recently approved fingolimod [311-313] are thought to largely function by modulating immune responses.

Experimental autoimmune encephalomyelitis (EAE): Many of the current therapies for MS and several emerging ones now in clinical trials, have been developed based on studies from diverse animal models (mostly rodent) of MS [289], collectively termed EAE. This is perhaps the strongest argument favoring the use of experimental models to understand and help treat human MS. There is no single model faithfully reflecting all heterogeneous features of MS. It is rather that different models resemble distinct aspects of the human disease, with respect to CNS histopathology, for instance demyelination, sclerotic plaques and axonal damage, as well as to immune-mediated disease mechanisms [305]. The genetic risk factors are also shared to a considerable extent between MS and EAE. The MHC II haplotype of experimental animals critically determines their susceptibility to disease [314], and IL-7Ra also confers susceptibility to EAE [315-316]. Gaining insights for MS from EAE is being greatly aided by growing technological advances that allow, among others, sophisticated genetic manipulations, cell targeting for therapeutic purposes, *in vivo* imaging of CNS cells and its interactions with inflammatory infiltrates. Conversely, animal models can be used to test the biological role of disease-relevant molecules identified in patients by similar advances and high-throughput screens, like recently shown [317-320]. It should be noted that animal models have been poor predictors of success and safety of a number of immunomodulating drugs tested in MS clinical trials [289]. This could be attributed to differences between the rodent and human immune systems, and to the failure of experimental animals to develop many of the adverse effects observed in humans, including the fatal progressive multifocal leukoencephalopathy caused by opportunistic infection [289, 305]. Despite these pitfalls, given the complexity of MS and the restricted accessibility to human CNS tissue (only post-mortem samples are used), it is current consensus that EAE models remain valuable tools for both drug discovery and dissecting pathogenesis of MS.

The first EAE-resembling model was described in early 1930s in Rhesus monkeys [321]. To date several EAE models have been developed mainly using rodents-susceptible inbred strains of mice and rats- owing to their easier handling and for allowing genetic manipulation. According to the mode of disease induction, these models can be grouped into actively induced EAE, passive EAE transfer and spontaneous EAE. Active disease is induced by immunization with a myelin-derived protein or peptide emulsified in adjuvant (complete Freund´s adjuvant; CFA). The most commonly used antigens are the frequent myelin components myelin basic protein (MBP) and proteolipid protein (30% and 50% of total myelin proteins, respectively), as well as the less frequent myelin oligodendrocyte protein (MOG; 0.05%). Notably, T cells and antibodies

reactive to these antigens have been reported in MS patients [283, 322], although non-myelin antigens may act as targets as well [314]. Commonly for most models, within 10 to 20 days after immunization animals begin displaying cumulative paralytic symptoms progressing from flaccid tail and impaired righting reflex to hind and front limb paralysis. Once full-blown symptoms develop, disease may be chronically maintained, followed by spontaneous remission or assume a relapsing-remitting course, with disease episodes succeeded by partial recovery periods. Whole lymphocyte populations or CD4 T cells isolated from secondary lymphoid organs of actively primed animals can cause EAE when adoptively transferred into naïve hosts. This is the so-called passive EAE induction. Transfer of MOG-reactive CD8 T cells isolated from MOG-primed animals, can also induce passive EAE, although this model is less frequently used [323]. Lastly, depending on the strain of mice or hygiene of housing conditions, spontaneous EAE or EAE syndromes can develop in certain transgenic mice carrying MBP-specific TCR [324] or in double transgenic mice with MOG-specific TCR and BCR [325-327]

In the current study, EAE was actively induced by immunization of C57BL/6 mice with a MOG-derived peptide (pMOG) in CFA, based on earlier work by Mendel *et al* [328]. Of note, studies on this EAE model have led to the development of some of the current MS treatments. Despite its low abundance among myelin proteins, MOG is part of the outer part of myelin sheath making it readily accessible to cells of the immune system [329]. MOG-induced EAE resembles CNS pathophysiological features of human MS in both mice [328] and rats [330], and is consistent with a MOG-response observed in some MS patients [331-333]. In mice, disease occurs at an 80% incidence or higher, is robust and the clinical course is highly reproducible. Within 20 days, immunization results in a single full-blown disease episode (as described above) followed in this case by a recovery phase, during which paralytic symptoms spontaneously resolve. This recovery phase can viewed as a relative mimic of the spontaneous remission occurring in RR-MS patients and thus makes this model attractive to study the endogenous mechanisms driving disease resolution and to thereby identify potential therapeutic targets. Considering that EAE is an immune system-driven disease, this model can be used in a broader sense to study how immune responses are naturally regulated.

1.4.1 Role of T cells in disease pathogenesis and regulation

Pathogenic contribution: MS symptoms are commonly believed to develop following entry of autoteactive T cells in the CNS [283]. Depletion of T cells (along with other immune cells) using

anti-CD52 antibody remarkably reduced relapse rate in early diagnosed, untreated RR-MS patients [288]. In addition, drugs interfering with the migration of lymphocytes in the CNS or their egress from peripheral lymphoid organs, natalizumab and fingolimod, respectively, were effective in MS patients [334-335]. In EAE, immunization initially induces the activation and expansion of myelin-specific T cells in peripheral lymphoid organs (draining lymph nodes and spleen) which then cross the BBB and enter the CNS. Once there, an effector phase ensues during which effector T cells orchestrate an inflammatory response via cytokine and chemokine secretion, promoting the activation of CNS-resident microglial cells (macrophage-like cells of the CNS) and recruitment of macrophages. Consequently, local tissue damage occurs resulting in the appearance of clinical symptoms. The size of inflammatory infiltrates, including T cells and phagocytic cells, progressively increases correlating with higher disease severity. Consistently, recovery from disease coincides with the disappearance of infiltrating cells from the CNS and resolution of local inflammation. Depletion of CD4 T cells prior to disease induction or during established EAE respectively prevents or ameliorates disease [336], demonstrating that CD4+ T cells are critical drivers of EAE.

The effector functions of autoreactive CD4 T cells are tightly associated with their contribution to disease. The predominant Th subsets currently linked with disease pathology are Th1 and Th17. Studies in late 1980s showed that encephalitogenic myelin-reactive CD4 T cells clones were of Th1 phenotype, as they secreted IFN-γ -and other Th1 associated cytokines, like TNF and lymphotoxin [337-339]. Consistently, administration of recombinant IFN-γ in MS patients worsened disease [340-341]. Furthermore, mice deficient in T-bet, the critical transcription factor for Th1 differentiation, or in IL-12p40, a subunit of the key Th1 polarizing cytokine IL-12, were resistant to EAE [342]. Curiously, however, lack or blockade of IFN-γ worsened EAE [343-344] and IL-12Rbeta2 deficient mice, which lack responsiveness to IL-12 were susceptible to EAE [345]. It was later reported that the IL12p40 subunit is shared by IL-23 [346], a cytokine involved in the survival and expansion of the recently identified Th17 subset [347-350]. Th17 cells secrete IL-17 and other cytokines [351], and their differentiation requires the transcription factor RORγt [352]. They can be primed in presence of IL-6 and TGF-β [353-355] IL-17- or IL-17R deficient mice develop milder EAE, as do mice treated with blocking anti-IL17 antibody, supporting a pathogenic role of IL-17 in this disease [350, 356-358]. However, overexpression of IL-17 specifically in CD4 T cells did not exacerbate EAE, calling into question a pathogenic contribution of Th17 cells [359]. Of note, IL-17 is also produced by NKT and γδ-T cells, which may also influence EAE [360-361] [362]. Other studies showed that Th1 and Th17

cells can independently transfer EAE and may even induce distinct CNS pathology [363-364], though the one induced by Th1 cells was more closely resembling the human MS lesions [363]. Transcriptome and immunohistochenical analysis of MS lesions revealed increased transcripts of IFN-γ, IL- 6 and IL-17-encoding genes, [317, 365], and elevated protein expression of IL-23p19, p40 [366] and IL-17 [367]. Thus, it is likely that both IFN-γ and IL-17 are involved in EAE and MS disease mechanisms, although the exact role of Th17 cells awaits further investigation. In the EAE model used in the present study, MOG-reactive IFN-g- or IL-17- producing CD4 T cells expand in peripheral lymphoid organs within 6 days after immunization, and are also found in the CNS upon disease onset and duration.

A role in MS and EAE pathogenesis is also attributed to CD8 T cells. Initial studies revealed that CD8 T cells often predominate over CD4 T cells in CNS lesions and their numbers correlate with axonal damage in MS plaques [301-302, 368-370]. Subsequent reports showed that MS lesions contained expanded CD8 T cell clones [371-372], implying that antigens in the CNS drove their local activation. Whether these cells are myelin-specific awaits confirmation. It is not clear how CD8 T cells contribute to MS pathology. Through cytokine production, like IFN-γ, and cytotoxicity, CD8 T cells could respectively, promote local inflammation and/or contribute to neuronal damage and/or oligodendrocyte death. In support of this notion, CD8 T clones isolated from MS patients produced IFN-γ, and killed cells transfected with myelin-derived antigens, albeit similar to CD8 T cells isolated form healthy controls [373]. Furthermore, according to a recent study of a viral infection of the CNS, CD8 T cells may contribute to pathogenesis by recruiting myeloid cells/monocytes to CNS [374]. Frequently expressed MHC I alleles have been identified in MS patients compared to controls, but their association with MS susceptibility is not as strong as observed for MHC II alleles [375]. Studies in EAE have also indicated a pathogenic role for CD8 T cells. MOG-reactive CD8 T cells are present both in peripheral lymphoid organs and in CNS of immunized mice, and secrete IFN-γ upon *in vitro* restimulation with MOG peptide [376]. The encephalitogenic capacity of MOG-reactive CD8 T cells was further demonstrated by their ability to cause EAE upon adoptive transfer in naive recipients [323]. Considering this evidence, MOG-reactive IFN-γ-producing CD8 T cells were assessed in the current study.

Regulatory role: A collection of studies has suggested that the autoimmune response in MS and EAE might result from defects in various regulatory T cell subsets that can control

convetional effector T cells. The CD4+CD25+ regulatory Tcells (Tregs) from subgroups of MS patients showed reduced *in vitro* suppressive activity compared to healthy individuals [377-378], or diminished expression of Foxp3 [379], whose optimal levels is critical for their stability and function [380-381]. A defect has also been reported for CD4+ T regulatory type 1 (Tr1) cells, which are thought to exert their function by secreting IL-10 or co-secreting IL-10 and IFN-γ [382]. This was associated with decreased IL-10 production [383-384], and a study implied this defect might be amplified by a reduced responsiveness of conventional $CD4^+$ T cells to IL-10 receptor signaling [384]. Additional indications come from observations that some of these populations might be increased by current MS treatments, including IFN beta and glatiramer acetate [385-386]. It should be noted though that it is unclear whether these abnormalities are causes or consequences of the disease. In EAE, depletion experiments of $CD4^+CD25^+$ Treg cells, have demonstrated that these cells control disease severity, are required for remission and, in particular in pMOG-induced EAE, they temporarily account for the resistance of recovered mice to subsequent disease re-induction [387-390]. These Treg cells accumulate in the CNS of EAE-induced mice with a similar temporal pattern as infiltrating effector T cells [388-389], however, their exact mode and place of regulation during disease remains enigmatic.

1.4.2 Role of B cells in disease pathogenesis and regulation

Pathogenic contribution: MS has been traditionally considered as T cell-mediated disease, despite the long known presence of B cells and plasma cells in CNS lesions, plasma blasts, and oligoclonal Ig bands in CSF of MS patients. This view has been recently challenged by growing evidence urging for re-assessment of the role of B cells in disease. Perhaps the most convincing evidence comes from a phase II clinical trial, which demonstrated that depletion of mature B cells using a monoclonal antibody, rituximab, remarkably reduced relapse rates in about 30-40 % of RR-MS patients treated [391]. This apparently pathogenic contribution of B cells may rely on different mechanisms, including antibodies, antigen presentation and cytokine production.

In the CSF, the intrathecal IgM and IgG have been recently suggested to arise from local plasma blasts, and both Ig and plasma cells seem to correlate with early MS onset and ongoing active inflammation in RR-MS, albeit not with disease activity [392-394]. Histopathological analyses of MS lesions and CSF, suggested that presumably myelin-specific antibodies are involved in plaque initiation and demyelination, by activating macrophages via Fc receptors and

complement. A recent study indicated that non-myelin-derived molecules may also be targeted by antibodies [319-320] Thus far, despite extensively addressed, neither the specificity nor the exact mechanisms of antibody-mediated pathogenesis have been clarified [395]. The role of antibodies in EAE is similarly unclear. Unlike myelin-specific T cells, myelin-reactive antibodies alone fail to transfer disease in naive hosts. On the other hand, they can worsen disease when administered at the time of disease induction [396-398], although not always [399]. In the pMOG–induced EAE, given that B cell-deficient mice are susceptible to EAE, antibodies are not required for disease development, but pMOG-reactive antibodies are generated upon immunization [398]. Moreover, Becher and colleagues showed that activatory-FcγR-deficient mice develop only mild EAE that worsened after transfer of exogenous MOG- specific IgG2b, possibly through a complement dependent manner [398]. Their study further implied that, despite its efficiency at activating complement, IgM might not be pathogenic at least in the absence FcγR. However, the role of individual antibody isotypes during EAE has not been dissected thus far.

Besides the CSF and parenchymal MS lesions, B cells can reside at the meninges, a set of membranes that surround, and thereby protect, the brain and spinal cord. There B cells have been found in organized germinal centre- like follicles, called ectopic B cell follicles [400-401]. It is thought that B cells derived from these follicles could result from local cognate B-T cell interactions, through which B cells further promote T cell responses via antigen presentation. Their function and contribution to disease pathogenesis remain unclear, as does whether the efficacy of rituximab in RR-MS patients [391] correlates with their elimination. Furthermore, ectopic follicles together with increased levels of BAFF and of the B cell chemoattractant CXCL13, in peripheral blood, MS lesions, or CSF correlate with early and active inflammation, albeit not with disease stage [402-405]. Similar observations have been reported in a relapsing-remitting EAE model [406].

The notion has been recently raised that B cells could contribute to MS pathology via cytokines. The cytokine profile of B cells from untreated MS patients appears to be skewed towards an inflammatory type, characterized by increased secretion of lymphotoxin, TNF and IL-6 [265, 276]. B cell depletion in some of these patients resulted in diminished peripheral Th1, Th17 and CD8+ T cell responses and this effect was reversed when supernatants of activated B cells from untreated patients were added to the T cell cultures [265] *in vitro*.

Collectively, these observations suggested that, via intrinsic cytokine deregulation or other mechanisms, B cells play pathogenic role in RR-MS, and B cell-targeting therapies have become an immense focus of ongoing developments.

Role in disease suppression: These developments however, might not be as straightforward to achieve. Rituximab exacerbated symptoms of ulcerative colitis patients, triggered psoriasis in SLE and RA-suspected subjects, certain infections, as well as PML [407]. In MS, B cell-depletion resulted in relapses in a patient [408], and a clinical MS trial testing a BAFF antagonist was terminated due to increased disease activity (**http://clinicaltrials.gov/ct2/show/NCT00642902)**. Therefore, B cells may both perpetuate and regulate disease. This notion was elegantly demonstrated in pMOG-induced EAE by B cell-depletion experiments. Rituximab exacerbated disease when administered before EAE induction, whereas the same treatment delivered after clinical onset had occurred, ameliorated disease [274]. A regulatory role for B cells in CNS autoimmunity had been earlier demonstrated by Fillatreau *et al* who showed that in contrast to wild-type mice, B cell deficient mice failed to recover from disease induced in the pMOG model [270]. That study further identified IL-10 as a cytokine required by B cells to suppress EAE: mice lacking IL-10 selectively in B cells suffered chronic disease, resembling B cell-deficient mice [270]. Further supporting a role of IL-10-producing B cells in disease regulation, B cells from mice treated with glatiramer acetate, were remarkably more potent at suppressing EAE upon transfer in naive hosts compared to those from untreated animals [409-411], and this effect correlated with increased IL-10 secretion [411].

IL-10-producing B cells have been reported in healthy individuals and notably, a few studies indicated that B cells from peripheral blood of MS patients make less IL-10 than B cells from healthy subjects [265, 275-276, 412]. The aforementioned increased production of inflammatory cytokines by B cells from untreated MS patients was accompanied by a parallel reduction in their secretion of IL-10. Moreover, when some of these patients were treated with mitoxantrone, their B cell production of IL-10 was restored [276]. Altogether, these findings advocate the notion that IL-10 producing B cells could have a disease-modulating effect, and importantly, they could be therapeutically targeted.

Search for signals inducing IL-10-production by B cells: Inducing or enhancing IL-10 production by B cells could be a beneficial therapeutic approach. However, little is known about the signals that drive the generation of IL-10-producing B cells from their naive precursors. B cells isolated from EAE-recovered mice made IL-10 *in vitro* after concomitant stimulation through BCR and CD40 [270] and inhibited disease development when transfered to naive hosts. Consistent with this, mice in which only B cells lack CD40 or in which B cell express a disease irrelevant antigen (hen-egg lysozyme) failed to recover from EAE, alike mice with IL-10-deficient B cells [270]. Thus, signals via antigen-specific BCR and CD40 are required for IL-10 production and *in vivo* suppressive capacity of B cells. These signals have also been shown to be involved in IL-10-production by B cells in CIA [271, 413]. A role for BCR in B cell-mediated suppression is further supported by the finding that mice deficient in CD19, a B cell co-receptor promoting BCR signaling [414-415] also develop severe EAE [416]. However, BCR and CD40 co-stimulation failed to induce IL-10 from naive B cells, and this correlated with their impaired ability to suppress EAE [270] or CIA [271] upon adoptive transfer. Taken together, these observations demonstrated that BCR and CD40 alone are insufficient to induce IL-10 production by naive B cells, suggesting that additional signals are necessary to endow B cells with potent regulatory functions.

2 Aims of thesis

B lymphocytes are functionally versatile cells: they have the unique ability to produce antibodies, are competent antigen presenting cells, and can secrete cytokines. In autoimmune diseases, B cells often play pathogenic role. In humans, B cell depletion, using rituximab, is currently an RA treatment and has showed beneficial effects in MS clinical trials. However, rituximab also aggravated disease in other MS and ulcerative colitis patients, triggered psoriasis in certain SLE and RA-suspected subjects or was followed by manifestation of non-tuberculus mycobacterial infections [407]. Altogether, these studies highlighted a complex role for B cells in immune responses and indicated that B cells can both fuel and counteract disease pathogenesis. Of note, rituximab did not alter circulating antibody titers of MS patients, implying that the pathogenic or suppressive functions of B cells in those diseases are mediated by antibody-independent mechanisms, such as antigen presentation and/or cytokine secretion. Indeed, B cells by provision of IL-10, a cytokine with anti-inflammatory actions, displayed potent regulatory activities in several models of autoimmune disorders, including EAE, RA and ulcerative colitis. Importantly, IL-10-producing B cells have been described in healthy humans and were defective in subgroups of MS and SLE patients. Defining the signals inducing IL-10 production by B cells would help discriminate pathogenic from protective B cells. This in turn, could help refine B cell-targeting therapies so that they selectively deplete pathogenic subsets and/or promote the regulatory functions of B cells. Set towards this long-term goal, the aims of the present thesis were:

- To identify the signals and corresponding receptor(s) inducing IL-10 production by naïve mouse B cells *in vitro*
- To evaluate the *in vivo* significance of this finding during EAE, by generating mice in which only B cells are unresponsive to these signals
- To elucidate the underlying mechanisms of the B cell-mediated suppression triggered by these signals.

3 Materials and Methods

3.1 Media and buffers

Tissue culture medium (complete RPMI)		
	RPMI 1640	Invitrogen/Life technologies
10%	FCS	Sigma
100 U/ml	Penicillin	PAA Laboratories
100 g/ml	Streptomycin	PAA Laboratories
50 µM	2-Mercaptoethanol	Invitrogen/Life technologies
PBS, pH 7.2		
137 mM	NaCl	Merck
2.7 mMl	KCl	Merck
1.5 mM	KH_2PO_4	Merck
8.0 mM	Na_2HPO_4 x2 H_2O	Merck
PBS/BSA, pH 7.2		
PBS		
0.02% (w/v)	Bovine serum albumin FrV	PAA Laboratories
Erythrocyte lysis buffer (EL-buffer)		
0.01 M	$KHCO_3$	Merck
0.155 M	NH_4Cl	Merck
0.1 mM	EDTA	Sigma

Carbonate Buffer pH 9		
0.05 M	Na_2CO_3,	Sigma

3.2 Antibodies

Enlisted below are the antibodies used for various cell stimulation assays, detection of cytokines and serum antibodies by ELISA, as well as for phenotypic and functional characterization of cells by flow cytometry. For ELISA, streptravidin (SA) -alkaline phosphatase conjugates (Sigma), served as secondary reagents when detection biotin-coupled antibodies were used, followed by addition of substrate PNPP (Sigma). Secondary reagents in flow cytometry used to detect staining by biotin-coupled antibodies, included SA-PE, SA-APC, SA-FITC or SA-PercP. The FITC-coupled IgM was used for immunofluorescence staining in spleen sections.

Specificity	Clone	Conjugate	Source
B cell stimulation			
CD40	FGK45		DRFZ
Igκ	187.1		DRFZ
Dendritic cell stimulation			
CD40	FGK45		DRFZ
T cell stimulation			
CD3	45-2C11		BD Biosciences
CD28	37.51		BD Biosciences
Blockade of IL-10 and IL-10 receptor			
IL-10	ES5-2A5		BD Biosciences
IL-10R	1B1.3a		BD Bioscience

Detection of serum antibodies by ELISA			
Ig (H+L) chain			Southern Biotech
Total IgG		Alkaline phosphatase	Southern Biotech
IgM		Alkaline phosphatase	Southern Biotech
Detection of cytokines by ELISA			
IL-10 (capture)	SXC1		DRFZ
IL-10 (detection)	JES5-2A5	Biotin	DRFZ
IL-6 (capture)	20F3		DRFZ
IL-6 (detection)	32.C11	Biotin	DRFZ
IFN-γ (capture)	R4-6A2		BD Biosciences
IFN-γ(detection)	AN18.17.24	Biotin	DRFZ
IL-17 (capture)	TC11-18H10		BD Biosciences
IL-17 (detection)	TC11-8H4	Biotin	BD Biosciences
IL-12 (capture)	C15.6		DRFZ
IL-12 (detection)	C17.8	Biotin	DRFZ

Fluorochrome or biotin conjugated antibodies for flow cytometry

Specificity	Clone	Conjugate	Source
CD19	1D3	APC	BD Biosciences
B220	RA3.6B2	Cy5	DRFZ
CD21	7G6	FITC	BD Biosciences
CD23	B3B4	PE	BD Biosciences
CD4	GK1.5	FITC,PE,Biotin	DRFZ
CD8a	53-6.7	PerCp	BD Biosciences
CD25	7D4	Biotin, APC	BD Biosciences
CD5	53-7.3	FITC, PE	BD Biosciences
FoxP3	FJK-16a	FITC,PE	eBiosciences
CD62L	MEL-14	PE	BD Biosciences
CD44	IM7	Cy5	DRFZ
CD11c	N418	FITC, PE, Cy5	DRFZ
CD11b	M1/70	Biotin, FITC, Cy5	DRFZ
MHC II	M5/114	Biotin, FITC, PE	DRFZ
CD45	LCA-Ly5	PE	BD Biosciences
CD40L	MR1	APC	Miltenyi Biotec
IFN-γ	XMG1.2	FITC, APC, PE	BD Biosciences
IL-17	TC11-18H10	PE, APC	BD Biosciences
TNF	MP6-XT22	PE	BD Biosciences

3.3 Recombinant cytokines

Recombinant cytokines were used as standards for ELISA assays according to manufacturer´s instructions or were supplemented in cell culture assays at a final concentration of 20ng/ml.

Cytokine	Assay	Source
IL-10	ELISA, Cell culture	R&D Systems
TGF-β	Cell culture	R&D Systems
IL-6	ELISA	R&D Systems
IFN-g	ELISA	R&D Systems
IL-17	ELISA	R&D Systems
TNF	ELISA	TNF-detection kit, eBiosciences
IL-23	ELISA	IL-23-detection kit, eBiosciences

3.4 Microbial products

Product	Microbe	Source
LPS	*E.coli 055:B5*	Sigma
LPS	*E.coli 026:B6*	Sigma
LPS	*Salmonella typhosa*	Invivogen
LPS	*Klebsiella pneumoniae*	Invivogen
CpG ODN 1826	Synthetic	TibMolBiol
CpG ODN 2006 control	Synthetic	TibMolBiol
M.tb whole cell extract	*Mycobacterium tuberculosis*	Dr. Neyrolles,

PGN-*spezies*	*Streptomyces spezies*	Fluka

3.5 Reagents used for EAE induction

Reagent	Source
MOG_{35-55} (MEVGWYRSPFSRVVHLYRNGK)	Institute for Medical Immunology, Charité, Berlin
Complete Freund´s Adjuvant	Sigma
Dessicated *Mycobacterium tuberculosis* H37Ra	BD/Difco
Pertussis Toxin (from *Bortedella pertussis*)	Sigma
D-PBS (without Ca^{2+}, Mg^{2+})	GIBCO

3.6 Devices and cell culture equipment

Device/equipment	Source
Heraeus® Multifuge 3L-R (rotor: 75006445)	Thermo Scientific
Incubator (Binder CB210)	Binder
AutoMACS	Miltenyi Biotec
FACSCalibur	BD Bioscience
FACSDiva	BD Bioscience
Camera	Nikon
ELISA reader (Emax microplate reader)	Molecular Devices

FilterMate Harvester	Perkin Elmer
Top-count NXT	Packard BioScience
Neubauer improved counting chamber	Carl Roth
96-well micro titre plates (u-, flat-bottomed)	Greiner bio-one
96-well micro titre plates (v-bottomed)	Corning, Costar
96-well micro titre plates (high binding EIA/RIA plates)	Corning, Costar
15ml and 50ml conical polypropylene tubes	Greiner bio-one
UniFilter-96 GF/B plates	Perkin Elmer
Preseparation filters	Miltenyi Biotec

3.7 Mice

C57BL/6, JHT, MyD88$^{-/-}$, TLR2/4$^{-/-}$, TLR4$^{-/-}$, TLR2$^{-/-}$, IL-10$^{-/-}$ and μS$^{-/-}$ mice were bred under specific pathogen free conditions at the Bundesinstitut fur Risikobewertung (Berlin, Germany). Germ-free mice were kept in isolators. Mice were used at 6-10 weeks of age. Animals were treated according to the requirements of the German legislation.

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3.8 Cell preparations

3.8.1 Preparation of single cell suspension of lymphoid organs

Mice were sacrificed by cervical dislocation unless otherwise stated. Spleens, lymph nodes (inguinal and popliteal) and thymi were aseptically removed and strained through a metal sieve. For isolation of bone marrow (BM) cells, bone marrow was obtained from aseptically removed femours and tibia by hydrostatic pressure using PBS. Single cell suspensions free of cell-aggregates were obtained by passing cells thought a 25G needle. Splenic and BM erythrocytes were lysed by osmotic shock following 3min incubation with the hypotonic EL-Buffer at room temperature (RT). Live cells, identified by trypan blue (Sigma) exclusion, were then counted and subjected to desired procedures. Cell suspensions were kept on ice.

3.8.2 Isolation of leukocytes from CNS (brain and spinal cord)

Mice were sacrificed by CO_2 asphyxiation and subjected to cardiac perfusion with cold PBS. Brains were collected following gentle rapture of the intracranial cavity and spinal cords were removed by intrathecal hydrostatic pressure. Both organs were cut into small pieces and digested with 1 mg/ml Collagenase type V (from *Clostridium histolyticum*; Sigma) and 0.5 mg/ml deoxyribonuclease I (from bovine pancreas; Sigma) for 24min at 37°C, with occasional mechanical disaggregation through an 18G needle. Single cell suspensions were washed in serum-free RPMI 1640. Brain-derived cell suspensions were resuspended in 10ml of 30% percoll and leukocytes were obtained as the cell pellet after centrifugation at for 20min at 2000rpm, RT. Leukocytes from spinal cords were collected from the interface of a 30:70% discontinuous percoll gradient after similar centrifugation. Live cells were counted and used in further assays. Digestion enzymes and Percoll were diluted in basal serum-free RPMI medium.

3.8.3 Cell isolation using magnetic cell sorting

The method of magnetic cells sorting (MACS, Miltenyi Biotec) is based on labeling of cells using small super-paramagnetic particles (microbeads, Miltenyi Biotech) and allows this way the separation of a frequent or rare cell population from a mixed cell suspension. These microbeads, made of dextran and iron oxide, are covalently conjugated with monoclonal antibodies that recognize a given surface marker specific for a cell type. Cells labeled with microbeads are retained on a magnetic column placed in a magnetic separator, that can be subsequently eluted, whereas non-labelled cells flow through “untouched”. The whole procedure is fast and efficient, the microbeads are extremely smalland biodegradable, and thus the separated cells (both positively labeled and non-labeled fraction) are viable, retain their functionality and can be further used for various assays. Purity can range between 90-99% depending on the frequency of cells to be isolated. Often MACS is used a first means to enrich for a given cell type within a cell suspension which can be then sorted with higher purity by fluorescence-activated cell sorting (see below). In the current study, magnetic cell sorting was performed using the automated AutoMACS (Miltenyi Biotec) benchtop instrument.

3.8.4 Isolation of splenic B cells

Splenic B cells were isolated by negative selection using anti-CD43 mircrobeads (Miltenyi Biotec). CD43 (Ly-48, Leukosialin) is expressed on all splenocytes, including splenic B1 B cells, except resting mature B2 cells. Whole splenocyte suspensions were incubated with anti-CD43 microbeads for 20min in the dark at 4°C, according to manufacturer´s instructions, subsequently washed to remove unbound beads and resuspended in PBS/BSA. Labeled cells were depleted using the program "deplete S" (S: sensitive mode) on the AutoMACS, and $CD43^-$ naïve B cells were collected as the negative non-labeled fraction. Depletion was performed twice and routinely yielded 97-99% pure B cells, as assessed by flow cytometry staining for the B cell specific marker CD19. In some cases, B cells were sorted from $CD43^-$ fraction using direct positively labeling with anti-CD19 microbeads (Miltenyi Biotec), resulting in a purity of ~99%. Contaminating cells included mostly $CD11c^+$ dendtitic cells. Cells were prepared and handled under sterile conditions.

3.8.5 Isolation of peritoneal cavity B cells

Peritoneal exudates were obtained using ice cold PBS/BSA. Briefly, 10ml PBS/BSA were gently injected into mouse peritoneal cavity through a 27G needle, peritoneum was gently "massaged" to promote even distribution of cells, and 8-9 ml of peritoneal lavage were recovered using a 25G needle. Peritoneal B cells were positively selected on AutoMACS via direct labeling with anti-CD19-coupled micerobeads (Miltenyi Biotec), yielding a purity of ~98%. Major contaminants were $CD5^+$ T cells.

3.8.6 Isolation of splenic dendritic cells

Spleens were cut into small pieces and digested with 1 mg/ml Collagenase type V and 0.5 mg/ml deoxyribonuclease; for 30min at 37°C, with occasional mechanical disaggregation by passing tissue pieces through a 18G needle. Single cell suspensions were extensively washed in serum-free RPMI 1640 and subjected to erythrocyte lysis as described in Section 3.8.1.1. Dendritic cells were labeled with anti-CD11c microbeads (Miltenyi) according to manufacturer's

instructions and positively selected on AutoMACS using the program “possel S”. The sorted DC population was ~98% pure, with B cells being the major contaminating cells.

3.8.7 Isolation of conventional $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ T regulatory cells

$CD4^+$ T cell subsets were purified from pooled spleens and lymph nodes (LN) of naïve C57BL/6 mice initially as total CD4+ T cell, from which CD25+ and CD25- cells were subsequently sorted. Single splenic/LN cell suspensions were first incubated with anti-CD4 FITC and anti-CD25-biotin antibodies (each at 2µg/ml) for 15min in the dark at 4°C. After washing, CD4 labeled cells were positively selected using anti-FITC-coupled microbeads (anti-FITC multisort Kit, Miltenyi Biotech) and subsequently “released” from the beads according to manufacturer´s instructions. The resulting total $CD4^+$ fraction was then incubated with anti-Biotin microbeads and further sorted into positively selected $CD4^+CD25^+$ and negative selected $CD4^+CD25^-$ fractions. Purities were routinely >98% for $CD4^+CD25^-$.and >95% CD4+CD25+ cells, as assessed by flow cytometry.

3.8.8 Isolation of B cell subsets via fluorescence activated cell sorting

Splenocytes and lymph node (LN) cells were initially enriched for resting B cells using anti-CD43 microbeads, as described in section 3.8.2.1. The splenic B cell-rich fraction was subsequently stained with flurorochome-conjugated antibodies to CD21, CD23 and CD19 to define the various B cell subsets whereas B cell-enriched LN cells were stained with fluorochrome-coupled anti-CD19. The various splenic B cell subsets and total LN B cells were then sorted by fluorescence-activated cell sorting (FACS) on a FACSDiVa (Becton Dickinson). The resulting sorted B cell populations were: total $CD19^+$ splenic B cells (according to CD19 expression), marginal zone [121] B cells ($CD19^+CD23^{lo}CD21^{hi}$), follicular (FO) B cells ($CD19^+CD23^{hi}CD21^{lo}$), and total $CD19^+$ LN B cells. Procedures were carried out under sterile conditions.

3.9 Flow cytometry

Flow cytometry is a technique that allows rapid measurements on particles or cells as they flow in a fluid stream one by one in a sensing point. The ability of laser based flow cytometers to measure multiple cellular parameters based on light scatter and fluorescence and to purify subpopulations of cells has lead to the increasing spread use of this application in immunology and medicine.

An essential feature of flow cytometry is the so-called hydrodynamic focussing, by which cells are individualized in a fluid stream and positioned at the observation point where they pass a laser beam of defined wavelength. First information on size and granularity of the cell is gained from light scattering when the cell passes the beam. This is detected as a forward scatter (FSC) and a sideward scatter (SSC), respectively.

For the identification of cell subsets from a heterogeneous population or for measuring activation states of individual cells monoclonal antibodies (mAb) are most frequently used. These antibodies have defined specificities for cell surface or intracellularly expressed molecules and can be detected by means of fluorescent dyes conjugated to them. Fluorescent dyes are characterized by a delocalised π-electron system and can be proteins (PE, PerCP, APC) or small organic molecules (FITC, Cy5). By absorption of a photon of appropriate wavelength electrons can be lifted from the occupied molecular orbital to a free molecular orbital. This procedure is termed as $\pi \rightarrow \pi^*$ crossover. Through relaxation from the exited level to the ground state a photon is emitted as fluorescence. The fluorescence intensity emitted from a cell is proportional to the amount of dye coupled to the cell and thus reflects the number of molecules present.

For flow cytometry, fluorescent dyes are used that can be excited by light of one specific wavelength but emit light at different wavelengths. For example, FITC and PE can both be excited by a 488 nm laser while their emission spectra peak at 520 nm and 575 nm, respectively. Using optical filters (530±15 nm filter for FITC and 585±21 nm filter for PE) emitted light of specific wavelengths is guided to different photomultiplier detectors. This allows the detection of fluorescence from different dyes using a single laser for excitation. The emission spectra of fluorescent dyes may however overlap. For meaningful detection of fluorescent intensities, this spectral overlay needs to be corrected by a compensation procedure. The number of parameters that can be detected simultaneously by a flow cytometer depends on the

number of lasers and detector it is equipped with. Becton Dickinson FACSCalibur uses a blue 488 nm argon laser (exciting FITC, PE and PerCP) and a red 635 nm laser (exciting APC) and allows the detection of the FSC, the SSC and four fluorescent dyes.

Besides the phenotypic and functional characterization of cells, Flow cytometry allows for the physical separation and sorting of different cell populations. Commonly used flow cytometers like FACSAria sort cells using the droplet sorting method. Here uniform droplets are generated from the fluid stream containing individual cells. When a cell is detected that fulfils the predefined criteria an electrical charge is applied to the droplet. The droplet then passes charged deflection plates and electrostatic forces deflect the droplet to the left or to the right where it is collected in a tube.

Acquired FACS data were analyzed using FlowJo software v7.5 (©Tree Star, Inc, 1997-2009)

3.9.1 Staining of cell-surface markers

Freshly isolated cells or following *in vitro* stimulation were stained for surface molecules. Cells were resuspended in PBS/BSA and placed in 96-well V-bottom plates (1-5 x10^5 cells/well). To avoid non-specific binding of staining antibodies to Fc Receptors, cells were first incubated in the presence of 30 µg/ml rat anti-mouse FcRγ antibodies (50 µl/well; clone 24.G2, DRFZ) for 15min on ice. Cells were centrifuged at 1500rpm for 3min, supernatants were decanted and cell pellets were then resuspended in 50µl of PBS/BSA containing the desired staining antibody combinations and incubated for another 15 min. Unbound antibodies were removed following two centrifugation-and-washing steps with PBS/BSA (200 µl/well). When biotinylated antibodies were used, a secondary staining step was carried out, in which cells were incubated with SA-conjugated to one of the fluorochromes, FITC, APC, PE or PerCp for 15min followed by washing as above. Finally, 200µl of PBS/BSA were added to each well and samples were acquired on a FACScalibur. The optimal concentration of staining antibodies was pre-determined in test experiments, and usually ranged between 1-5 µg/ml. All staining steps were performed at 4°C in the dark.

3.9.2 Intracellular staining

Functional characterization of cells entailed the determination of their cytokine production after short-term *in vitro* stimulation. Cytokines were detected in protein form in the cell cytoplasm by intracellular staining with specific antibodies. To this end, cells were cultured in the presence of antigenic stimuli and a chemical inhibitor (Gogli stop Kit, BD Pharmingen) which "immobilizes" proteins at the golgi apparatus, and thereby, blocks their release from the cells. After re-stimulation, cells were harvested for flow cytometry and stained for surface markers (as decribed in 3.9.1). Cells were then fixed with a paraformaldehyde-based solution and subsequently permeabilized with a detergent containing buffer, both provided in the Golgi stop Kit, according to manufacturer´s instruction. Anti-cytokine antibodies were added for 30 min at 4°C in the dark. Cells were extensively washed and resuspended in PBS/BSA prior to acquisition on a FACScalibur.

Intracellular staining was used also as means to identify natural regulatory T cells among freshly isolated cells, by staining for their intracellular marker FoxP3. For this purpose, cells were first surface-stained for CD4 as previously described (section 3.9.1) and then stained for intracellular FoxP3, using the FoxP3 kit (eBiosciences), according to manufacturer´s instructions.

3.10 *In vitro* functional assays

3.10.1 Stimulation of B cells and dendritic cells

Purified B cells or dendritic cells (DC) were re-suspended in complete RPMI medium and seeded at $5x10^5$ cells/100µl/ well in triplicates in flat-bottom 96-well tissue culture plates. Various stimuli, including anti-CD40, anti-Igκ (for B cells only) and TLR agonists, were added alone or in combinations in a total volume of 100µl at the concentrations indicated. Cultures were incubated at 37°C / 5% CO_2 –rich atmosphere and monitored microscopically on a daily basis for cell growth and potential contamination. Cytokine production by B cells and DC was determined by cell-based ELISA (see section 3.12.1) after 72h and 48h, respectively. Alternatively, supernatants of B cells stimulated with TLR agonists LPS or CpG were collected at the indicate time points and used in T cell proliferation and suppression assays, as wells as

DC cytokine suppression assay (see below). For the same purpose, supernatants of LPS-activated DC were collected after 48h of culture. Solutions of stimulating antibodies or TLR agonists (also in complete RPMI) were filter-sterilized using 0.2µm filters prior to use. Pictures of B cell cultures after were taken at 72h using a Niccamera at a 20x magnification.

3.10.2 $CD4^+$T cell suppression assays

A T cell proliferation assay was set up to assess the direct effect of soluble factors secreted by TLR-activated B cells on T cell proliferation. Purified $CD4^+CD25^-$ T cells were seeded at $1x10^5$ cells/100µl/well in flat-bottom 96-well plates previously coated with 1 µg/ml anti-CD3 (for 2h at 37°C). Sets of triplicate wells were supplemented or not with an equal volume of supernatants from B cells or DC that had been stimulated with LPS (2 µg/ml) for 72h and 48h, respectively or with an equal volume of recombinant mouse IL-10 or IL-6 (each at 20ng/ml final concentration). In order to further address the role of B cell-derived IL-6, some wells containing B cell supernatants received neutralizing anti-IL-6 antibody at 20µg/ml. T cell proliferation was determined after 48h by ^{3}H-thymidine-incorporation assay, as described in section 3.10.3 below.

The effect of soluble factors secreted by TLR-activated B cells on T cell proliferation was also assessed when T cells were activated in the presence of DC. For this purpose, $3x10^3$ TLR2/4$^{-/-}$ DC were incubated for 2 h in 96-well U-bottom plates in presence of anti-CD3 (0,1 µg/ml) together with either 7.5x 104 CD4+CD25+ T cells; supernatants from LPS-activated B cells supplemented or not with anti-IL-10 and anti-IL-10 receptor antibodies (each at 20µg/ml final concentration); supernatants from LPS-activated wild-type DC or with recombinant mouse IL-10 (20 ng/ml final concentration). Following incubation, 1.5 $x10^5$ naïve CD4+CD25- T cells were added alone or together with CpG ODN 1826 (1 µg/ml final concentration). T cell proliferation was measured 48h later by ^{3}H-thymidine-incorporation assay. Supernantants were collected from B cells and DC (2µg/ml for 72hr) previously cultured with 2µg/ml LPS for 72h and 48h, respectively.

3.10.3 DC cytokine suppression assay

This assay was set up in order to evaluate the effect of soluble factors secreted by TLR-activated B cells on cytokine production by DC. Splenic TLR2/4$^{-/-}$ DC, were plated out at 5x10^5 cells/50µl/ in flat bottom 96-well plates and were initially incubated for 2h with 100µl of either medium, supernatants from LPS-activated B cells supplemented or not with blocking anti-IL-10 and anti-IL-10 receptor antibodies (each at 20µg/ml final concentration) or with recombinant mouse Il-10 (20 ng/ml final concentration). Following incubation, 50µl of CpG ODN 1826 (1 µg/ml final concentration) or medium were added as indicated and cultures were further incubated for 48h. At this time-point supernatants were collected and assayed for their content in TNF, IL-12, IL-6 and IL-23 by ELISA (see below).

3.10.4 Ex vivo re-stimulation of splenocytes and lymph node cells

Splenocytes or LN cells prepared at the indicated time-points after EAE induction, were cultured at 8 x10^5 cells/well in flat-bottom 96 well plates in the presence of titrating amounts of MOG_{35-55}. After 48h, autoantigen-specific proliferation and cytokine production was determined by ^{3}H-thymidine-incorporation assay and cell-based ELISA, respectively.

To determine ex-vivo cytokine production by T cells and quantify antigen-specific CD4 T cells, 4x10^6 splenocytes or LN cells were separately re-stimulated with 20 µg/ml MOG_{35-55} in 48-well plates in a total volume of 1 ml in the presence of Golgi stop (BD Pharmingen). After a 6h-incubation, cells were harvested for surface and intracellular cytokine staining as earlier described.

3.10.5 ^{3}H-thymidine-incorporation assay

^{3}H.Thymidine incorporation is commonly used as a means to measure cell proliferation by monitoring DNA synthesis. The assay utilizes a strategy wherein a radioactive nucleoside, ^{3}H-thymidine is incorporated into new strands of chromosomal DNA during mitotic cell division. A scintillation beta-counter is used to measure the radioactivity in DNA recovered from the cells in order to determine the extent of cell division that has occurred in response to a stimulus/antigen. 1µCi/well ^{3}H-Thymidine was added to the cultures after 48h allowing its

incorporation into newly synthesized DNA by dividing cells. Following 16-18h, cells were harvested onto filter-bottom UniFilter-96 GF/B plates using a FilterMate cell harvester. The free, not incorporated ^{3}H-Thymidine was washed out. Scintillation fluid was added (45 µl) to the cells harvested on the filter plate. The scintillation fluid contains fluorescent compounds that emit a flash of light when absorbing energy that is released during the decay of tritium. These flashes of light are measured by a Top-count NXT liquid scintillation counter as counts per minute (cpm). The cpm value is proportional to the proliferative activity in the cell culture during the incubation period with ^{3}H-Thymidine.

3.10.6 Ex vivo re-stimulation of CNS-infiltrating leukocytes

Leukocytes isolated from the spinal cord and brain of EAE-immunized mice were stimulated at 1x10^{6} cells/well in 96 well plates with soluble anti-CD3 and anti-CD28 (at a final concentration of 0.1 µg/ml and 1µg/ml, respectively) in the presence of Golgi stop. After a 6h-incubation period, cells were harvested for surface and intracellular cytokine staining.

3.11 Assessment of germinal centers by *in situ* immunoflurocensce

Spleens from immunised mice were embedded in O.C.T compound (cryoprotection medium; Sakura) in tissue-tek, snap-frozen in dry ice and stored at -80°C until use. Using a cryostat (machine), 5µm-thick splenic cryo-sections were obtained and mounted on Superfrost Plus slides (Fisher Scientific). Sections were fixed in cold acetone for 8min, then air-dried for 40min at RT and stored at -20°C until further use. Prior to staining, sections were adjusted to RT for 10min and re-hydrated for 20min in PBS. Staining was subsequently carried out in a moist light-protected chamber for 90min at RT. Sections were directly stained for germinal centres with biotinylated PNA (penagglutinin; Vector Laboratories) and counterstained for B cell follicles with IgM-FITC (BD Pharmingen). Slides were subsequently washed in PBS for three-10min intervals, incubated with SA-Rhodamine red conjugates for 30min to detect PNA and washed as above. Finally sections were mounted in fluoromount G (company) and visualised on a flurorescence microscope Zeiss 7082. Optimal concentrations of primary and secondary staining reagents were pre-determined in test experiments.

3.12 ELISA

Enzyme-Linked Immunosorbent Assay (ELISA) is a method to detect the presence of an antigen or an antibody in a sample. Usually at least one antibody with specificity for a particular antigen is used to perform the ELISA. The capture antibody or an antigen is coated to the surface of high binding 96-EIA/RIA microtitre plate. Free binding sites are blocked by incubation with blocking buffer. The sample is added and the antigen-specific antibodies are bounded to the plate. A separate labelled antibody that recognizes a different epitope to the immobilized first antibody is then used to detect the bound antigen. The detection antibody can be covalently linked to an enzyme or can itself be detected by a secondary antibody which is linked to biotin. Biotin is then bound by streptavidin coupled to an enzyme that catalyses a colorimetric reaction of its substrate. In this study the detection antibodies were conjugated to the enzyme alkaline phosphatise (AP) that hydrolyzes the para-nitrophenyl phosphate (pNPP) into yellow coloured para-nitrophenol and phosphate. Light absorption by Para-nitrophenol was measured at 405nm on an ELISA plate reader (Emax).

3.12.1 Mesurement of cytokine production

Cytokine production was generally measured using cell-based ELISA- except for DC suppression experiments- as previously described [417]. B cells, DC, splenocytes or LN cells stimulated for 48h with the indicated antigens in 96-well tissue cultures plates were gently re-suspended, and 150µl were transferred to 96-well ELISA/RIA plates previously coated with a capture anti-cytokine antibody and blocked with PBS/3%BSA. Transferred cell cultures were incubated at 37°C / 5%CO_2 for a further 18-24h to allow for binding of the cytokine present in the supernatant and/or directly produced by the cells. Onto the same plates known concentrations of the recombinant cytokine to be detected were added in serial dilutions in complete RPMI medium, at 100µl/well. These steps were carried out under sterile conditions. Plates were then extensively washed with PBS/0.1% Tween 20 to remove cells and unbound soluble factors, and incubated with 100µl of PBS containing a biotin-conjugated secondary antibody for 2h, RT. Before and after the addition of 100µl of ExA-AP (Sigma) for 30min, RT, plates were extensively washed as above, and antibody-cytokine complexes were detected following incubation with the AP substrate, PNPP. Cytokine concentration in the test samples were calculated using SoftMaxPro software.

For the DC suppression assays. cytokines were detected in previously collected culture supernatants. To this end, 50µl of supernatant were assayed for cytokines by sandwich ELISA using pairs of capture-and-detection anti-cytokine antibodies. Supernatants were incubated on RIA/ELISA plates pre-coated with capture antibody for 2h, RT. Cytokine detection and quantification were carried out as described for cell-based ELISA.

3.12.2 Serum antibody ELISA

ELISA was also used as a method to measure antigen-specific or total antibody isotype titers in sera prepared from naïve or treated animals at the indicated points after immunization. For the detection of antigen-specific antibodies, high binding 96 well EIA/RIA plates were coated with 50µl of MOG_{35-55} (20µg/ml) or DNP-BSA (50 µg/ml) in PBS overnight at 4°C. Plates were washed in PBS and blocked with PBS/1%BSA at 4°C overnight or for 3h at 37°C. Then a standard sample and sera pre-diluted in PBS was added and two fold dilution series were performed and incubated for 2h at RT.. As detection antibody an alkaline phosphatase conjugated IgM, or pan-IgG diluted 1:1000 in PBS/0.05% Tween were used and incubated for 1h at RT. All incubations steps were followed by repeated washing with PBS/0.1%Tween followed by a last wash in PBS. Detection was carried out using ExA-AP and PNPP as described in previous section. For the detection of total circulating IgM, plates were coated with 2µg/ml anti- (H+L) chain. The same standard sample was applied on all plates for each experiment. This allowed for the calculations of the relative antibody titers to be made by comparing the relative dilutions of the samples to the one of the standard sample for a given optical density (chosen to fall within the linear part of dilution curves) according to the following formula: (dilution of sample - dilution of standard) x 100/ dilution of standard .

3.13 Animal experiments

3.13.1 Generation of mixed bone-marrow chimera mice

Mixed bone marrow chimera mice were generated in order to restrict genetic deficiencies solely in B cells, as previously described [270]. Recipient wild-type mice received 1150cGy of γ-irradiation via a cesium source. One day later, irradiated mice were reconstituted with 5 x10^6 donor bone marrow cells. All bone marrow cell preparations were depleted of T cells by labeling with anti-Thy1.2-coated magnetic microbeads (Miltenyi) followed by negative selection on AutoMaCS. To restrict genetic deficiency to B cells, the bone marrow inoculums consisted of 80% B cell-deficient bone marrow (μMT) and 20% bone marrow cells from either MyD88$^{-/-}$, TLR2/4$^{-/-}$, TLR4$^{-/-}$ or TLR2$^{-/-}$ mice as schematically shown in Figure 3-1. Control groups received either 20% wild-type bone marrow cells to generate chimeric mice with a wild-type B cell compartment, or were reconstituted with 100% μMT bone to generate chimeric B cell-deficient mice. The efficiency of irradiation-induced bone-marrow ablation in recipient mice was confirmed by the absence of B cells in recipients of 100% μMT bone marrow cells, as in these mice CD19$^+$ cells were undetectable by flow cytometry. In contrast, all other groups of chimeric mice harbored CD19$^+$ cells at frequencies comparable to those of wild-type mice. All chimeric mice were allowed to reconstitute their peripheral immune system over a period of 6-8 weeks before used in experiments.

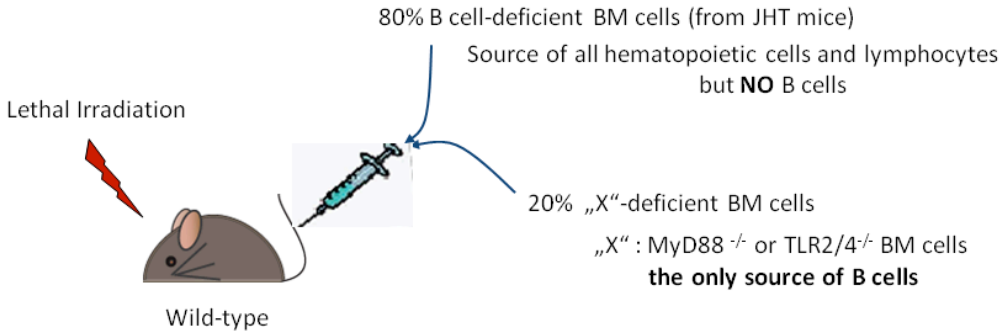

Figure 3.1 Generation of mixed bone marrow chimeras
Irradiated recipient mice were reconstituted with a mixture of BM cells from JHT and and from mice deficient in the gene of interest (noted as X; for example MyD88$^{-/-}$, TLR2/4$^{-/-}$, or TLR9$^{-/-}$), at the indicated ratio. The resulting chimeric mice contain WT cells except B cells, deriving from the JHT BM, and B cells deriving from X BM and are thus deficient in X. The effect of non-B cells deriving from the 20% of X BM was controled for, by reconstituning an additional group of recipients with WT-derived (80%) and X-derived (20%) BM cells (not shown)

3.13.2 EAE induction and clinical assessment

Each mouse received 50µg of MOG_{35-55} peptide emulsified in CFA supplemented with 400µg dessicated *Mycobacterium tuberculosis* H37Ra, in a total volume of 100µl. The peptide/CFA emulsion was administered as two 50µl subcutaneous injections, one into each hind leg. On the day of immunization and two days later, each mouse received via the tail vein 240ng of pertussis toxin diluted in 200µl D-PBS. Clinical signs of EAE were assessed daily according to the following 0-6 scoring system: 0 no signs of disease, 1 flaccid tail, 2 impaired righting reflex and/or gait, 3 partial hind limb paralysis; 4 total hind limb paralysis, 5 hind limb paralysis with partial fore limb paralysis; 6 moribund or dead.

3.13.3 Immunisation with model antigen in alum

Mice were immunized via the intraperitoneal route with 100µg DNP-KLH mixed in alum (Company) in a total volume of 200µl. Mice were bled at the indicated time-points, sera were prepared and titers of DNP-specific IgM and total IgG were determined by sandwich ELISA.

3.13.4 Serum treatment

In serum transfer experiment, each $\mu S^{-/-}$ mouse received via the intraperitoneal route doses of 500µl serum prepared from a pool of gender and age matched wild-type naïve syngeneic animals. Serum was first administered one day prior to EAE induction and on days 1, 3, 5 and 7 thereafter. As assessed by ELISA using commercially available IgM as standard, each dose of serum contained ~200µg of IgM. Independently prepared pools of sera were used in replicate experiments. Pooled sera were stored in -20°C for no longer than 6 months and retained their content in natural IgM throughout this period, as determined by ELISA.

3.14 Statistical analysis

Statistical significance was evaluated using student´s T-test (unpaired, two-tailed within 95% confidence intervals). For the statistical analysis of EAE curves, the same test was applied

to compare average disease scores between different groups at selected time-points throughout the disease course. The T-tests were performed using Prism software (Graphpad Software, Inc.).

4 Results

4.1 Role of TLR/MyD88 signaling in B cells during T cell-mediated inflammation of CNS

Previous studies have demonstrated that IL-10 –producing B cells elicit suppressive functions in several experimental models of autoimmune inflammation including collagen-induced arthritis (CIA), ulcerative colitis (UC) and experimental autoimmune encephalomyelitis (EAE). Moreover, a subgroup of MS patients presented with reduced IL-10 production by B cells, suggesting a regulatory role of these cells in human autoimmune disease and thus, raising the possibility to exploit them as potential therapeutic tools for the suppression of undesired responses. However, despite the reported involvement of certain molecules in IL-10-mediated B cell-suppression, the signals required for the generation of IL-10-producing B cells from their naïve precursors have not been characterized. The experiments described below were performed in order to identify these signals and investigate their relevance in the suppressive functions of B cells *in vitro* and *in vivo* during EAE.

4.1.1 TLR agonists induce IL-10 production by naïve B cells

Previous work in our laboratory showed that mice in which B cells carry a BCR specific for a disease- irrelevant antigen, hen-egg lysozyme, suffer a non-remitting chronic form of EAE, similar to mice with IL-10-deficient B cells. Furthermore, *in vitro* IL-10 production by ex vivo isolated B cells from EAE-recovered mice required stimulation through BCR. Besides the BCR, CD40 expression by B cells was also necessary for both, recovery from EAE and for inducing, concomitantly with BCR stimulation, re-call IL-10 production by the ex vivo isolated B cells. These findings suggested the involvement of antigen-specific BCR and CD40 signals in the suppressive functions of IL-10-producing B cells. To address whether stimulation through these receptors can trigger IL-10 secretion by naïve B cells, purified splenic naïve B cells were cultured in the presence of soluble agonistic antibodies to BCR (αIgκ) and CD40 (αCD40) either alone or in combination. Quantification of IL-10 protein by ELISA in the culture supernatants after 72h revealed that BCR and CD40 co-stimulation failed to induce IL-10 by naïve B cells, although it potently induced B cell proliferation (Fig.4.1A and data not shown). These results were not due to suboptimal signals delivered by the αCD40 antibody used here, since co-culture

of B cells with a fibroblast cell line expressing the natural ligand for CD40, CD40 ligand (CD154), also failed to induce IL-10 (data not shown).

Apart from BCR for capturing antigen, and CD40 for receiving T cell help, B cells express a diverse array of PRRs mediating the recognition of conserved microbial structures. Among these receptors are the TLR upon stimulation of which B cells have been shown to proliferate and produce antibodies. Although an implication of TLR in the suppressive function of B cells has not been reported thus far, TLR agonists are present in the aforementioned disease models regulated by IL-10- producing B cells, either as constituents of the adjuvant used to induce disease (CIA and EAE) and as products from the commensal flora (UC). Therefore, the capacity of TLR agonists to induce IL-10 production by B cells was assessed *in vitro*. In contrast to BCR and CD40, the TLR4 agonist LPS and the TLR9 agonist CpG potently induced IL-10 secretion by splenic naïve B cells (Fig. 4.1A). Time-course experiments performed in our laboratory to follow the kinetics of IL-10 production by B cells upon LPS and CpG stimulation, showed earliest detection of IL-10 after 24h, followed by a peak at 72h and a plateau thereafter until 96h. Because previous studies on dendritic cells (DC) demonstrated that different LPS molecules induce distinct cytokine responses by DC [418], various bacterial sources of LPS were used here to stimulate B cells. However, they all induced similar amounts of IL-10 (Fig. 4.1A). Interleukin 10 secretion by B cells in response to CpG specifically required the presence of the characteristic methylated motif, since the unmethylated control sequence, CpG control, induced only low amounts of IL-10 (Fig. 4.1A). TLR4- and -9 –induced IL-10 secretion by B cells was dependent on expression of MyD88, the major signaling adaptor downstream of these TLR (Fig. 4.1B). As expected, B cell production of IL-10 upon LPS, but not CpG, stimulation was TLR2/4 dependent.

Consistent with previous reports [419-420], both LPS and CpG, but not CpG control, induced B cell proliferation (Fig.4.2). As observed with IL-10 production, proliferation in response to LPS required TLR2/4 expression by B cells, whereas this was dispensable for CpG-induced proliferation (Fig.4.2). These results further supported that the effects of LPS and CpG in B cells were, respectively, TLR4- and most likely TLR9-specific. Altogether, these results demonstrated that, in contrast to BCR and CD40, the TLR agonists LPS and CpG are potent inducers of IL-10 by naïve B cells *in vitro*.

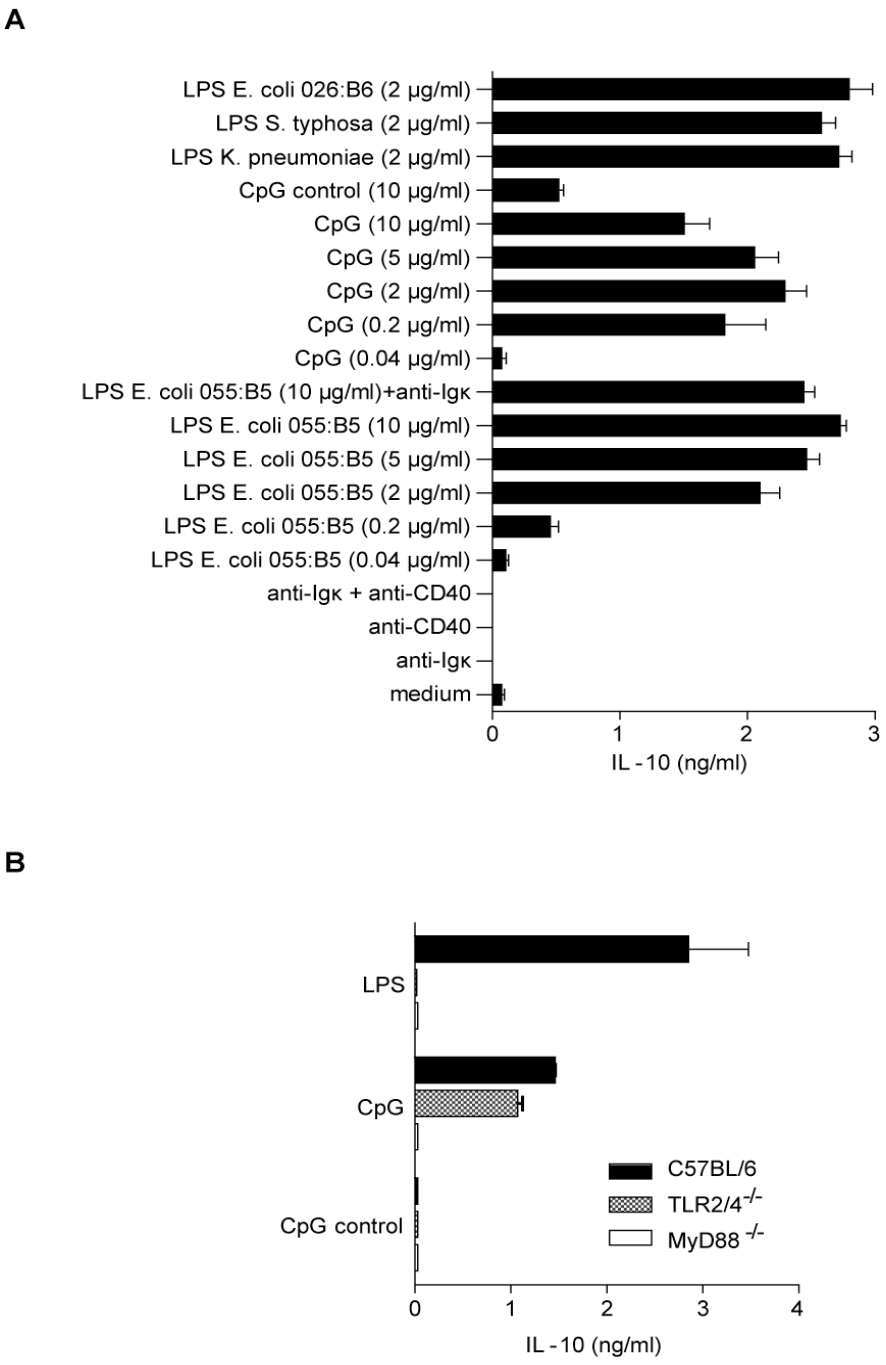

Figure 4.1 TLR-4 and -9 agonists induce IL-10 production by naïve B cells in a TLR/MyD88-dependent manner.

(A) $5x10^5$ purified splenic naïve C57BL/6 B cells were cultured in medium alone or in the presence of indicated amounts of LPS from different bacterial origins, CpG and its control form CpG control or of agonistic antibodies to BCR (anti-Igκ) and CD40 (anti-CD40). (B) Purified splenic naïve B cells from C57BL/6, MyD88$^{-/-}$ or TLR2/4$^{-/-}$ mice were cultured in the presence of LPS, CpG or CpG control, each at 10µg/ml. [298] After 72hr IL-10 protein in the cultures was quantified by cell-based ELISA. Graphs show mean±SEM. Results are representative of at least four independent experiments.

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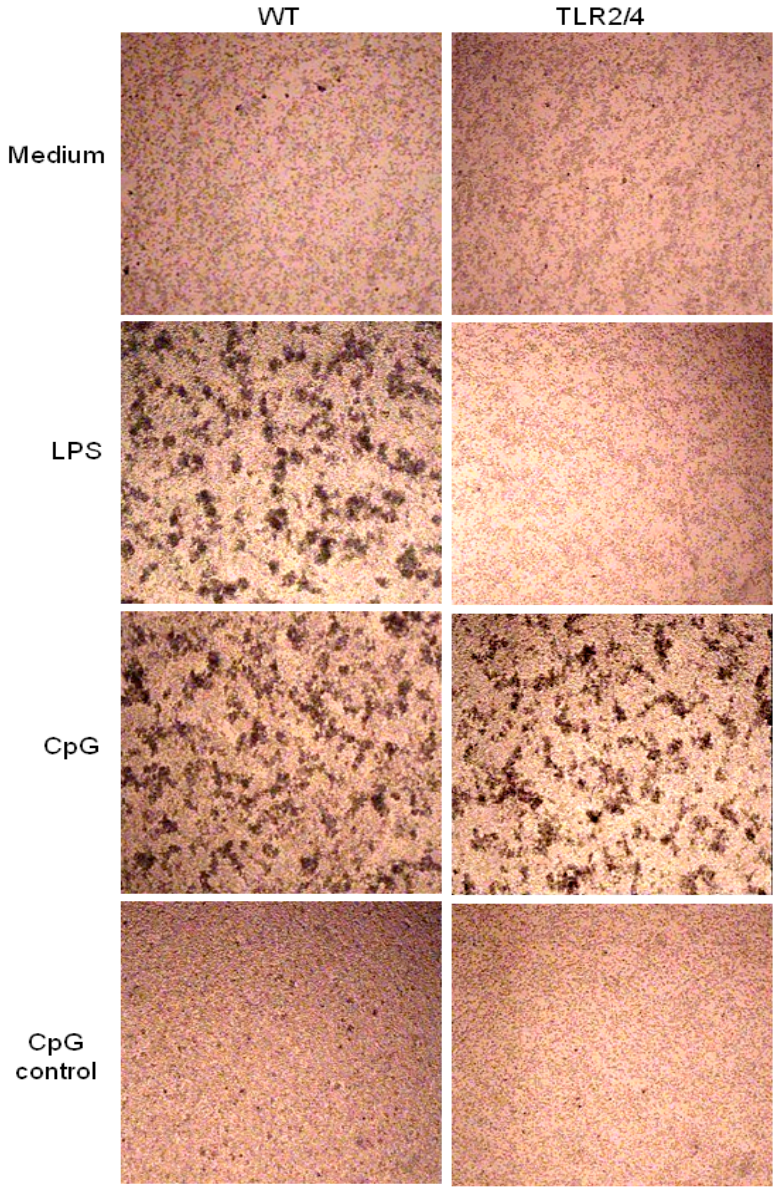

Figure 4.2 Microscopy image of LPS and CpG induced- B cell proliferation

Purified splenic naïve B cells from C57BL/6 or TLR2/4$^{-/-}$ mice were cultured in medium alone or in the presence of LPS, CpG or CpG control, each at 10µg/ml. After 72hr images of the cultures were obtained using a Nikon camera (magnification 20x). Wild-type B cells incubated with LPS or CpG, but not CpG control or medium alone, formed clusters indicative of activation. These clusters were absent in LPS- but not CpG-stimulated TLR2/4$^{-/-}$ B cells. Data are representative of two independent experiments.

4.1.2 Splenic B cell subsets, lymph node and peritoneal cavity B cells can all make IL-10 in response to LPS

Splenic B cells are a heterogeneous population, comprising phenotypically, functionally and anatomically distinct subpopulations. Among the main B cell subsets in mouse spleen are the follicular B cells (FO) and marginal zone B cells [121], both belonging to the B2 cells originating in the bone marrow. In contrast, LN B cells (also B2 type) are considered a homogeneous population resembling in phenotype and function splenic FO B cells. To test whether IL-10-secretion in response to LPS is a property restricted to a particular subset, naïve FO, MZ and LN B cells were FACS-sorted from splenic or LN preparations according to their distinct expression of surface markers shown in Fig.4.3A. Briefly, FO B cells were defined as $CD19^{+}CD23^{hi}CD21^{lo}$, MZ B cells were $CD19+CD23^{lo/neg}CD21^{hi}$ whereas LN B cell were sorted as CD19+ cells. In addition, total (unsorted) splenic $CD19^{+}$ B cells were also obtained and used as a control. These different populations were subsequently cultured in the presence of LPS. Quantification of IL-10 protein in the cultures demonstrated that all B cell subsets were able to make IL-10 after LPS stimulation (Fig.4.3B). Microscopic day-to-day monitoring of the cultures confirmed the previously reported proliferative capacity of all B cell subsets to this mitogen, as well as the more rapid response of MZ B cells [421], which showed the earliest proliferation. This latter observation correlated with the tendency of MZ B cells to produce the highest amounts of IL-10 (Fig. 4.3B).

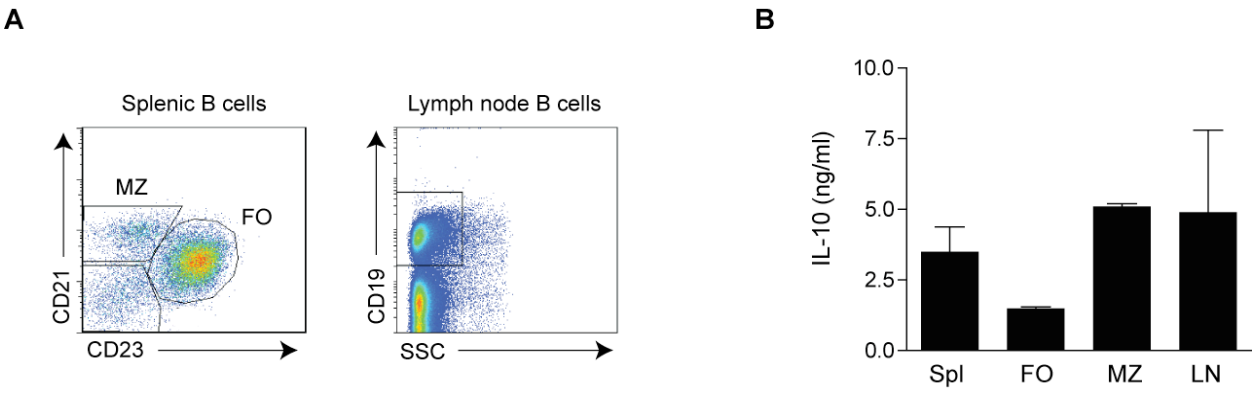

Figure 4.3 Splenic FO and MZ B cell subsets and LN B cells can make IL-10 in response to LPS
(A) Splenic (left plot;gated on live $CD19^{+}$ B cells) follicular (FO) and marginal zone [121] B cells were FACS-purified from C57BL/6 mice according to their surface expression of CD21 and CD23. FO were $CD19^{+}CD23^{hi}CD21^{lo}$ whereas MZ were $CD19^{+}CD23^{lo/neg}CD21^{hi}$. Lymph node (LN) B cells (right plot; gated on live LN cells) were FACS-purified as $CD19^{+}$ cells from LN of C57BL/6 mice. (B) Sorted B cell subsets were cultured with 10µg/ml LPS and 72h later IL-10 secreted in the cultures was measured by cell-based ELISA. "spl" denotes total unsorted $CD19^{+}$ B cells from spleen. Graphs show mean±SEM. Pooled results of three independent experiments are shown.

In addition to B2 cells, another population of B cells exist, the so-called B1 subset, which originates mainly in fetal liver and resides mostly in the peritoneal and pleural cavities. In the peritoneal cavity, B1 cells co-exist with FO-like B2 cells at a 1:1 ratio. Similar to their splenic counterparts, purified peritoneal cavity (PeC) B cells were also able to make IL-10 in response to LPS and CpG in a MyD88-dependent manner, albeit to a higher extent (fig 4.4A and B).This elevated IL-10 production by PeC B cells could be due to the enriched presence of B1 B cells compared to splenic B cells, an assumption supported by a previous report showing that B1 cells were the main source of IL-10 among splenic B cells upon TLR stimulation [422].
Altogether, these findings demonstrated that IL-10 production in response to the TLR agonists used here could be elicited by all B cell subsets tested.

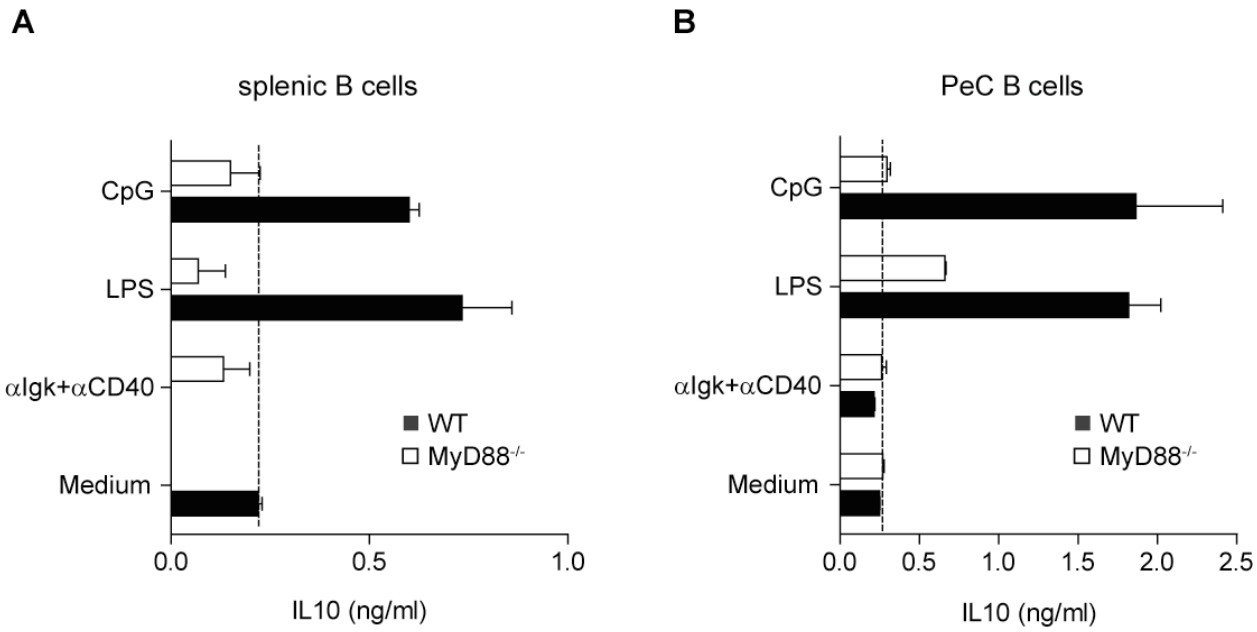

Figure 4.4 Peritoneal cavity B cells make IL-10 following LPS or CpG stimulation.
CD19+ B cells were isolated from spleen (A) and peritoneal cavity (B) of naïve C57BL/6 and MyD88$^{-/-}$ mice via magnetic cell sorting with anti-CD19-coated microbeads. Cells were then cultured with medium alone or stimulated with LPS or CpG (each at 10 µg/ml), or co-stimulated with agonistic antibodies to BCR (anti-Igκ) and CD40 (anti-CD40).After 72h IL-10 present in the cultures was quantified using cell-based ELISA. Graphs show mean±SEM. Results are representative of three independent experiments.

4.1.3 Marginal IL-10 production by total splenic dendritic cells upon LPS and CpG stimulation

DC and macrophages also express TLR and produce cytokines upon stimulation with TLR agonists [117]. Therefore, the ability of DC to make IL-10 in response to the TLR agonists LPS and CpG was tested *in vitro*. In order to minimize IL-10 production from contaminating B cells, DC were isolated from wild-type splenocytes that have been previously depleted of B cells. Dendritic cells cultured in the presence of LPS or CpG produced low levels of IL-10 (~0.2 ng/ml, above background; Fig 4.5, dotted line) that remained unaltered upon simultaneous engagement of CD40. It should be noted here that this IL-10 production could have been produced by the few contaminating B cells, which proliferated in the TLR-stimulated DC cultures, reaching frequencies of ~20% by the end of incubation period. (Fig.4.5). Similar results were obtained following identical stimulation of bone-marrow-derived DC and macrophages (data not shown). Thus, IL-10 production upon stimulation with the LPS and CpG used here was a response uniquely elicited by B cells, compared to other antigen presenting cells.

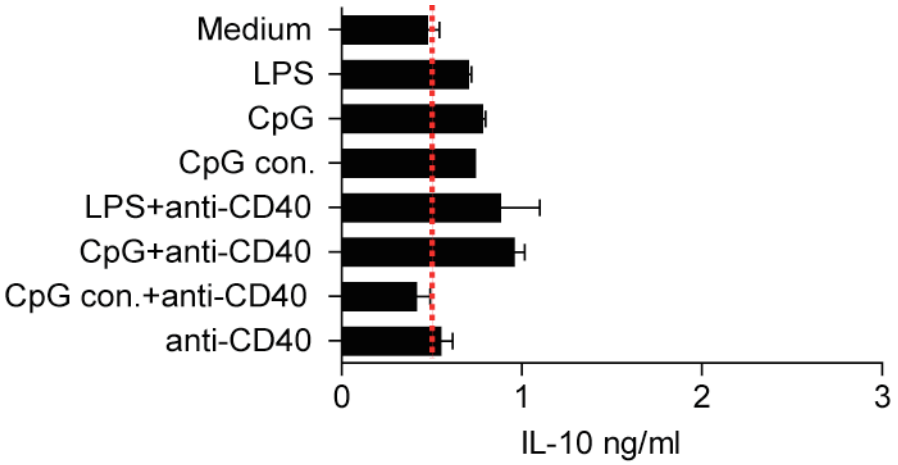

Figure 4.5 CpG and LPS induce only low IL-10 levels by splenic dendritic cells.
CD11c+ DC were purified from the spleen of C57BL/6 mice via magnetic cell sorting with anti-CD11c-coated microbeads and cultured under the indicated conditions for 48h. Cultures were then assayed for their content in IL-10 using cell-based ELISA. Red dotted line indicates background IL-10 levels in the absence of stimulation (medium). Graphs show mean±SEM. Results are representative of at least three independent experiments.

4.1.4 TLR-stimulated B cells limit CD4$^+$ T cell proliferation and production of IFN-γ *in vitro*

The finding that TLR ligation induces naïve B cells to produce IL-10, together with the reported suppressive role of IL-10-producing B cell in EAE prompted the investigation of whether the TLR-induced IL-10-producing B cells could suppress T cell responses. This hypothesis was first addressed *in vitro*. For this purpose an *in vitro* suppression assay was established in which purified naïve CD4$^+$CD25$^-$ T cells (referred to as CD4 T cells hereafter) were stimulated with anti-CD3 in the presence of purified splenic DC. As expected, under this culture condition CD4 T cells readily proliferate, whereas addition of CD4$^+$CD25$^+$ Treg cells (referred to as Tregs hereafter) suppressed this response (Fig.4.6A white bars). Consistent with previous studies [423], addition of the TLR agonist CpG abrogated this Treg-mediated inhibition. Remarkably, supernatants obtained from B cells activated with LPS suppressed CD4 T cell proliferation (Fig.4.6A, and B $_{(LPS)}$) even in the presence of CpG. In contrast, supernatants from LPS-activated DC had no suppressive effect and further promoted T cell proliferation (Fig.4.6A DC$_{(LPS)}$). Supernatants from CpG-stimulated B cells exhibited similar suppressive effects (Fig.4.6B). The extent of suppression by both LPS-activated and CpG-activated B cell supernatants was proportional to the amounts of LPS and CpG, respectively, that had been used to stimulate B cells (Fig.4.6B). Furthermore, in the case of LPS, the degree of suppression correlated with the duration of B cell cultures: CD4 T cell proliferation progressively declined as longer-term B cell culture supernatants were used (Fig.4.6C). Because the amount of IL-10 secreted by B cells was previously found to be proportional to the dose of LPS or CpG used, and to peak at 72h of culture, these latter two observations indicated that T cell inhibition correlated with increasing amounts of IL-10 present in the B cell supernatants. The role of IL-10 in suppression was directly tested by blocking IL-10 signaling in the assay. Indeed, concomitant blockade of IL-10 and its receptor abrogated the suppression by LPS-activated B cell supernatants (Fig.4.6 D). In addition, supernatants from IL-10-deficient B cells stimulated with LPS failed to display such inhibitory effect, demonstrating that IL-10 is critical for the suppressive functions of TLR-activated B cells *in vitro*.

Besides proliferation, TLR-activated B cells also suppressed CD4 T cell production of IFN-γ, a cytokine signature of type 1 T helper cells (Th1) (Fig.4.7). Suppression of IFN-γ, similar to that of proliferation, was also abrogated upon blockade of IL-10. Finally, recombinant IL-10 alone mimicked the B cell-mediated suppression of IFN-γ, whereas recombinant TGFβ1, a

cytokine shown to inhibit T cell responses [266], had only a moderate effect. Altogether, these findings demonstrated that TLR stimulation induces suppressive functions in B cells *in vitro*, by endowing them with the capacity to limit, at least partly via IL-10, both CD4 T cell proliferation and Th1 differentiation. They further suggested that TLR activation of DC and B cells results in opposing effects on T cell activation: TLR-stimulated DC produced a cytokine milieu poor in IL-10 that augmented T cell activation, whereas TLR-stimulated B cells produced a cytokine milieu rich in IL-10 that dampened T cell responses.

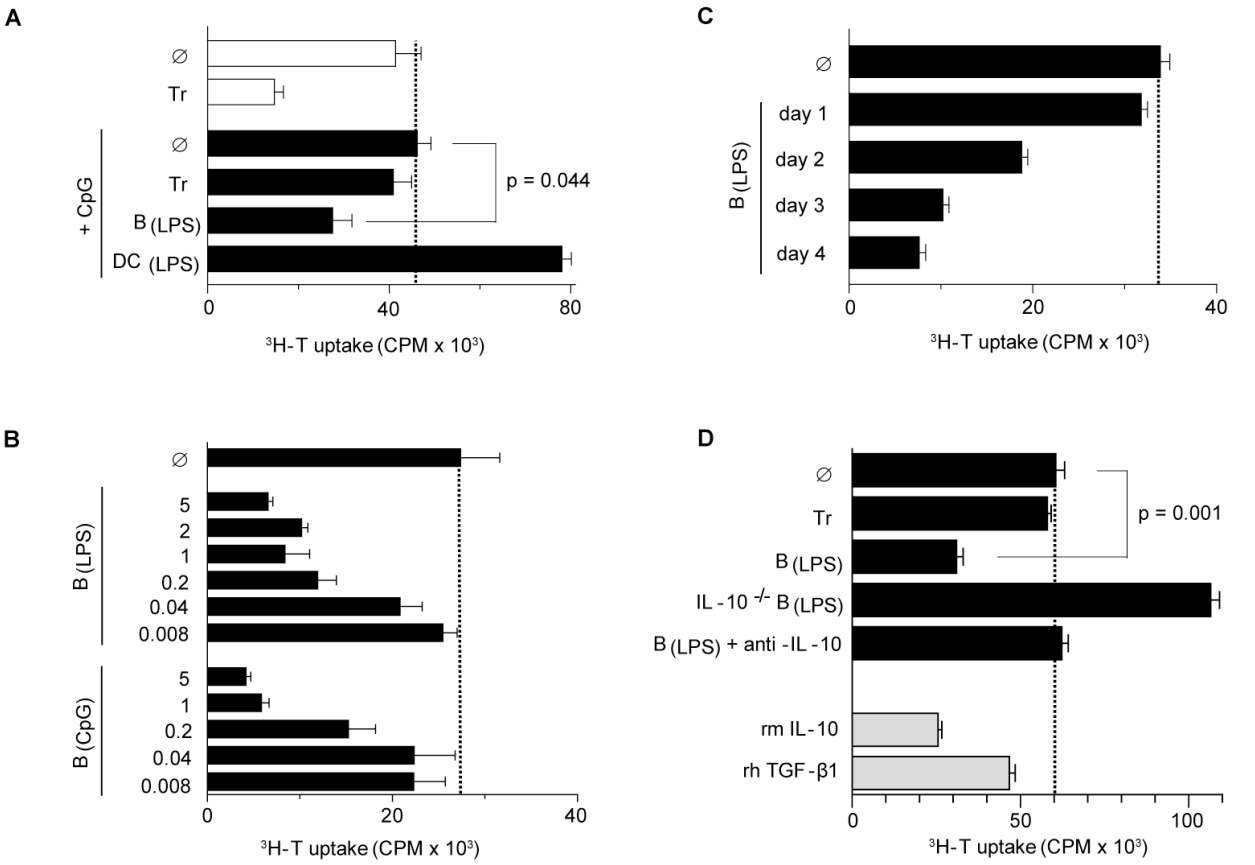

Figure 4.6 IL-10 derived from TLR-activated B cells suppresses *in vitro* CD4 T cell proliferation induced by TLR-stimulated DC.

Purified $CD4^+CD25^-$ T cells were co-cultured with $TLR2/4^{-/-}$ DC in the presence of anti-CD3. Proliferation was measured 48h later by ^{3}H-thymidine incorporation. (A) Cultures were additionally supplemented with: medium (Ø); purified $CD4^+CD25^+$ T regulatory cells (Tr); supernatants from B cells or DC activated with LPS (2μg/ml), denoted as $B_{(LPS)}$ and $DC_{(LPS)}$, respectively. Cultures indicated by the black bars also received CpG (1μg/ml) (B-C) Proliferation assay was set up with $CD4^+CD25^-$ T cells, $TLR2/4^{-/-}$ DC and anti-CD3 in the presence of CpG and of (B) supernatants from B cells stimulated with the indicated amounts (in μg/ml) of LPS or CpG or (C) supernatants from LPS-stimulated B cells collected at the indicated time points. (D) Proliferation assay performed as in A, including the following conditions: supernatants from LPS-activated B cells in the presence of blocking anti-IL-10 and IL-10 receptor antibodies (anti-IL-10); supernatants from LPS-activated $IL\text{-}10^{-/-}$ B cells ($IL\text{-}10^{-/-}$ $B_{(LPS)}$); recombinant mouse IL-10 (rIL-10) or human TGF-β1 (rhTGF-β1). Graphs show mean±SEM. *, P<0.05; **, P<0.01; Results are representative of three independent experiments.

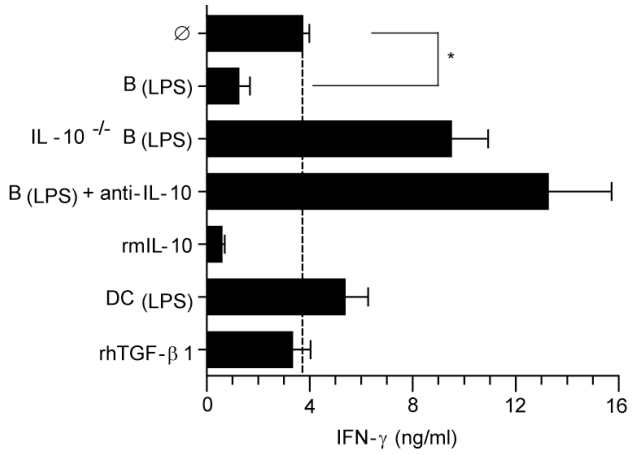

Figure 4.7 IL-10 from LPS-activated B cells suppresses *in vitro* IFN-γ production by CD4 T cells activated in the presence of CpG-stimulated DC.

Purified naïve $CD4^+CD25^-$ T cells were co-cultured with TLR2/4$^{-/-}$ DC in the presence of anti-CD3 (0.1 µg/ml).Cultures were supplemented with : medium (Ø); supernatants from LPS-stimulated wild-type B cells ($B_{(LPS)}$) either alone or in the presence of blocking anti- IL-10 and IL-10 receptor antibodies (anti-IL-10); supernatants from LPS-activated wild-type DC ($DC_{(LPS)}$); supernatants from LPS-activated IL-10$^{-/-}$ B cells; recombinant mouse IL-10 or recombinant human TGF-β1 (rh TGF-β1). All cultures received CpG (1 µg/ml) 2h later. After 48h culture supernatants were collected and assayed for IFN-γ by ELISA. Graphs show mean±SEM. *, P<0.05. Results are representative of three independent experiments.

4.1.5 MyD88 in B cells is required for recovery from EAE

The suppressive effects of TLR/MyD88-activated B cells on T cell responses observed *in vitro* suggested that MyD88 could be involved in the regulatory functions of B cells *in vivo* during EAE. To investigate the role of MyD88 specifically in B cells, mice lacking MyD88 selectively in B cells were generated using a previously described bone marrow chimera approach ([270]; see Materials and Methods). Using this system, the following chimera mice were generated: a) B-MyD88$^{-/-}$ mice, in which only B cells lack MyD88, b) B cell- deficient mice, c) B-WT control mice, in which hematopoietic cells are mostly wild-type and d) WT control mice with 100% wild-type hematopoietic cells. Following reconstitution, EAE was induced in all groups by immunization with a myelin oligodendrocyte-derived peptide (MOG_{35-55}) emulsified in adjuvant, and disease symptoms were daily assessed. Control WT chimeric mice displayed a monophasing EAE course, and recovered from disease, whereas, B cell-deficient mice displayed more severe, non-remitting disease, consistent with previous studies [269-270]. B-MyD88 mice, resembling B cell-deficient mice, suffered an exacerbated and chronic EAE, contrary to control mice that

recovered from disease (Fig.4.8A). This finding demonstrated that MyD88 is required for the regulatory functions of B cells during EAE. Considering that antigen-specific CD4 T helper cells of Th1 and Th17 types are involved in the inflammatory process driving this disease, the influence of MyD88 expression in B cells on CD4 T cell responses was then examined. On day 30 post EAE induction, antigen-specific recall responses were assessed *in vitro* following re-stimulation of splenocytes with the autoantigen. Compared to their wild type counterparts, splenocytes from mice lacking MyD88 in B cells displayed enhanced autoantigen-induced proliferation (Fig.4.8B) and produced increased amounts of IL-17 and IFN-γ (Fig.4.8C and D), the cytokine signatures of Th17 and Th1 cells, respectively. Likewise, B cell-deficient mice also showed elevated *in vitro* recall responses. Thus, MyD88 signaling in B cells limited disease severity and promoted recovery by regulating autoreactive Th1 and Th17 cells.

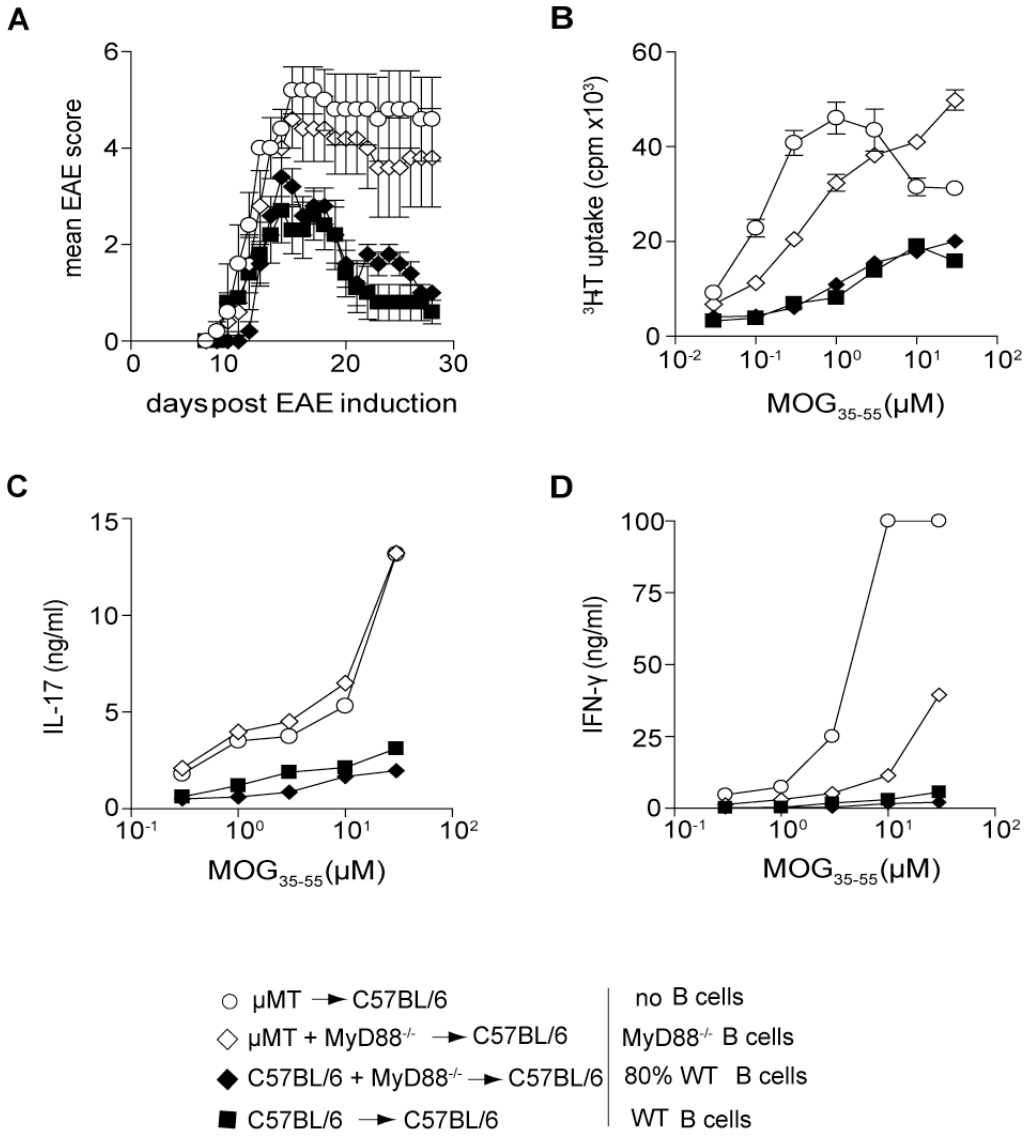

Figure 4.8 MyD88 is required in B cells for recovery from EAE.
EAE was induced in the indicated chimera mice. (A) Clinical disease score (mean±SEM) throughout a 30-day period. (B-D) On day 30 post-EAE induction, splenocytes prepared from each group were stimulated with titrating doses of MOG_{35-55} .After 48h (B) cellular proliferation was assessed by ^{3}H-thymidine incorporation and the concentration of IL-17 (C) and IFN-γ (D) in culture supernatants were measured by cell-based ELISA. Graphs show mean±SEM. Results are representative of two independent experiments.

In agreement with earlier studies [424], MyD88-deficient mice were completely resistant to EAE induction (Fig. 4.9A). Resistance to disease correlated with decreased antigen-specific proliferation and IFN-γ levels, and with severely impaired IL-17 production compared to control mice, as determined after splenocyte re-stimulation with MOG35-55 on day30 post EAE

induction (Fig. 4.9 B and D). Altogether, these results revealed a dual role for MyD88 in initiation and resolution of EAE: MyD88-signalling in cells other than B cells, presumably DC and macrophages, orchestrates inflammatory Th1 and Th17 responses that drive disease, whereas in B cells it triggers anti-inflammatory cascades that limit Th1 and Th17 responses and thereby promotes recovery from disease.

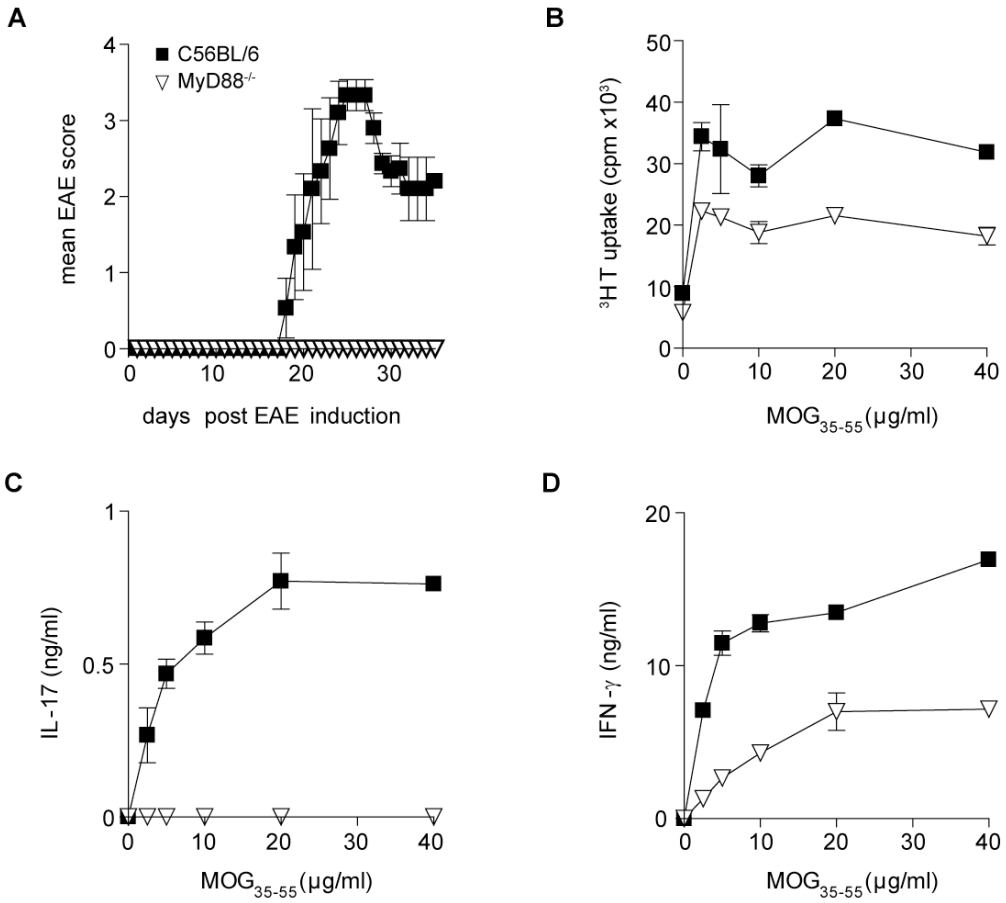

Figure 4.9 Resistance of MyD88$^{-/-}$ mice to EAE correlates with lack of MOG$_{35-55}$-specific IL-17 production. EAE was induced in C57BL/6 and MyD88$^{-/-}$ mice. (A) Clinical disease score (mean±SEM) assessed throughout a 35-day period. (B-D) On day 30 post EAE induction, splenocytes prepared from each group of mice were stimulated with titrating doses of MOG$_{35-55}$ .After 48h cellular proliferation (B) was assessed by ^{3}H-thymidine incorporationand the concentration of IL-17 (C) and IFN-γ (D) in the cultures was measured by cell-based ELISA. Graphs show mean±SEM. Results are representative of two independent experiments.

4.1.6 Role of TLRs to disease development and resolution

The observation that MyD88 in B cells is required for recovery from EAE prompted the investigation of the TLR that triggered the protective MyD88-signalling in B cells. Because both LPS and CpG induced MyD88-dependent IL-10-production by B cells *in vitro*, the role of their corresponding TLR was examined *in vivo*. This was pursued by generating chimera mice with TLR2/4-deficient or TLR9-deficient B cells, denoted as B-TLR2/4$^{-/-}$ and B-TLR9$^{-/-}$ mice, respectively. Following EAE induction, B-TLR9$^{-/-}$ mice displayed a wild-type-like disease course, achieving recovery after a short paralysis phase (Fig4.10A), and showed *in vitro* IL-17 and IFN-γ recall responses comparable to control mice (Fig.4.10B and C). This indicated that TLR9 is dispensable for the regulatory functions of B cells *in vivo* that promote disease resolution in this EAE model. Nevertheless, disease onset occurred earlier in complete TLR9$^{-/-}$ mice compared to controls, pointing to a pathogenic role of TLR-9 in disease development (Fig.4.11). In contrast, B-TLR2/4$^{-/-}$ mice, similar to B-MyD88$^{-/-}$mice, developed more severe and chronic EAE. This exacerbated disease was also characterized by increased autoantigen-induced production of IL17 and IFN-γ (Fig.4.10B and C), suggesting that limitation of Th1 and Th17 responses was a common mechanism of suppression by TLR2/4- and MyD88- mediated signaling in B cells. However, complete absence of TLR2/4 only led to delayed EAE onset whereas it had no effect on the severity of clinical symptoms (Table 4.1). This finding suggested that signals via TLR2/4 in cells other than B cells have pathogenic effects and, therefore, they contribute to both disease initiation and resolution. Collectively, these data demonstrated that distinct TLR differentially contribute to disease induction and remission: TLR2 and/or TLR4 are required for the suppressive functions of B cells that control disease resolution, whereas along with TLR9 they promote disease initiation via cells other than B cells.

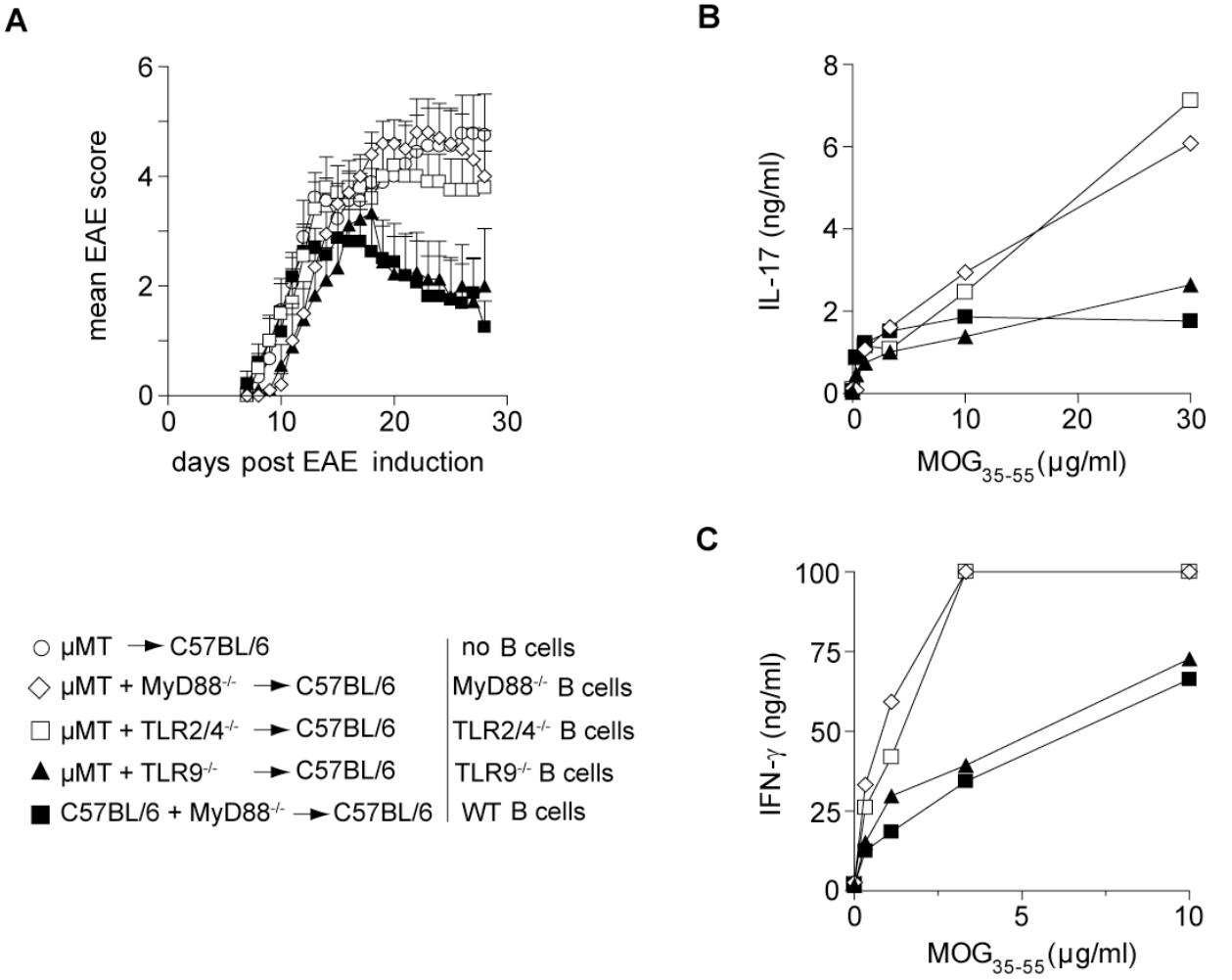

Figure 4.10 TLR-2 and/or TLR-4, but not TLR-9, in B cells is required for their suppressive functions during EAE.

EAE was induced in the indicated chimera mice. (A) Clinical disease score (mean±SEM) assessed throughout a 30-day period. (B and C) On day 30 post-EAE induction, splenocytes prepared from each group were stimulated with titrating doses of MOG_{35-55}. After 48h the concentration of IL-17 (B) and IFN-γ (C) in the cultures was measured by cell-based ELISA. Graphs show mean±SEM. Results are representative of two independent experiments.

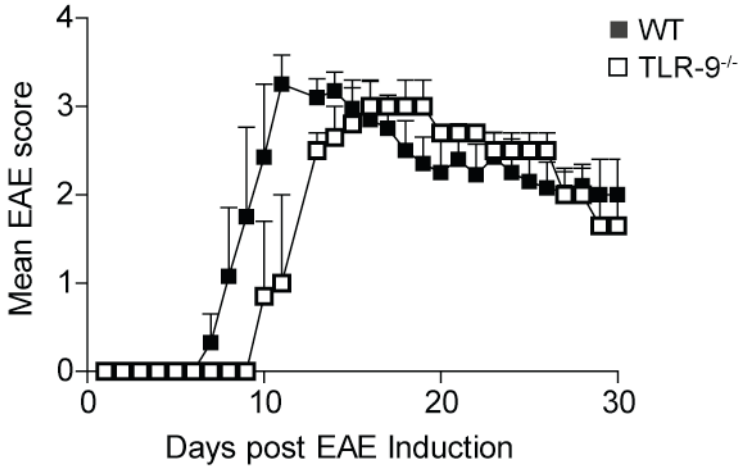

Figure 4.11 TLR9 accelerates onset of EAE.
Clinical disease score (mean+SEM) as assessed for C57BL/6 wild-type (WT) and TLR9$^{-/-}$ mice for 30 days after EAE induction. Data from one experiment are shown.

Table 4.1 Disease parameters in TLR2/4$^{-/-}$ and WT mice[a]

Mouse group	Disease incidence	Mean day of disease onset	Mean maximum Clinical score
C57BL/6	11/11 (100%)	12.40 ± 1.91	3.80 ± 1.10
TLR2/4$^{-/-}$	13/14 (92.8%)	15.10 ± 2.44	3.50 ± 0.26
Statistical significance	ns (P>0.05)	* (P<0.05)	ns (P>0.05)

[a] EAE was induced by immunisation with MOG_{35-55} emulsified in CFA and disease course was monitored thereafter.Student´s t-test was used for statistical analysis.

4.1.7 Sources of TLR agonists controlling the suppressive functions of B cells during EAE.

The next step was to determine the source(s) providing the agonists for these distinct TLR during disease. Two potential candidates were examined: the commensal flora and components of the adjuvant used to induce EAE. A previous study demonstrated that gut microorganisms are recognized by TLR in the steady state and that, in particular, TLR4 engagement contributes to local tissue repair and homeostasis [425]. In addition, intestinal microbiota were recently implicated in predisposition to experimental autoimmune type 1 diabetes via a mechanism involving MyD88 [426], and that their recognition in the gut can prime systemic immunity [427]. Collectivelly, these studies suggested that TLR agonists derived from commensal flora could influence the outcome of immune responses, both systemic and at affected organs distal to the gut. Therefore, EAE course was compared between germ-free (GF) mice and regular specific-pathogen free (SPF) C57BL/6 mice. Absence of commensal microorgamisns hardly altered disease severity or recovery phase of mice (Fig4.12A). Consistently, splenocytes from GF mice showed similar proliferative responses and only slightly reduced IL-17 and IFN-γ production in response to autoantigen, compared to SPF mice (Fig.4.12B, C and D). Therefore, it was unlikely that TLR agonists were provided from the commensal the gut flora, pointing to a possible role for the adjuvant used in the EAE induction protocol. This adjuvant is a water- in-oil emulsion enriched with heat-killed desiccated *Mycobacterium tuberculosis* (*Mtb*) and is necessary for disease to develop. Extracts of *Mtb* have been shown to stimulate TLR-dependent inflammatory cytokine production by DC [428]. Thus, it was plausible to hypothesize that *Mtb* components could also stimulate TLR-MyD88 signalling in B cells and thereby trigger their suppressive functions. This hypothesis was supported by the findings that whole cell *Mtb* extract induced IL-10 secretion by naïve B cells *in vitro* in a dose-dependent manner and that this response was abrogated when B cells lacked MyD88 or TLR2/4 (Fig.4.13). Collectively, these results strengthened the notion that adjuvant –derived TLR agonists triggered the regulatory functions of B cells during EAE. Nevertheless, a possible role for endogenous TLR ligands (of non –commensal origin) or for the pertussin toxin used in EAE induction protocol, which has been reported to act via TLR4 [429], could not be formally excluded.

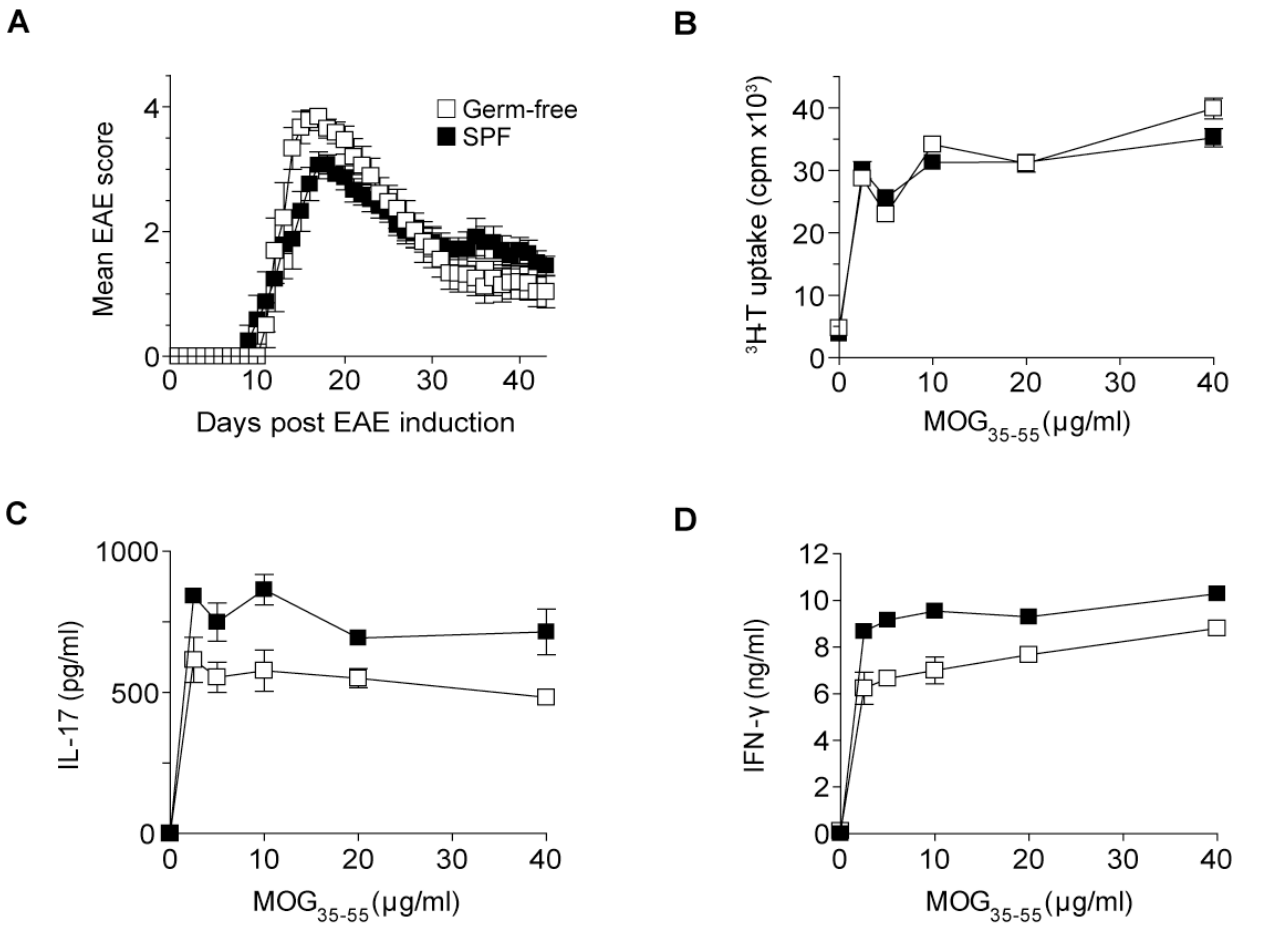

Figure 4.12 Gut microflora has marginal influence on the course of EAE.

EAE was induced in specific pathogen –free (SPF) and germ-free (GF) C57BL/6 mice. (A) Clinical disease score (mean±SEM) assessed throughout a 30-day period. (B-D) On day 30 post EAE induction, splenocytes prepared from each group were stimulated with titrating doses of MOG_{35-55} . After 48h cellular proliferation (B) was assessed by ^{3}H-thymidine incorporation and the concentrations of IL-17 (C) and IFN-γ (D) in the cultures were measured by cell-based ELISA. Graphs show mean±SEM. Results are representative of two independent experiments.

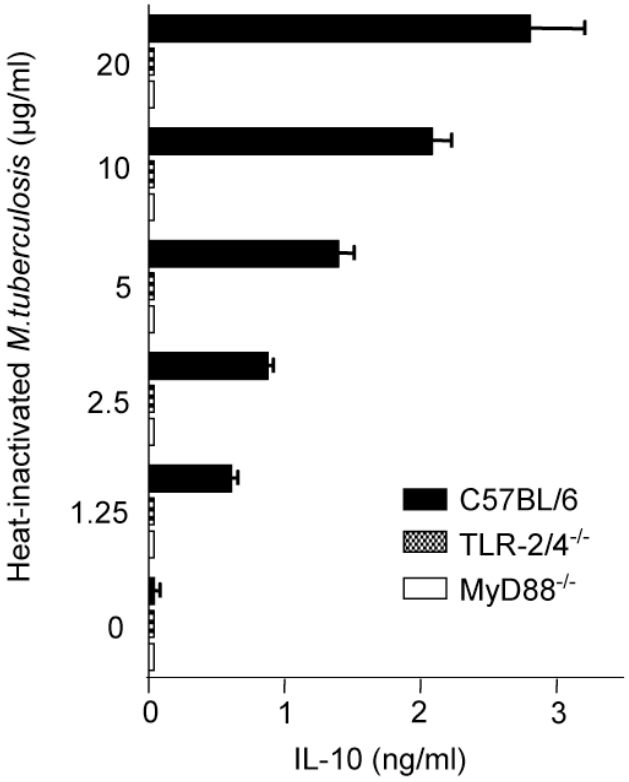

Figure 4.13 *Mycobacterium tuberculosis* extract triggers IL-10 production by naïve B cells *in vitro* in TLR2/4- and MyD88-dependent manner.

B cells were isolated via magnetic cell sorting from spleens of naïve C57BL/6, TLR2/4$^{-/-}$ and MyD88$^{-/-}$ mice and were cultured in the presence of titrating amounts of heat-inactivated Mycobacterium *tuberculosis* whole cell extract. After 72h, production of IL-10 in the cultures was quantified by cell-based ELISA. Graphs show mean±SEM. Results are representative of two experiments.

4.1.8 TLR-activated B cells limit T cell activation by interfering with DC function

In order to gain insight on how TLR-activated B cells could dampen CD4 T cell responses, the previously observed suppressive effects of B cell supernatants were further dissected. In the *in vitro* assay performed, B cell supernatants could either directly target T cells or indirectly by influencing DC. To test a possible direct effect, culture supernatants from LPS-activated B cells were applied on CD4 T cells stimulated with plate-bound anti-CD3 in the absence of DC. In this experimental setting, B cell supernatants failed to suppress and instead, enhanced T cell proliferation, resembling the influence of DC-supernatants, albeit to a lower extent (Fig.4.14A). This was not due to a direct effect of LPS present in the supernatants, since purified LPS alone minimally affected T cell proliferation (Fig.4.14A). Similar results were obtained when recombinant IL-10 was added to the T cell cultures (Fig.4.14A). These findings suggested that LPS induced B cell production of soluble factors, other than IL-10 that promoted T cell expansion. Indeed, B cells cultured with LPS or CpG made IL-6 in a dose-dependent manner (Fig.4.14B). Other cytokines known to be secreted by DC upon LPS stimulation, including IL-12, TNF, IL-1β and IL-23 [93, 430] were undetectable in the supernatants from LPS-activated B cells, as determined by ELISA (data not shown). Furthermore, IL-6 blockade restored T cell proliferation levels whereas recombinant IL-6 alone mimicked the effect of the supernatants from LPS-activated B cells, demonstrating that in the absence of DC the latter augmented T cell proliferation via IL-6.

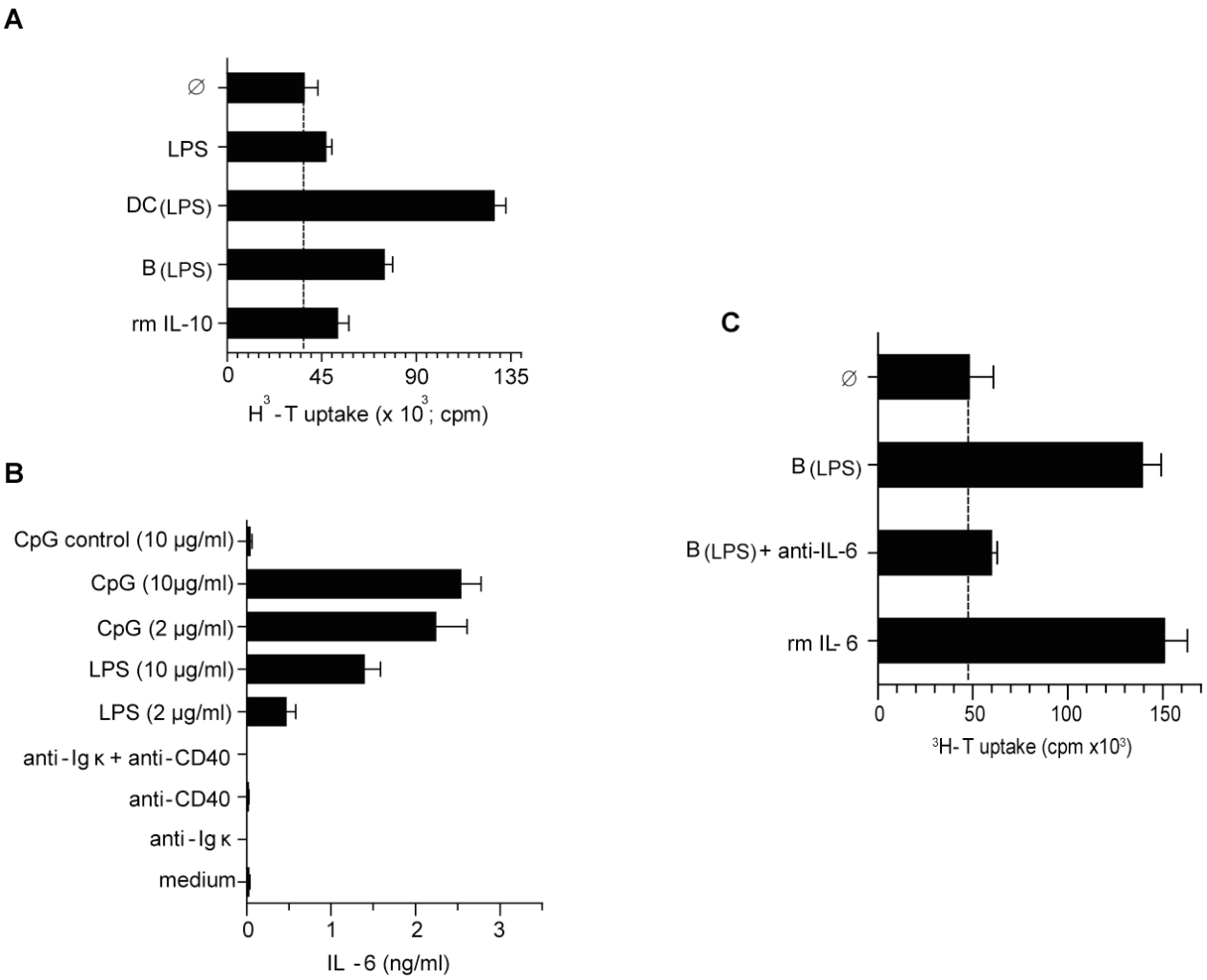

Figure 4.14 IL-10 from LPS-activated B cells does not suppress CD4 T cells directly *in vitro*.
(A) Purified naïve CD4+CD25- T cells were cultured in the presence of plate-bound anti-CD3. Proliferation was measured 48h later by 3H-thymidine incorporation. Cultures were additionally supplemented with: medium (Ø); LPS; supernatants from B cells or DC activated with LPS (2µg/ml; for 72h and 48h, respectively), denoted as B(LPS) and DC(LPS), respectively, or with recombinant mouse IL-10 (rmIL-10). (B) Splenic naïve B cells from C56BL/6 mice were stimulated with the indicated amounts of LPS or CpG, with agonistic antibodies to BCR (anti-Igκ) and CD40 (anti-CD40) alone or in combination. Following 72h of incubation IL-6 was quantified in the cultures using cell-based ELISA. (C) T cell proliferation assay was set up as in A. Cultures additionally received medium (Ø) supernatants from LPS -activated B cells (B(LPS)) alone or in the presence of blocking anti-IL-6 and anti-IL-6 receptor antibodies (anti-IL-6; 20 µg/ml each); recombinant mouse IL-6 (rmIL-6; 20 ng/ml). Graphs show mean±SEM. Results are representative of two independent experiments.

In light of these observations, the IL-10-dependent inhibition of T cell proliferation by B cell supernatants required the presence of DC, indicating the latter as the direct target of suppression. To address this possibility, purified splenic DC were cultured in the presence of supernatants from LPS-stimulated B cells and then activated with CpG. In response to CpG alone, DC made several cytokines including the pro-inflammatory TNF and IL-6, which augment

T cell expansion, IL-12 that drives Th1 differentiation, and IL-23 that promotes survival of Th17 cells (Fig. 4.15). Addition of B cell supernatants resulted in reduced production of these cytokines by DC and this effect involved IL-10, since blocking IL-10 and its receptor reversed suppression (Fig.415). Of note, the magnitude of suppression as well as of its abrogation upon IL-10 blockade, varied among the cytokines, implying that these were differentially regulated by IL-10, and that factors other than IL-10 in the B cell supernatants were also involved in suppression. Collectively, these data indicated that *in vivo* TLR-activated B cells could regulate, partly via IL-10 production, EAE by limiting DC-derived cytokines that promote T cell growth, Th1 differentiation and survival of Th17 cells.

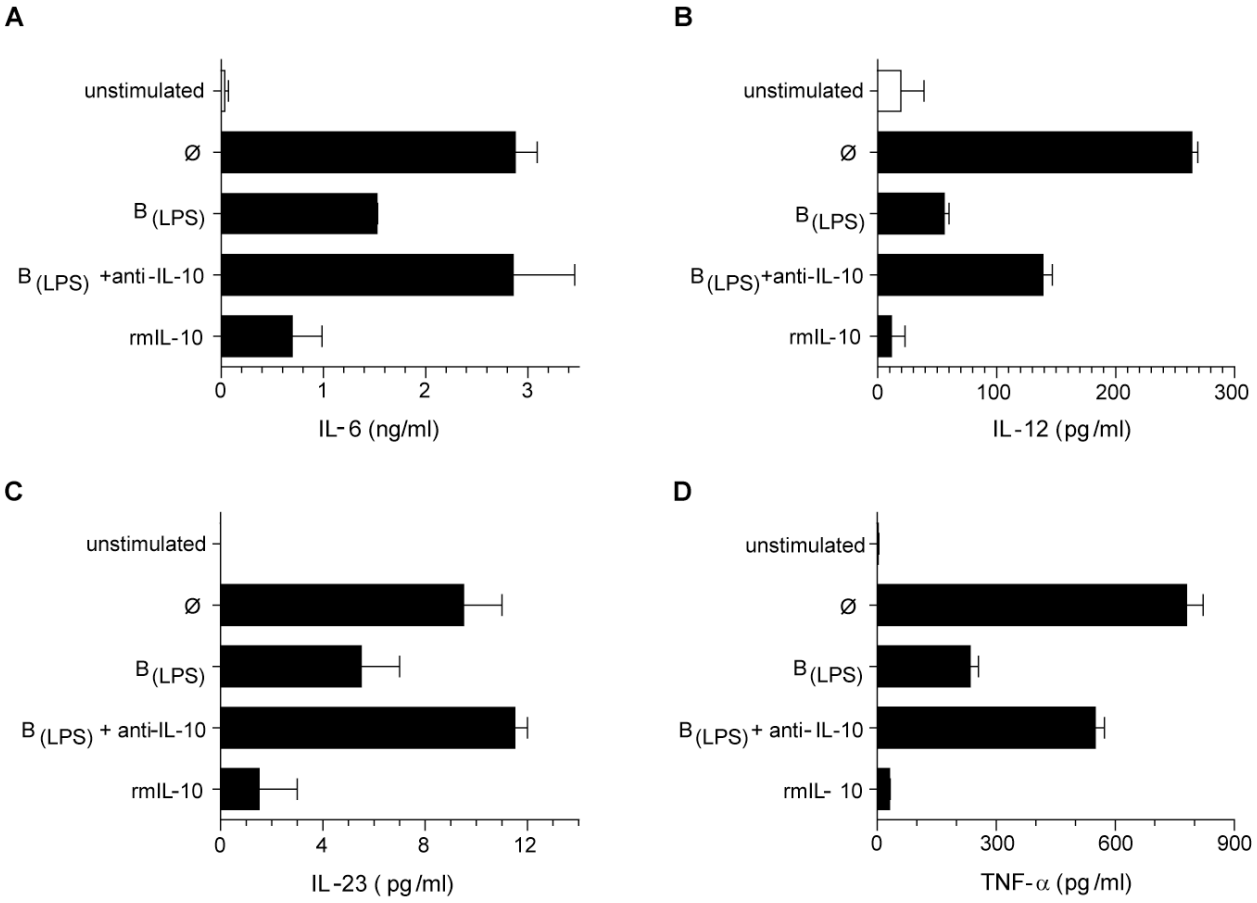

Figure 4.15 IL-10 from LPS-activated B cells limits cytokine production by CpG-stimulated DC.
CD11c+ DC purified from the spleen of TLR2/4-/- mice via magnetic cell sorting were cultured under the following conditions: with medium alone (Ø); with supernatants from LPS-activated B cells ($B_{(LPS)}$) either alone or in the presence of blocking anti-IL-10 and anti-IL-10 receptor antibodies (anti-IL-10; 20µg/ml each);with recombinant mouse IL-10 (rm IL-10, 20 ng/ml). Culture conditions indicated with black bars additionally received CpG (1µg/ml) 2h later, while unstimulated cells were solely supplemented with medium and no CpG (white bars). After 48h culture supernatants were collected and their content in IL-6 (A), IL-12 (B), IL-23 (C) and TNFα (D) was quantified by ELISA. Graphs show mean±SEM. Results from one and only experiment are shown.

4.1.9 Absence of MyD88 in B cells does not impair antigen-specific B cell responses

Although B cell-intrinsic MyD88 signaling is generally considered as an amplifier of humoral immune responses, there is still considerable controversy about its importance in B cell activation and antibody production [152-153]. Thus, arguably, lack of MyD88 expression might have rendered B cells unable to become activated *in vivo*, so that the exacerbated disease observed in B-MyD88$^{-/-}$ mice was due to an intrinsic defect of the cells, rather than a mere inactivation of their regulatory function. To test this possibility B-MyD88$^{-/-}$ and B-WT chimera mice were immunized with the model antigen DNP-KLH and their DNP-specific B cell responses were assessed. Histological analysis of spleen sections revealed that germinal center formation, a process whereby antigen-specific B cells proliferate and improve their BCR via affinity maturation and somatic hypermutation, occurred normally in the absence of MyD88 in B cells, although GC appeared somewhat smaller compared to control mice (Fig 4.16A). Furthermore, B-MyD88$^{-/-}$ mice mounted DNP-specific IgM and IgG responses of similar magnitude to those of B-WT mice (Fig.4.16B and C). It should be noted however, that the natural DNP-reactive IgM detected in B-WT mice before immunization, was completely absent in mice with MyD88-deficicient B cells (Fig.4.16.B, day 0). Collectively, these findings showed that in the absence of MyD88-signalling B cells the hallmarks of B cell activation, that is antibody production and GC formation, remained largely unaffected, and hence, reduced the likelihood that the severe EAE observed in B-MyD88$^{-/-}$ mice was due to a B cell-intrinsic defect.

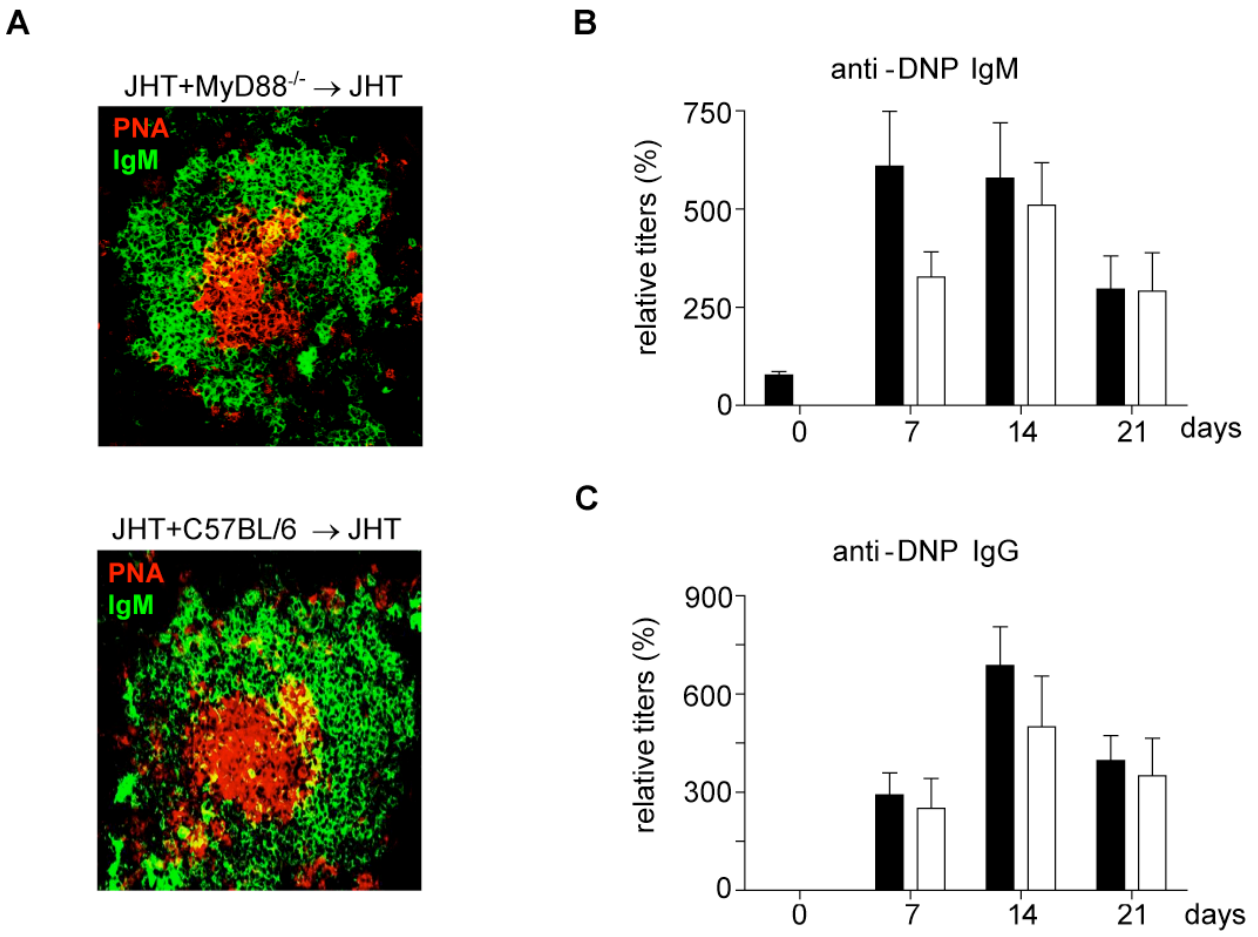

Figure 4.16 Absence of MyD88 in B cells does not abrogate humoral immunity to model antigen.
Chimera mice with wild-type B cells (JHT+ C56BL/6→JHT; black bars) or with MyD88$^{-/-}$ B cells (JHT+ MyD88$^{-/-}$ →JHT; white bars) were immunized via the intraperitoneal route with 200µg DNP-KLH in alum. (A) On day 21 post immunization germinal centers were assessed by histology in splenic cryo-sections using PNA (red) and IgM (green) immunefluorescence staining. (B and C) The relative titers of circulating DNP-reactive IgM (B) and IgG (C) were determined by ELISA in sera collected at the indicated time points. Graphs shown mean±SEM.Results are representative of two independent experiments.

4.1.10 Reduced natural IgM in mice lacking MyD88 in B cells

The difference observed in the natural DNP-reactive IgM between naïve B-MyD88$^{-/-}$ and B-WT mice (Fig.4.16B, day 0) implied that total natural IgM levels might have been impaired in B-MyD88$^{-/-}$ mice and therefore, prompted the measurement of total serum IgM. Indeed, the titers of circulating natural IgM in naive B-MyD88 mice were reduced by 3-fold in comparison to naïve controls (Fig.4.17A). To better describe this phenomenon, total IgM levels in both groups of

chimera mice were monitored in two-week intervals throughout their reconstitution period. Natural IgM titers were markedly diminished in mice lacking MyD88 in B cells at all time-points tested (Fig.4.17B).

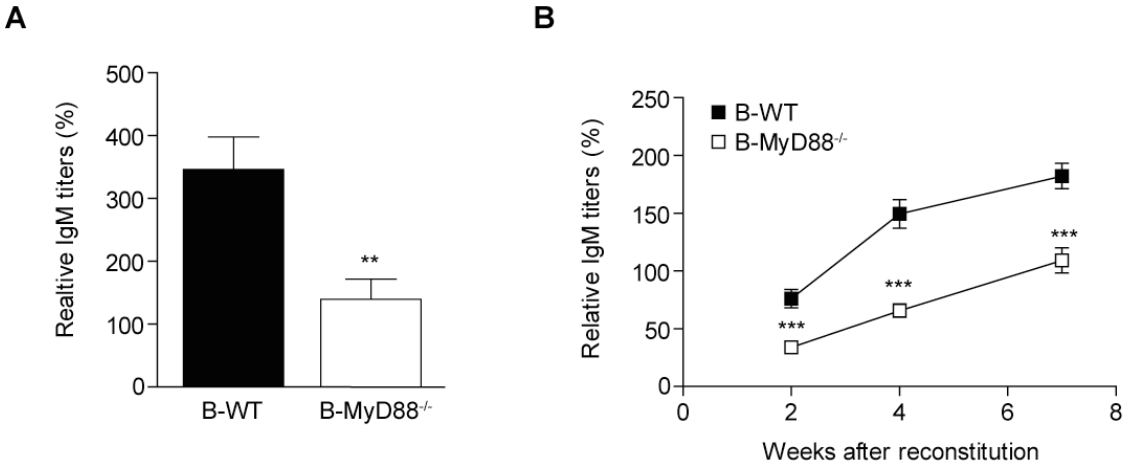

Figure 4.17 Reduced natural IgM titers in the absence of MyD88 in B cells.
(A) Relative titers of natural total IgM were determined by ELISA in the sera of naïve chimera B-WT (wild-type B cells; n=12) and B-MyD88$^{-/-}$ mice (MyD88-/- B cells; n=10) at eight weeks after bone marrow (BM) reconstitution. (B) Kinetics of circulating natural IgM following BM reconstitution were monitored by measuring IgM (as in A) in the sera of B-WT (n=20) and B-MyD88$^{-/-}$ (n=20) mice collected at the indicated time-points. Results show mean±SEM. ***, P<0.001; **, P<0.01.

4.2 Role of secreted IgM in EAE

The observation that natural IgM was diminished in mice with MyD88$^{-/-}$ B cells raised the question whether the reduced IgM titers also contributed to the exacerbated disease observed in B-MyD88$^{-/-}$ mice. The following experiments addressed this possibility indirectly by investigating the role of secreted IgM during EAE using genetically modified mice that cannot secrete this antibody. In these so-called μs$^{-/-}$ mice the exon encoding for the secretory tail piece of IgM has been mutated by targeted gene disruption, so that B cells cannot synthesize selectively the secreted form of IgM, but do express IgM on their surface [183-184]. Consistent with previous studies [183, 196], μs$^{-/-}$ displayed disturbed B cell development: MZ B cells were expanded over FO B cells in the spleen whereas B1 B cells were expanded in the peritoneal

cavity and spleen (Fig.4.18A and B). In contrast, they harbored in their spleen and lymph nodes normal frequencies (and numbers) of CD4 and CD8 T cells, as well as $CD4^{+}FoxP3^{+}$ T regulatory cells (Fig.4.18Cand D;data not shown).

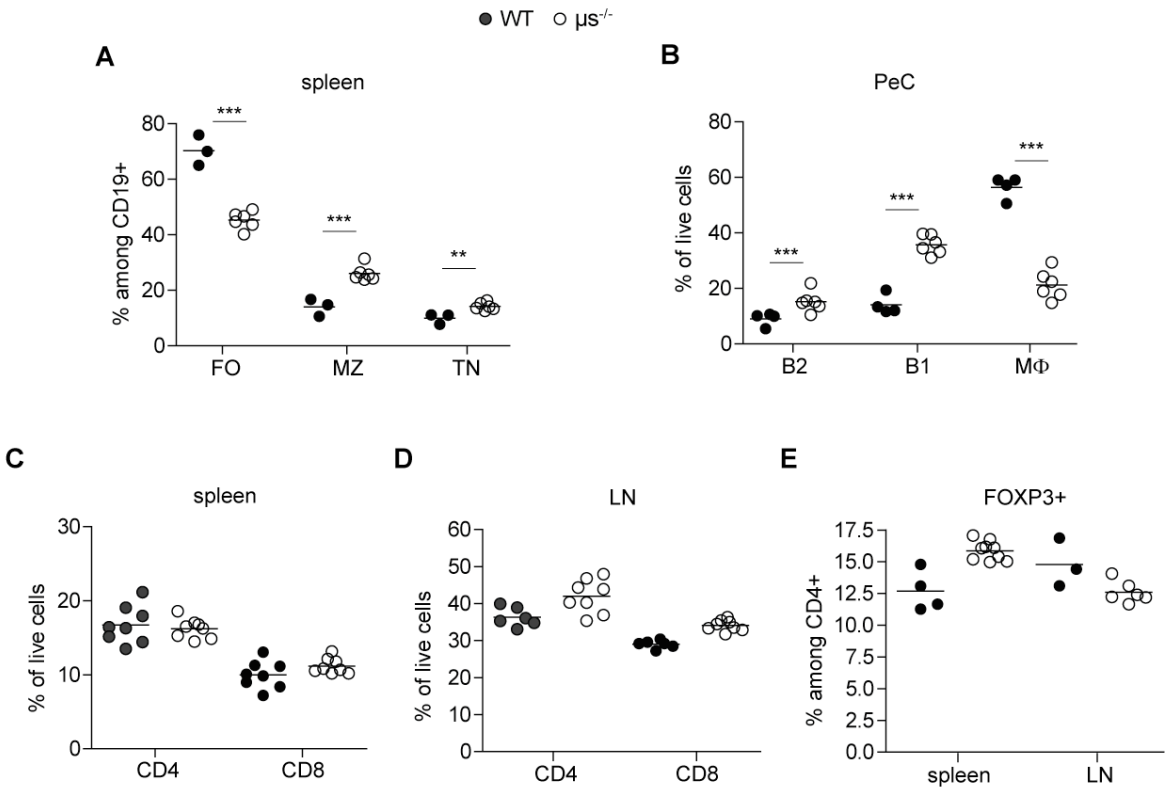

Figure 4.18 B cell and T cell compartment in mice lacking secreted IgM.
Comparison of B cell- and T cell- subsets between wild-type (WT) and $\mu s^{-/-}$ mice as determined by flow cytometry. (A) Frequencies of splenic FO, MZ and transitional (TN) B cells. These subsets were defined as described in Fig.4.3A, left plot. (B) frequencies of B1 , B2 cells and macrophages (MΦ) in the peritoneal cavity, determined by flowcytometry. B1 cells were defined as $B220^{low}CD11b^{+}$, B2 cells as $B220^{hi}CD11b^{-}$ and macrophages as $B220^{-}$ $CD11b^{hi}$ cells. (C and D) Frequencies of splenic and lymph node (LN) CD4 and CD8 T cells among live cells; defined according to expression of CD4 and CD8, respectively. (E) Frequency of splenic and LN T regulatory cells among live CD4 T cells, defined by surface expression of CD4 and intracellular expression of FoxP3. Pooled results from two independent experiments are shown. *** P<0.001; **P<0.01.

4.2.1 Accelerated and exacerbated EAE in the absence of secreted IgM

To assess the influence of secreted IgM (sIgM) on EAE course, $\mu s^{-/-}$ and wild-type mice were immunized with MOG_{35-55} emulsified in adjuvant. Remarkably, $\mu s^{-/-}$ mice displayed clinical symptoms significantly earlier than wild-type mice and developed more severe disease (Fig.4.19). Thus, secreted IgM has protective functions in EAE, interfering with both disease initiation and the magnitude of pathogenic responses. In contrast, the recovery phase was only marginally affected in the absence of sIgM, implying that endogenous disease-regulating mechanisms were still able to control the exacerbated response. Thus, the reduced circulating IgM levels could have accounted for the earlier disease onset that occurred in B-$MyD88^{-/-}$ mice (Fig.4.20) and partially contributed to their disease severity rather than to disease persistence.

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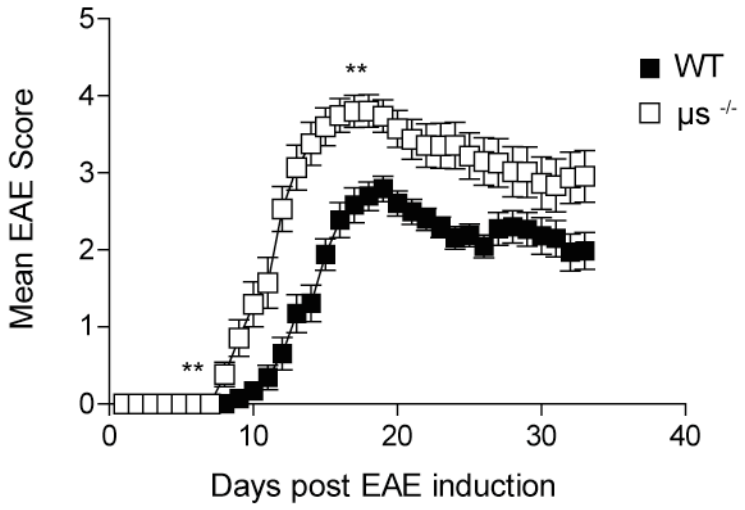

Figure 4.19 Early EAE onset and exacerbated disease in the absence of secreted IgM.
Clinical disease score (mean+SEM) as assessed for wild-type (WT; n=30) and $\mu s^{-/-}$ mice (n=35) for 30 days after EAE induction. Data are pooled from four independent experiments. **P<0.01.

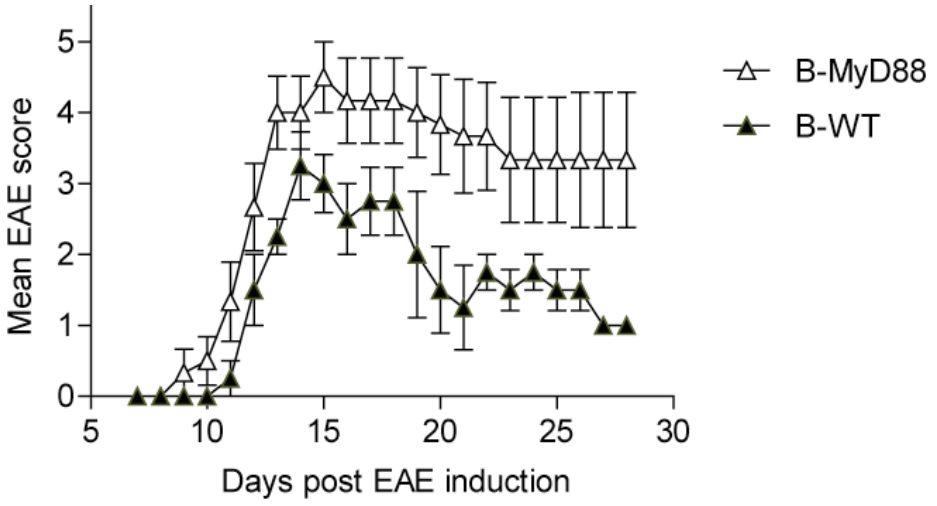

Figure 4.20. Lack of MyD88 in B cells results in accelerated and more severe EAE.
EAE was induced in mice lacking MyD88 in B cells (B-MyD88$^{-/-}$) and in mice with wild-type B cells (B-WT) and disease symptoms were daily monitored. These results were extracted from fig4.8A to present more clearly the earlier onset of and more severe disease displayed by B-MyD88$^{-/-}$ mice, which have reduced levels of natural IgM.

4.2.2 Earlier disease onset in μs$^{-/-}$ mice correlates with increased inflammatory T cell responses at the inflammatory site and peripheral lymphoid organs.

The accelerated EAE in μs$^{-/-}$ mice was in line with a previous study reporting early onset of lupus pathogenesis in lupus-prone mice carrying the secreted IgM mutation [198], pointing to a protective role for IgM in autoimmunity. In that disease model early pathogenesis correlated with increased titers of anti-DNA IgG autoantibodies that led to elevated deposits of immune complexes on renal glomeruli. In addition, lack of secreted IgM predisposed naïve C57BL/6 mice to autoantibody development, suggesting a contribution of IgM in restriction of autoreactive B cell repertoire. Despite that antibodies in this model of EAE are not required for disease development -B cell-deficient mice are susceptible [269-270]- they can contribute to pathogenesis upon transfer [396-398]. Therefore, MOG_{35-55}-reactive IgG autoantibodies in μs$^{-/-}$ and control mice were assessed during EAE. Following one week after EAE induction circulating MOG_{35-55}-reactive antibodies of IgG2a, IgG1, and IgG2b isotypes were upregulated in both groups of mice (Fig.4.21 and data not shown). Notably, by days 21 and 30, mice lacking secreted IgM carried increased levels of MOG_{35-55} reactive IgG2a antibodies compared to wild-type mice (Fig.4.21). However, these differences occurred rather late to account for the

accelerated clinical symptoms or the aggravated disease that peaked around day 18 in μs$^{-/-}$ mice.

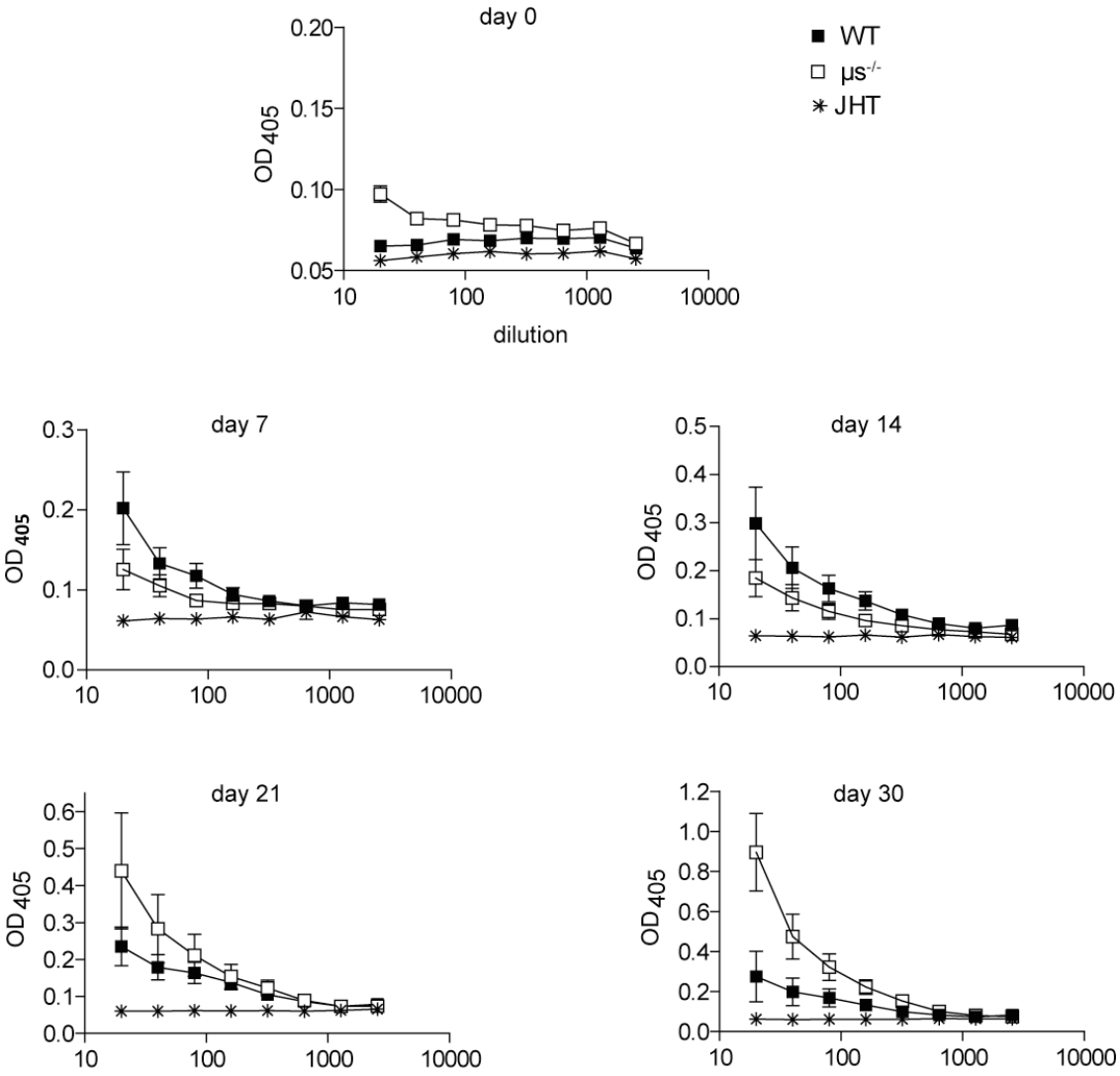

Figure 4.21 Increased levels of MOG$_{35-55}$-reactive IgG2a in μs$^{-/-}$ mice during EAE.
Circulating MOG$_{35-55}$-reactive IgG2a in wild-type (WT) μs$^{-/-}$ mice were determined by ELISA in sera collected before (day 0) and at the indicated time points after EAE induction. Graphs shown mean±SEM. Results are representative of two independent experiments.

In order to identify the immune parameters involved in early disease onset in the absence of secreted IgM, immune responses were analyzed on day 10 post EAE induction, when μs$^{-/-}$ mice

were already sick, while wild-type mice only began to display the first clinical symptoms (Fig. 4.22A). Lack of secreted IgM resulted in increased accumulation of CNS-infiltrating cells, both in the brain and spinal cord, and according to cellular characterization of the infiltrates, this was due, at least partly, to higher numbers of CD4 T cells and CD8 T cells (Fig.4.22 B, C and D). The functional properties of these T cells were evaluated by measuring their production of cytokines after short-term re-stimulation with anti-CD3 and anti-CD28. Analysis revealed the presence of inflammatory populations among CD4 T cells in both groups of mice, evidenced by their production of IL-17 and IFN-γ following short-term TCR-re-stimulation (Fig.4.22E). However, these cells were strikingly more abundant in $\mu s^{-/-}$ mice compared to controls (Fig.4.22F). Thus, secreted-IgM deficiency resulted in augmented Th1 and Th17 responses in the CNS that correlated with early disease onset. Augmented inflammatory CD4 T cell responses in the absence of secreted IgM were also observed in the spleen upon restimulation with MOG_{35-55}. In particular, $\mu s^{-/-}$ mice harbored in their spleen 2-fold higher numbers of antigen-specific CD4 T cells compared to controls, as assessed by co-expression of CD4 and CD154 (CD40L) (Fig. 4.23A). Moreover, they had more IFN-γ-, IL-17- and TNF-producing CD4 T cells (Fig. 4.23B) than wild-type mice. Identical assays performed at this time point with lymph node cells draining the immunization site (dLN) showed comparable CD4 T cell responses between the two groups of mice (data not shown). Therefore, early appearance of clinical symptoms in mice lacking secreted IgM correlated with enhanced autoreactive Th1 and Th17 responses at both the inflamed site and the spleen.

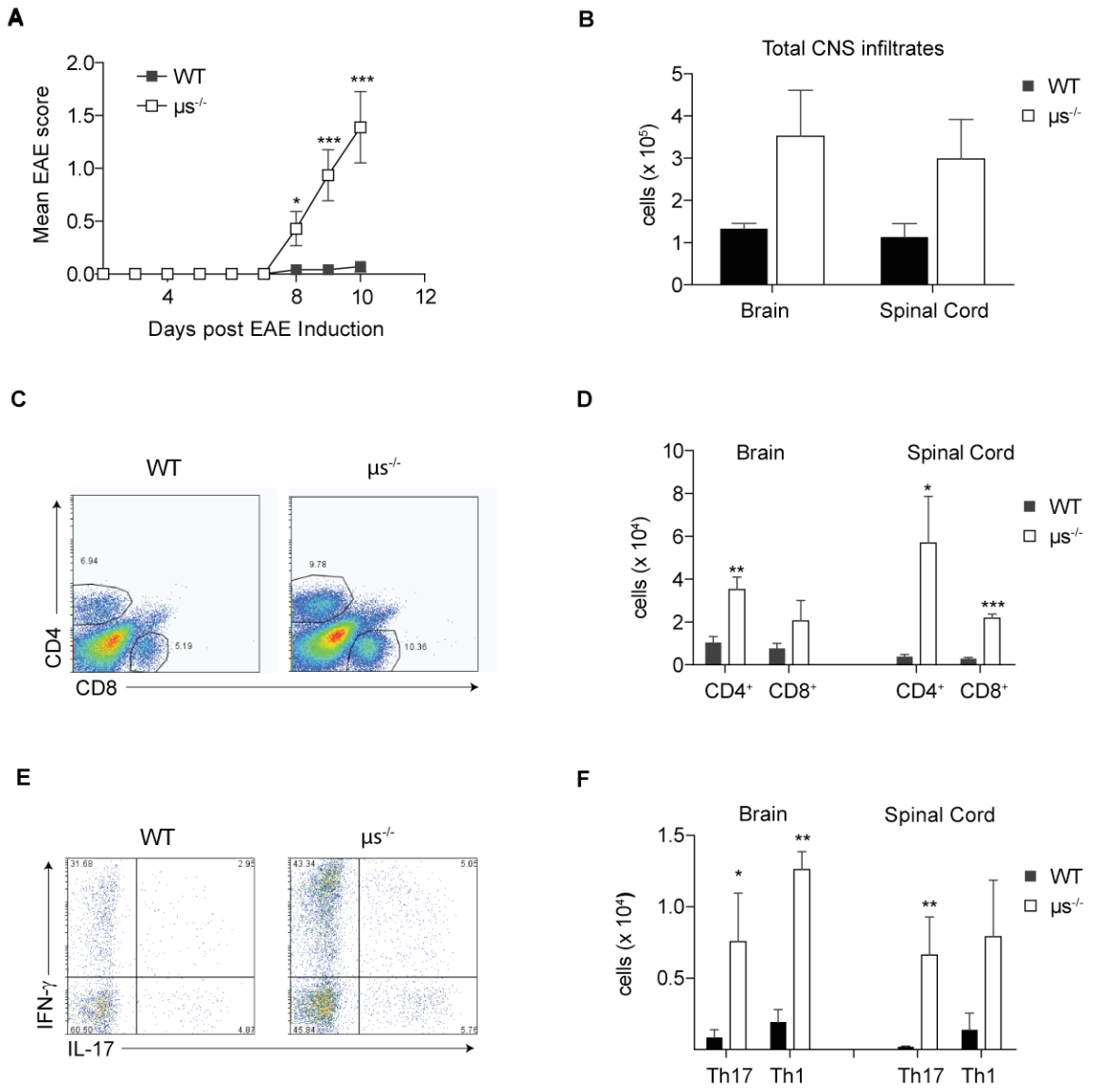

Figure 4.22 Early EAE onset in the absence of secreted IgM correlates with increased inflammatory T cells in the CNS.

EAE was induced in WT and μs$^{-/-}$ mice and ten days later once disease onset occured, CNS-infiltrating leukocytes were isolated and characterized by flow cytometry. (A) Clinical disease score (mean±SEM) by day 10. (B) Total numbers of live brain- and spinal cord -infiltrating cells (C) Flow cytometric analysis of CD4 and CD8 T cells, as defined by surface expression of CD4 and CD8 Plots (gated on CD45^{+} leukocytes), respectively. Shown are representative stainings from the spinal cord. (D) Numbers of CD4 and CD8 T cells determined in C. (E) Total leukocytes isolated from the CNS were cultured for 6h in the presence of soluble anti-CD3 and anti-CD28 and then co-stained for surface CD4 and intracellular IFN-γ or IL-17. Shown are representative stainings from spinal cord, gated on CD4+ cells. (F) Numbers of IFN-γ^{+} (Th1) and IL-17^{+} (Th17) CD4 T cells. Graphs in B, D and F show mean±SEM. Pooled results of two independent experiments are presented. ***, P<0.001; **, P<0.01; *, P<0.05.

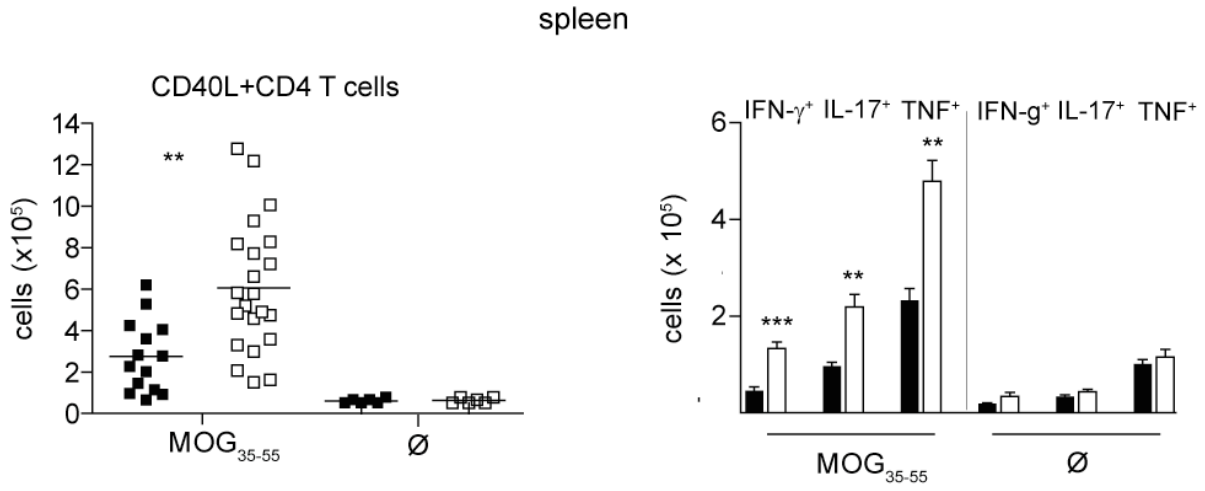

Figure 4.23 Early EAE onset in the absence of secreted IgM correlates with augmented autoreactive CD4 T cell responses in the spleen.

Ten days following EAE induction, splenocytes from μs$^{-/-}$ (n=14) and wild-type (WT; n=22) mice were prepared and re-stimulated *in vitro* with MOG_{35-55} for 6h.Cells were then co-stained for surface CD4 and (A) intracellular CD40L (CD154;) or (B) the indicated cytokines. Absolute numbers in both graphs are presented. Data show mean (A) or mean±SEM (B). Pooled results from two independent experiments are shown. *** P<0.001; **P<0.01.

4.2.3 Lack of secreted IgM results in increased magnitude of Th1 and Th17 responses before disease onset in draining lymph nodes

The observation that early disease onset in μs$^{-/-}$ mice correlated with increased Th1 and Th17 cells in the CNS, suggested that secreted IgM might have affected activation of CD4 T cells before they reached the CNS. For this reason the influence of secreted IgM in the development of autoreactive CD4 T cell response was assessed before clinical symptoms appeared. EAE was induced in μs$^{-/-}$ and wild-type mice and their immune responses were analyzed six days later, in dLN and spleen. Cells from dLN proliferated in both groups upon *in vitro* re-stimulation with MOG_{35-55} in a dose-dependent manner, indicating that initiation of antigen-specific immune responses had taken place at that time point (Fig. 4.24A). However, dLN cells from μs$^{-/-}$ mice exhibited significantly higher proliferative response (Fig. 4.24A). In contrast, re-stimulation of LN cells from naïve μs$^{-/-}$ and wild-type mice induced no proliferation (data not shown), thereby confirming that antigen-specific cells had expanded *in vivo* upon immunization. Because the proliferation assay employed here does not allow for the identification of the proliferating cells, this increased proliferation could not be attributed to a

particular cell type. The effect of secreted IgM on the initiation and priming of T cell responses were further characterized by measuring T cell activation and T helper cell effector functions. The activation status of CD4 T cells was determined ex vivo by flow cytometry according to upregulation of the activation marker CD44 and loss of the LN- and spleen-homing molecule L-selectin (CD62L) (Fig.4.24B). The analysis showed that immunization induced CD4 T cell activation in both groups of mice (Fig.4.24C). However, LN from $\mu s^{-/-}$ mice contained more than 2-fold higher numbers of activated CD44+CD62L- CD4+ T cells compared to their wild-type counterparts (Fig.4.24C). The potential role of secreted IgM in the effector functions of autoreactive CD4 T cells in the dLN was examined following short-term *in vitro* re-stimulation of dLN cells with the autoantigen. Analysis revealed higher numbers of CD4 T cells producing IFN-γ or IL-17 in $\mu s^{-/-}$ mice compared to controls (Fig.4.24D), whereas their frequencies were similar (Fig.4.24E). At this time point in the spleen, neither MOG_{35-55} -induced proliferation, nor the numbers of activated and cytokine-producing CD4 T cells differed between the groups (data not shown). Collectively these findings indicated that the protective functions of secreted IgM in EAE were in effect before disease onset and resulted in the limitation of autoreactive Th1 and Th7 responses in dLN.

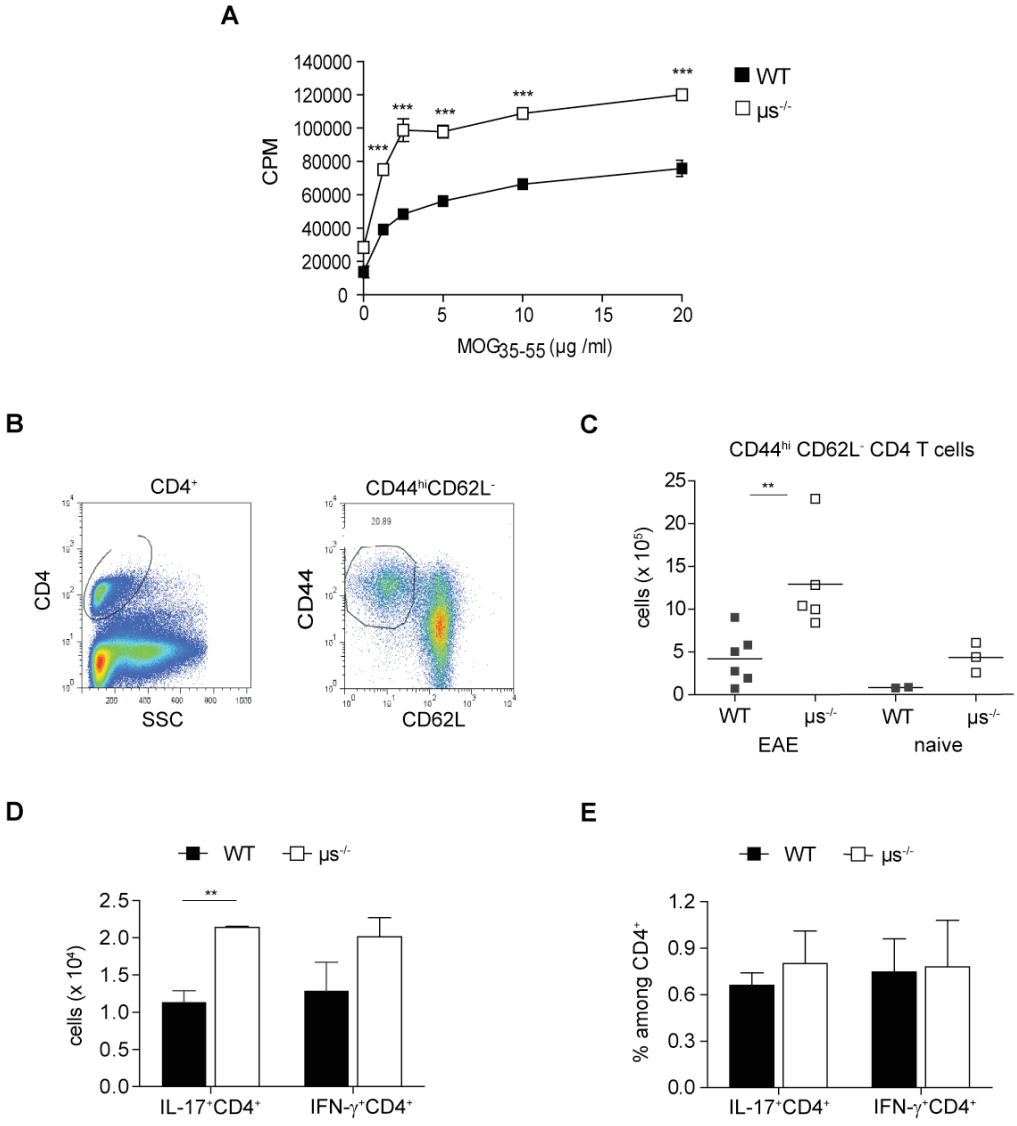

Figure 4.24 Lack of secreted IgM leads to amplified inflammatory CD4 T cell responses before disease onset. EAE was induced in µs$^{-/-}$ (n=5) and wild-type (WT; n=6) mice and six days later their CD4 T cell response in dLN were assessed. (A) LN cells were restimulated *in vitro* with MOG_{35-55}. After 48h proliferation was measured by ^{3}H-thymdine incorporation. (B) Flow cytometry plots of CD4 (left), CD44 and CD62L co-staining of live LN cells (right; gated on CD4^{+} cells shown in left plot).Gates indicate total CD4^{+} cells and activated CD44^{hi}CD62L^{-} CD4 T cells, respectively. (C) Absolute numbers of activated CD44hiCD62L- CD4 T cells (D) Absolute numbers of IL-17^{+} and IFN-γ^{+} CD4 T cells, determined by intracellular cytokine staining following 6h-restimulation of LN cells with MOG_{35-55}. (E) Frequency of the cell populations described in (D). Graphs in A, D and E show mean±SEM; in C mean is shown. *** P<0.0001; ** P<0.01. Results are representative of three independent experiments.

4.2.4 Administration of wild-type serum restores EAE onset and severity in μs$^{-/-}$ mice

Secreted IgM exists as a natural antibody in non-immune naïve mice but it is also the first isotype to be produced upon antigen exposure. Indeed, monitoring of circulating MOG_{35-55} – reactive IgM before and after EAE induction revealed that MOG_{35-55}-reactive IgM antibodies were present in naïve wild-type mice and were upregulated by days 7 and 14 post-immunization, after which they remained stable up to day 35 (Fig.4.25). Therefore, it was unclear whether the early and exacerbated EAE exhibited by μs$^{-/-}$ mice was due to lack of natural or MOG-induced IgM, or both. To address this issue, natural IgM was reconstituted in μs$^{-/-}$ mice by transferring normal serum from naïve wild-type mice. Normal serum was administered one day before EAE induction and every two days thereafter until day7, before clinical disease onset had occurred in μs$^{-/-}$ mice (Fig.4.26A). This short-term application of naïve serum restored both the disease onset and severity, so that the recipient μs$^{-/-}$ mice exhibited a disease course comparable to wild-type mice (Fig.4.26 and Table 4.2). Thus, natural IgM was sufficient to reverse the EAE phenotype in mice lacking sIgM, despite the latter´s inability to mount *de novo* MOG-specific IgM responses upon disease induction. Nevertheless, considering that MOG-reactive IgM was detectable in the transferred serum, a requirement for autoantigen specific-mediated suppression cannot be excluded. Altogether, these observations indicated that natural antibodies can provide protection from CNS autoimmunity.

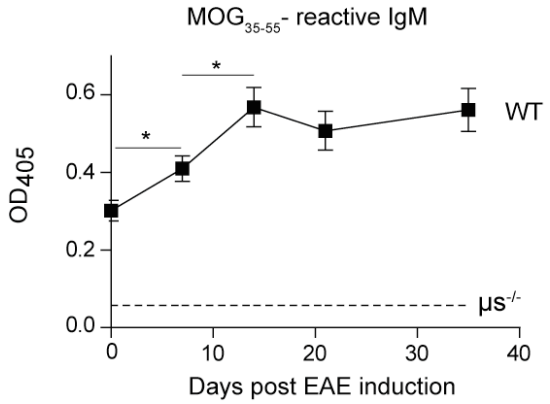

Figure 4.25 MOG-reactive IgM exists as both natural antibody and as MOG-induced antibody upon EAE induction.
Sera were collected from wild-type mice before and after EAE induction at the indicated time points to determine MOG_{35-55} reactive IgM by ELISA. Graphs show mean±SEM. *, P<0.05.

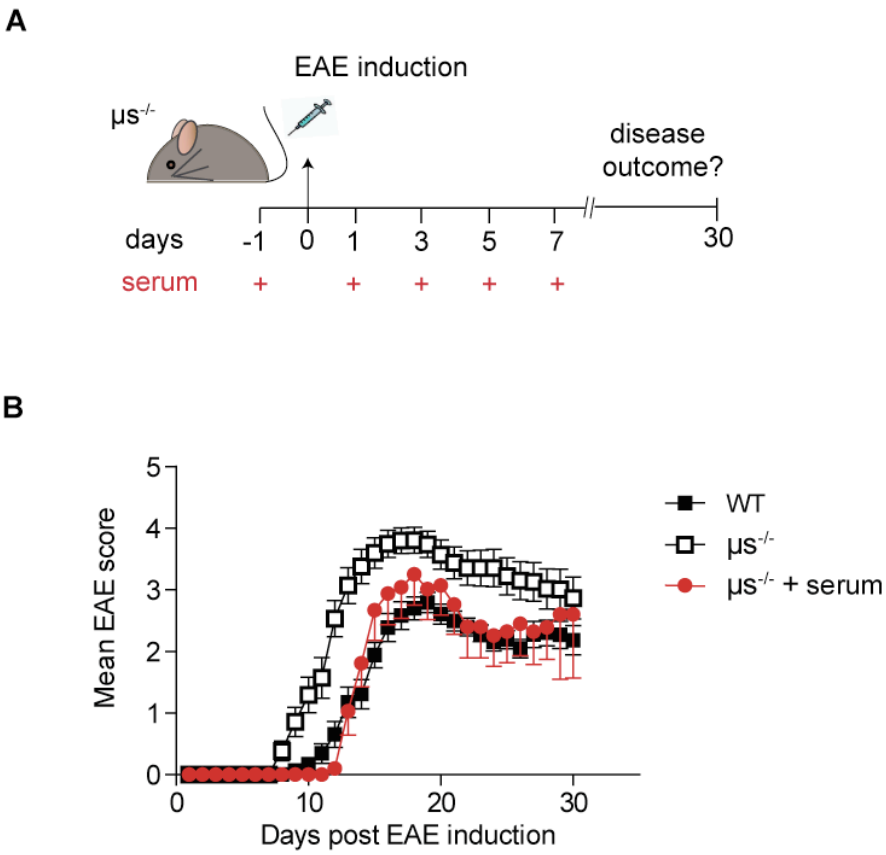

Figure 4.26 Application of normal serum IgM restores EAE onset and severity in μs$^{-/-}$ mice

(A) Schematic chronicle of serum treatment. 500μl serum-doses each containing 100μg total IgM was administered via the intraperitoneal route in μs$^{-/-}$ mice (n=9) at the time points indicated, starting one day before EAE induction. (B) Clinical disease score (mean±SEM) as assessed daily throughout a 30-day period. Pooled results from two independent experiments are shown.

Table 4.2. Disease parameters altered upon serum transfer

Mouse group	Mean day of disease onset	Mean maximum Clinical score
μs$^{-/-}$	10.83 ± 0.36	4.12 ± 0.17
μs$^{-/-}$ + serum	13.33 ± 0.28	3.30 ± 0.12
Statistical significance	*** (0.0005)	** (0.009)

5 Discussion

B cells can influence immune responses via antibody production, antigen presentation, and cytokine secretion. Antibodies with neutralization, opsonization and cellular cytotoxicity functions contribute to host defense against pathogens. B cells also promote T cell responses to pathogens by presenting cognate antigen to CD4 T cells. In turn, the latter provide B cells with "help" signals that direct germinal center reaction, antibody class switch, affinity maturation, formation of memory B cell and long-lived plasma cells, which mediate protective immunity to re-infection. In addition, by virtue of their expression of PRRs, like TLR, B cells can recognize conserved microbial structures and rapidly respond to pathogens, in a T cell- independent manner though secretion of antibodies and cytokines.

In the context of autoimmune diseases, B cells often play pathogenic role attributed to autoantibody production and autoantigen presentation. In humans, B cell-depletion using Rituximab, an antibody specific for the mature-B cell surface protein CD20, has been recently approved as an RA treatment and has also shown considerable efficacy in subgroups of RR-MS and advanced-SLE patients [119, 391, 431-433]. However, rituximab also exacerbated disease in cases of MS [408] and ulcerative colitis patients [277], whereas it triggered psoriasis in certain SLE- or RA-suspected subjects [434]. These findings suggest that, apart from their contribution to disease pathogenesis, B cells can also regulate immune responses and, thereby, ameliorate disease. Consistent with this, interleukin 10-producing B cells have emerged as regulators of several experimental models of autoimmune inflammation, including CIA, ulcerative colitis and EAE [123, 270-271, 274]. Human IL-10-producing B cells have also been described in healthy subjects and intriguingly, were defective in subgroups of MS [276] and SLE patients [278]. These studies provide evidence that B cells with suppressive functions are relevant in human disease and raise the need to therapeutically exploit these cells to limit undesired immune responses.

A first step towards this goal would be to determine the signals driving the generation/differentiation of IL-10-secreting B cells from their naïve precursors, which have remained elusive. The current study identified TLR-MyD88 as a signaling pathway inducing IL-10 by naïve mouse B cells *in vitro* and demonstrated for the first time its requirement for the regulatory functions of B cells *in vivo* during inflammation of the CNS. Apart from shedding light into the molecular requirements of B cell-mediated suppression, these findings revealed an unexpected role for TLR/MyD88 pathway in regulation of immune responses,

5.1 TLR/MyD88 signaling triggers anti-inflammatory functions in B cells: dual role for MyD88 in immune responses.

Thusfar, the role of TLR in immune responses has been extensively explored in macrophages and dendritic cells, the immune system´s cellular sentinels of danger signals. Engagement of TLR elicits rapid pro-inflammatory cascades in these cells enabling them to orchestrate appropriate antigen specific T cell responses. Indeed, germline mutation of MyD88 renders mice susceptible to several bacterial and viral infections, and confers resistant to diverse experimental autoimmune diseases, including EAE, autoimmune type 1 diabetes, antibody-induced arthritis, and SLE [424, 426, 435-438]. The current study provides compelling evidence that this pathway can also propagate inhibitory signals when activated in B cells. Using a mixed bone marrow chimera system devoid of leakiness, we showed that mice lacking MyD88 or TLR2/4 selectively in B cells suffered a more severe chronic form of EAE, a mouse model of multiple sclerosis. The exacerbated disease observed in mice with MyD88-deficient B cells correlated with increased autoreactive Th1 and Th17 cells, suggesting that MyD88 in B cells promoted disease resolution by limiting inflammatory T cell responses. We confirmed [424] that, by contrast, MyD88-deficient mice were resistant to disease, and further showed that resistance correlated with their impaired ability to mount Th1 and Th17 responses. Thus, our findings suggest a dual role for MyD88 during EAE: MyD88 signaling in cells other than B cells, presumably DC and macrophages, is critical for the initiation of inflammation and T cell responses, whereas MyD88-signalling in B cells antagonizes this effect, thereby controlling overt inflammatory responses. The notion that TLR triggering can confer suppressive properties to B cells is supported by earlier studies employing exogenously activated B cells to restrain undesired immune responses. Almost twenty years ago, Fuchs and Matzinger observed that when adoptively transferred into naïve female mice, male LPS-activated B cells were three times more potent than resting B cells at inducing tolerance to male splenocytes [439]. Later, Scott and colleagues showed that adoptive transfer of B cells stimulated with LPS could ameliorate spontaneous diabetes in NOD mice, MOG-induced and MPB-induced EAE, experimental autoimmune uveitis, and adjuvant-induced arthritis in rats, when expressing the disease-relevant autoantigens [440-443]. Inhibition correlated with diminished autoantigen-specific T cell responses and, in some cases, autoantibody production, as well as with milder pathology of the respective target organ. In an independent study, adoptive transfer of LPS-activated B cells prevented spontaneous diabetes in 80% of recipient mice, and inhibited diabetogenic T cells from transferring diabetes in prone lymphopenic hosts [444]. In both cases,

recipient mice displayed strongly reduced autoreactive Th1 immunity, suggesting that LPS-activated B cells conferred protection by limiting inflammatory T cells. Responsiveness of B cells to LPS was likely also required to limit Th2-mediated autoreactivity in mouse model of spontaneous UC caused by genetic ablation of Galphai2, a subunit of the G protein-coupled receptors. In this model, cotransfer of $CD8^+$ T cells and wt B cells normally rescues Galphai2-deficient mice from spontaneous colitis, and this required IL-10 sufficient B cells [241, 445]. When, however, transferred B cells lacked intrinsic Gaplhai2, protection was abrogated [241]. It is notable, that upon LPS stimulation, Gaplhai2-deficient B cells displayed impaired proliferative capacity and IL-10 production [446], which could account for the ineffectiveness ofGalphai2 deficient B cells, although the involvement of TLR signaling was not determined in those studies. Besides LPS, a few studies have demonstrated that the TLR9 ligand CpG can also elicit anti-inflammatory responses via B cells. For example, when CpG was administered in allergen-sensitized mice it ameliorated experimental allergic conjunctivitis after challenge, by inducing IL-10 producing B cells, which could then transfer protection to newly sensitized recipients [447]. Furthermore, during neonatal immune responses, GpG elicited IL-10-production by CD5+ B cells that conferred neonate mice resistance to CpG- induced inflammation by inhibiting Th1 responses [448]. In vitro experiments in a later study by the same group indicated that agonists of other TLR, including TLR2, TLR3 and TLR8, or RNA viruses targeting TLR7, might also induce suppressive IL-10-producing B cells [449], though this awaits *in vivo* demonstration during both neonatal and adult immune responses.

Despite the evidence provided by the current and other studies that B cells can acquire suppressive functions upon intrinsic TLR/MyD88 signaling, the latter may also confer B cells disease promoting activities. *In vitro*, LPS and CpG also induced IL-6 secretion by B cells that enhanced T cell proliferation in the absence of DC. Mice deficient for IL-6 are resistant to actively induced EAE [450-451] and this may be linked to a requirement for IL-6 in the differentiation of Th17 cells and/or to its augmenting effect in T cell survival [452-453]. Although the contribution of B cell-derived IL-6 to disease severity remains to be addressed, our observations suggest that depending on the cellular microenvironment in which T-B cell dialogue takes place, TLR-activated B cells may also promote pathogenic T cell responses.

It is unlikely that the exacerbated EAE phenotype in the absence of MyD88 expression was due to a complete block of B cell activation. Absence of MyD88 in B cells was dispensable for germinal centre formation and antigen-induced antibody production in response to a TD-antigen, arguing against a requirement for MyD88 in B cell activation. Similar observations have

been reported in responses to viral infections [150, 454]. This is consistent with a previous report from Nemazze and colleagues, who showed that TRIF/MyD88 double deficient mice were still able to mount antibody responses comparable to wt mice upon immunization with various adjuvants [153]. We observed that mice with MyD88-definicient B cells GC displayed smaller GC, supporting the finding from Barr *et al*, [455] that mice with MyD88-deficiency in B cells developed significantly smaller GC after immunization with a TD-antigen together with LPS. Thus, although TLR/MyD88 signaling in B cells amplifies GC responses, it is not required for B cell activation.

5.2 Delineating the contibution of MyD88 and individual TLR in disease initiation and regulation

In an attempt to delineate the contribution of TLR the MyD88-dependent suppressive function of B cells, we induced EAE in mice which B cells lacked TLR2/4 or TLR9. We found that disease resolution required B cell expression of TLR2/4. Similar to mice lacking MyD88 in B cells, mice with TLR2/4 -deficient B cells suffered severe and chronic EAE and displayed uncontrolled autoreactive T cell responses. Moreover, LPS-induced IL-10 by naïve B cells dependent on both TLR2/4 and MyD88. Preliminary, experiments carried out after completion of the current study, further compared the disease outcome among chimera mice with either wild-type B cells, TLR2- or TLR4-deficient B cells (denoted, respectively, as B-WT, B-TLR2$^{-/-}$). Both B-TLR2$^{-/-}$ and B-TLR4$^{-/-}$ mice displayed exacerbated and non remitting EAE with B-TLR4$^{-/-}$ mice showing the most severe symptoms (Appendix 7.1; Fillatreau and Anderton, unpublished observations). These observations revealed a synergistic effect of TLR2 and -4 signaling in B cell-mediated suppression, and highlighted a more protective role for TLR4. Consistent with a protective role for TLR2, preliminary experiments showed that, like TLR4-LPS ligation, the TLR2 agonist peptidoglycan [456] also elicited IL-10 production by naïve B cells. Considering that TLR2 signals through MyD88, these observations advocate for a concerted involvement of TLR2, TLR4 and MyD88 in the regulatory functions of B cells *in vivo,* possibly by triggering their secretion of IL-10. These data however do not exclude a possible contribution of TRIF, another TLR adaptor, which mediates a MyD88-independent pathway downstream TLR4 (and TLR3). In fact, the finding that B cell-expression of TLR4 was more protective over TLR2, whose exclusive adaptor is MyD88, is consistent with a role for TRIF. As shown by Kagan *et al*, the MyD88 and TRIF pathways share signaling components and can be “physically “ coupled downstream TLR4

[81]. Conceivably, such cross-talk is likely to have functional consequences. In support of this, TRIF and MyD88 were required for optimal IL-10 secretion by TLR-activated myeloid cells *in vitro* [457-458] and for systemic IL-10 prodution *in vivo* during infection with LPS-containing bacteria [459]. Therefore, *in vivo*, stimulation of TLR4 in B cells could result in more IL-10 production, compared to stimulation of TLR2, and this could account for the more pronounced phenotype of the B-TLR4$^{-/-}$ mice. Furthermore, according to several studies, engagement of TLR4 can promote upregulation of TLR2 [460-462], raising the possibility that TLR4$^{-/-}$ B cells may express suboptimal levels of TLR2 and thus, make suboptimal IL-10 in response to TLR2 stimulation.

Our data indicated that lack of TLR2/4 signaling in B cells could account for the exacerbated EAE in mice with MyD88-deficient B cells. However, MyD88 may have additionally TLR-independent signaling functions. First, MyD88 acts as a critical signaling adaptor for type 1 IL-1R and IL-18R [463], both of which are required for EAE induction [464-465]. Resting naïve B cells do not respond to IL-1, however, along with other cytokines IL-1 can promote proliferation of activated B cells [466-468]. Our finding that mice with MyD88-deficient B cells might develop smaller GC in response to TD antigen, could reflect reduced B cell proliferation, and thus, imply an involvement of lack of IL-1R signaling in MyD88-deficient B cells, even though IL-1R-deficient mice mount normal B cell responses [469]. Similar to IL-1R, naïve B cells express low levels of IL-18R, which can be upregulated by IL-12 [470-471]. Expression of this receptor on B cells may important for their production of IFN-γ, as IL-18, IL-12 and CD40 signals can synergistically induce IFN-g production by B cells *in vitro* [470-471]. It has been reported that B cells can produce IFN-γ *in vivo* [255] and IFN-γ signaling is critical for recovery from EAE [343, 472]. It is plausible that MyD88 could be required in B cells for their production of IFN-γ, subsequently, for their contribution to disease resolution. This could explain why both CD40 and MyD88 are required on B cells for recovery from EAE. MyD88 could additionally potentiate IFNg signaling by acting downstream of IFNgR to stabilize IFNg-inducible cytokine and chemokine transcripts, as recently shown for macrophages [473]. Altogether, further experiments are necessary to uncouple the TLR-dependent and independent functions of MyD88, for example, investigating EAE progression in mice lacking IL-1R, IL-18R or IFN-γ selectively in B cells. Lastly, determining whether absence of TLR2/4 in B cells also affects GC numbers and size would help discern the TLR-dependent and -independent role of MyD88 in these responses.

TLR9 expression in B cells was dispensable for recovery from disease, despite the ability of CpG to induce IL-10 production by B cells *in vitro*. In addition, TLR9 could not compensate for the lack of TLR2/4 in B-TLR2/4 mice. Our findings suggested that TLR involved in disease induction and resolution likely derive from the adjuvant CFA used to induce disease. Notably, lack of TLR9 in all cells affected EAE onset, indicating that TLR9 agonists were present in the *M.tuberculosis (M.tb)*-containing CFA. Consistently, DNase treatment of mycobacterial components in CFA reduced the severity of adjuvant-induced arthritis and re-supplementing CpG DNA restored full-blown disease [474]. Furthermore, CFA immunization resulted in the release of mycobacterial DNA that was detectable in the BM, draining LN and spleen [474], indicating that it was accessible to B cells. Several studies have demonstrated that different GpG oligonucleotides differ in their ability to stimulate DC and B cells [475-476]. Thus, in contrast to the CpG used in our *in vitro* studies, which stimulated both cell types, it is possible that the CFA-derived CpG induced a response only by DC but not by B cells. DC were responsive to *M.tb*-derived genomic DNA in a TLR9-dependent manner [477]. We found that B cell IL-10 production in response to heat- inactivated *M.tb* strictly required TLR2/4, implying that TLR9 expression was insufficient to drive IL-10 production by B cell in response the to heat-inactivated *M.tb* present in CFA. Comparing ex vivo IL-10 production by B cells from B-TLR9, B-TLR2/4 and B-WT mice after disease induction would help precise the contribution of these TLR in B cell-derived IL-10.

Irrespective of the degree of their contributions, these findings demonstrated that specific TLR on B cells regulate disease resolution. By contrast, disease induction could not be attributed to any of the TLR tested, as TLR2/4, and TLR9 knockout mice were susceptible to disease, despite that MyD88-deficeint mice are resistant to disease. Similar findings have been reported in other disease models in which CFA is used as adjuvant, including experimental autoimmune uveitis [478] and experimental autoimmune myocarditis (EAM) [479]. In all cases, including EAE, resistance to disease induction in absence of MyD88 could be attributed to lack of IL-1R signaling, and particularly in EAE, also to impaired IL-18R signaling [464-465] and was associated with defective Th1 and Th17 responses. These findings suggest that TLR2/4 and 9, might be either unnecessary or have redundant roles in EAE induction. Triple TLR2.4.9 knockout mice need to be generated to better document their role. Alternatively, other TLR not tested here, could contribute to disease induction, as shown recently for TLR7 in EAM [480]. Independently of these possibilities, our findings showed that MyD88 and individual TLR have differential effects in disease induction and resolution. They further implied that these effects

were likely mediated via distinct cell types, namely B cells, and presumably DC and macrophages. Yet, considering that TLR2/4 expression by B cells was required for disease resolution, it appeared puzzling that TLR2/4-deficient mice normally recovered. It is possible that the protective effects of TLR2/4 signaling via B cells counteract the pathogenic effects of TLR2/4 signaling via other cells, so that the latter further perpetuate disease when B cells lack TLR2/4. Conversely, in the absence of pathogenic TLR2/4 signals, B cells may need not to control disease via TLR2/4, and hence, recovery would not rely on B cell expression of TLR2/4. If so, it may follow that B cell-mediated regulation is dispensable for disease resolution in TLR2/4$^{-/-}$ mice. This issue could be addressed by assessing the EAE outcome in JHT.TLR2/4$^{-/-}$ mice, which lack both B cells and TLR2/4, or in TLR2/4$^{-/-}$ mice treated with a B cell-depleting antibody.

5.3 Sources of TLR agonists regulating EAE

Our results demonstrated the involvement of TLR-MyD88 signaling in disease development and resolution; however the identification of specific TLR agonists triggering these processes remains a challenging task. The findings that, absence of microflora marginally affected the disease course, and that, *M.tb* extract induced IL-10 by B cells *in vitro* via TLR2/4 /MyD88 pathway, suggested that mycobacteria-derived structures were the likely candidate agonists. Pinpointing down to specific TLR ligands was hampered by the fact that the exact molecular mechanisms mediating the *M.tb*-host immunity interplay are currently unclear. Although mycobacterial antigens, stimulating TLR2 and TLR4 have been identified *in vitro* - for example the 19kD protein- ablation of these receptors, as well as TLR9 or MyD88, has generated conflicting results about their role in anti-mycobacterial immunity *in vivo*. They further implied that PRR other than TLR may participate in recognition of *M.tb*. Indeed, members of the C-type lectin family DC-SIGN, which signal in MyD88-indepedent manner, have been recently implicated in host defense to *M.tb* [481]. Nevertheless, C-type lectins can influence cellular responses in co-operation with TLR (see section 1.2.1). DC-SIGN is thought to be a DC-restricted receptor [482] and its expression in mouse B cells has not been documented. These observations indicate that expression of DC-SIGN may determine the structure of the antigen recognized, so that, the TLR-dependent protective functions of B cells, and the pro-inflammatory functions of DC are triggered by distinct CFA-derived agonists.

Development of EAE in the mouse model we used requires administration of pertussis toxin (PTX), which is reported to aid disease initiation through multiple ways . Studies by Kerfoot *et al*. showed that PTX can elicit signaling events through TLR4 in a macrophage cell line [429]. Therefore, PTX might serve as an alternative or additional TLR agonist triggering disease initiation through DC/macrophages or protection via B cells. It should be noted though, that the toxin is administered intravenously and has probably a short half-life *in vivo*, suggesting that its potential effects through TLR4 are likely to occur early after disease induction. The notion that PTX could act as an additional signal triggering suppressive functions in B cells via TLR4 during EAE, would support the more prominent role of B cell-expression of TLR4 in disease resolution compared to TLR2. Nevertheless, PTX is unlikely to provide a strictly required signal for the induction of IL-10 by B cells in general, because IL-10-producing B cells in CIA are generated in the absence of PTX. Similar to EAE, CIA is induced using CFA immunization, thus, it is plausible that CFA-derived TLR agonists might trigger IL-10 production by B cells also in this case.

Lastly, in addition to recognition of microbial structures, TLR4, among others, is involved in sensing endogenous “danger” signals arising during tissue injury, collectively called danger-associated molecular patterns (DAMPs). These include proteins or polysaccharide structures that become accessible upon injury- induced degradation of extracellular matrix, like hyaluronic acid and heparin sulfate, or that are released from damaged/necrotic cells, like the high-mobility group box 1. The responses elicited by engagement of TLR4 (or TLR2) by these molecules can be pro-inflammatory, mediated by cytokines like TNF, or anti-inflammatory, promoting tissue repair [483-487]. Thus, during EAE, apart from CFA-derived agonists, DAMPs released at the target organ may also trigger protective functions in B cells via TLR4.

5.4 Involvement of TLR, CD40 and BCR signals in B cell-mediated suppression: parallel pathways or concerted action?

Previous studies demonstrated that CD40 and antigen-specific BCR are also required on B cells to promote recovery from EAE [270], because mice with CD40-deficient B cells, and mice in which B cells carry disease irrelevant BCR, suffer chronic EAE, resembling those lacking TLR2/4 or MyD88 in B cells. Moreover, IL-10 production by B cells isolated from EAE recovered mice, required concomitant stimulation through CD40 and BCR. Therefore, all these

three signals share at least one common “end product” -IL-10 production-, and their individual contribution is non- redundant. This raises then the question as to how do the signals via these receptors operate relative to each other to finally confer B cells disease limiting properties. One possibility, is that they act in parallel: TLR may induce an innate pathway of suppression in a given B cell population, whereas CD40 and BCR would trigger an adaptive pathway mediated by a different B cell subset. Alternatively, these receptors may propagate signals in a stepwise, temporally concerted manner within the same B cell population. The findings of the current and previous studies would seem to support the latter hypothesis, by which the immunosuppesive functions of B cells are initially triggered by TLR and subsequently re-enforced though CD40 and BCR signaling. Regulation of autoreactive T cells by B cell-derived IL-10 takes place already within the first 10 days upon EAE induction, as mice with IL-10-deficient B cells displayed increased inflammatory T cells at this timepoint. Notably, when B cells lacked CD40, differences in the T cell response appeared later than day 10. Hence, B cells begin to acquire anti-inflammatory properties independently of CD40. Consistently, we found that CD40 and BCR stimulation, contrary to TLR, failed to elicit IL-10 secretion by naïve B cells. Furthermore, IL-10 produced by B cells within three days of TLR activation, was sufficient to significantly restrict T cell proliferation and cytokine production *in vitro*. B cell-driven suppression was indirect, entailing partial inhibition of proinflammatory cytokines by TLR-exposed DC. Thus, TLR engagement could provide the very first signals endowing B cells with immunosuppressive capacities *in vivo*.

The suppressive functions of B cells appear to persist beyond day 10, as advocated by the following observations: a) T cell responses become uncontrolled after day 10 when B cells lack CD40, and b) recovered wild-type mice harbored splenic B cells that produced IL-10 when co-stimulated with agonistic anti-CD40 and the autoantigen/anti-BCR. Therefore, following an initial stage of suppression, a second phase might ensue, during which the regulatory activities of B cells, originally triggered by TLR, become antigen specific and dependent on CD40. Assuming that T cells are the providers of CD40L *in vivo*, it is possible that antigen-specific B cells, previously primed to produce IL-10 by TLR, are selectively expanded though cognate interaction with T cells. Such interaction would sustain their provision of IL-10 and allow them to promote recovery once auto-reactive T cell responses and hence, disease symptoms, are fully developed. Cognate B-T cell interactions at this stage may also permit a direct inhibition of T cells by IL-10-producing B cells, besides their indirect suppression via DC. Nevertheless, we cannot exclude that TLR signals remain important during this CD40-dependent phase. This

issue could be addressed via inducible gene deletion of TLR2/4 or MyD88 in B cells a few days after disease induction. This model of stepwise integration of TLR, CD40 and BCR signal in the initiation and maintenance of the regulatory functions B cells *in vivo*, may as well apply to CIA, which is also regulated by IL-10-producing B cells in a CD40- and BCR- dependent manner [271]. Consistent with a "delayed" contribution of CD40, agonistic antibody to CD40 ameliorates arthritis only after immunization, whereas its preventive administration before disease induction is ineffective [413]. The success or failure of the anti-CD40 could be accounted for, respectively, by the presence or absence of B cells primed to produce IL-10 at the time of treatment. Although it is yet to be determined, TLR/MyD88 signaling might also serve here as an IL-10-priming signal, considering that, CIA, similar to EAE, is induced using CFA, that could provide the TLR agonists.

5.5 Mechanism of suppression mediated by TLR2/4/MyD88 signaling in B cells

Overall, our results pointed to IL-10 from TLR-activated B cells as a mediator of their suppressive function. Mice with MyD88- or TLR2/4-deficient B cells resembled mice with IL-10-deficient B cells in both, disease phenotype and enhanced Th1 cell responses [270]. In addition, upon *in vitro* stimulation with TLR4 or TLR9 agonists, naïve B cells produced the anti-inflammatory cytokine IL-10 in a MyD88-dependent manner, and supernatants from TLR4-activated B cells limited T cell proliferation and IFN-g production, but not upon blockade of IL-10 signaling. B cell-derived IL-10 inhibited T cell responses indirectly by limiting the production of proinflammatory cytokines by DC, including IL-12, IL-6, TNF and IL-23. This finding is consistent with previous studies showing that B cells can influence the activation of DC. DC from B cell-deficient mice produced more IL-12 relative to controls, resulting in stronger Th1 responses *in vivo*, both upon immunization with TD antigen in CFA, or after their adoptive transfer in wild-type recipients [272]. Notably, in both cases this Th1-enhancing effect was detectable within the first six days, following immunization or DC transfer. Moreover, B cell-derived IL-10 inhibited IL-12 secretion by DC and thereby, prevented Th1 immunity in neonate mice [448]. IL-6 and TNF have also been shown to be suppressed by IL-10, albeit in experimental settings not involving B cells [488]. The former cytokine is critical for induction of EAE [450-451] and has recently emerged as a key factor driving Th17 differentiation. Therefore, besides Th1, IL-10 from TLR-activated B cells can inhibit Th17 responses. The role of IL-10 in inhibiting IL-23 is not well

documented. It should be noted that suppression of IL-12 and to a lesser extent of TNF, production by DC in the presence of B cell supernatants was only partially abrogated upon blockade of IL-10-signalling. This implied that TLR-activated B cells secreted inhibitory factors other than IL-10, yet to be determined. An alternative/additional explanation for the case of IL-12 may derive from the known ability of TNF to inhibit IL-12 [430, 489]. Thus, TNF from CpG-activated DC could have acted in a autocrine manner and synergized with IL-10 present in the B cell supernatant to reduce IL-12, so that blocking IL-10 had only a partial effect. Concomitant TNF and IL-10 blockade would help address this possibility.

Besides DC, IL-10 from TLR activated B cells might have additional cellular targets *in vivo*. A recent study demonstrated that upon exposure to LPS peritoneal macrophages from B cell deficient mice displayed enhanced expression of pro-inflammatory cytokines and chemokines including TNF, compared to those from wild-type mice, indicating that B cells can also regulate macrophage function. The authors further showed that co-culture of WT, but not IL-10 – deficient, B cells and macrophages in the presence of LPS, rendered the latter refractory to LPS activation, evidenced by their reduced production of pro-inflammatory cytokine and chemokines, and up-regulation of IL-10. Notably, macrophages with anti-inflammatory phenotype have been described in both human [490-491] and rodent [492-494] inflamed CNS during MS/EAE, residing in the perivascular space [491], where B cells have also been reported to reside [495]. Macrophages on their part, by provision of BAFF [496-497], could promote survival of the neighboring IL-10-producing B cells. In support of this possibility, IL-10 has been shown to enhance BAFF expression by human macrophages [498]**.** These observations support the possibility that B cell-derived IL-10 may exert immunosuppressive actions in CNS autoimmunity also via macrophages.

Another cell target of B cell-derived IL-10 could be B cells themselves. B cells express IL-10R and IL-10 can act in an autocrine manner to promote B cell proliferation factor either directly and/or by promoting the B cell-survival factor BAFF [496], which can be made by TLR-activated B cells [499]. Thus, B cell-derived IL-10 could help expand IL-10-producing B cells. However, our unpublished observations suggested that IL-10 is not a critical factor for TLR-induced B cell proliferation at least *in vitro*, as IL-10-deficient B cells readily proliferated in response to LPS. Alternatively, IL-10 may act on B cell themselves to promote its own production. The ability of IL-10 to promote its own production, either alone or in combination with other factors, has been described for several cell types. Repetitive treatment of DC with IL-10 resulted in IL-10 production by these cells [500] that in turn induced the differentiation of IL-

10 producing Tr1 cells. In addition, as mentioned earlier, IL-10 from LPS-activated B cells can promote IL-10 production by macrophages. Furthermore, IL-10 is one of the factors involved in the differentiation of the so-called -alternatively activated M2 macrophages [501-503] which also produce IL-10. Nevertheless, altogether, these observations raise the possibility that IL-10 from TLR-activated B cells may act on B cells and DC to respectively, amplify or induce its own production. Thus, it may be relevant to test whether supernatant from TLR-activated IL-10R-decificient B cells retains its capacity to interfere with DC function and to limit T cell proliferation, as well as whether TLR-activated B cells induce IL-10 production by DC themselves. Finally, as previously mentioned, T cells may be another target of B cell-derived IL-10 *in vivo*.

Another question arising from our findings relates to the identidy of the B cells producing IL-10, considering that the B cell compartment comprises distinct subsets. Studies by Tedder and colleagues [274], have indicated a role for the spleen-resident $CD1d^{hi}CD5^{+}$ B cell subset in promoting recovery from EAE. They showed that upon induction of EAE, IL-10 mRNA transcripts were up-regulated exclusively within this population, but not for example in FO B cells, which are $CD1d^{lo}CD5^{-}$. They further demonstrated that transfer of WT, but not IL-10-deficient,$CD1d^{hi}CD5^{+}$ B cells, restored resolution of EAE in B cell-depleted mice, which otherwise suffer non-remitting disease. It should be noted though that transfer of these cells did not reduce the severity of disease. This observation might indicate that not all of the transferred cells are protective. Indeed, when naïve $CD1d^{hi}CD5^{+}$ B cells were cultured in the presence of a stimulation cocktail including LPS, only around 20% of these made IL-10. It would be interesting to test whether TLR/MyD88 sigaling is required for their *in vivo* protective function. Alternatively, -or additionally- it could be that this B cell subset alone is insufficient to control disease, and that other subsets are involved too. Consistent with previous reports, our data demonstrated that FO, MZ and B1 B cell subsets, as well as $CD1d^{hi}$ B cells (shown recently by others in our laboratory) can make IL-10 in response to LPS. It is thus plausible that different B cell subpopulations participate in regulating the immune response evolving along the course of disease. Yet, whether all B cell subsets make IL-10 *in vivo* during EAE remains to be determined. These subsets reside in distinct locations in the body (mouse and human) and thus are likely to have different access to TLR agonists provided by the adjuvants (CFA and PTX) or by endogenous sources, as well as different access to various cellular microenvironments *in vivo*. In addition, certain B cell subsets appear to be more responsive to TLR stimulation over others. For example, B1 cells, MZ and $CD1d^{hi}$ B cells more readily proliferated and tended to make more IL-10 in response to LPS, relative to other subsets. In view of these parameters, it is

at current difficult to predict the identity of the TLR-primed IL-10-producing B cell susbset(s) regulating disease or where this regulation takes place *in vivo*. A potentially informative approach to address this issue would be the use of the so-called “IL-10.eGFP” reporter mice. In these mice, cells that transcribe *Il-10* gene start express cytoplasmic green fluorescent protein, and that is detectable by flow cytometry. Conceivably, these mice would allow for the detection and phenotyping of B cell populations that up-regulate IL-10 upon EAE induction and throughout the course of disease.

Our findings do not rule out the possibility that IL-10-independent mechanisms are also involved in suppression by TLR-activated B cells. Scott and colleagues observed that LPS-activated B cells inhibited autoimmune responses in a CD86 and MHC II- dependent manner [218, 504]. This could be of particular relevance to our studies as MyD88-signalling has been shown to regulate expression of MHC II and co-stimulatory molecules [505]. It is therefore possible that MyD88-deficient B cells expressed *in vivo* insufficient or altered levels of such molecules. Furthermore, engagement of TLR has also been shown to induce a distinct array of chemokine and chemokine receptors by various cell types, including B cells [506-508], which can regulate their migration. Thus, we cannot exclude that B cells lacking MyD88 were somehow “misplaced” *in vivo*, so that they could not reach the appropriate location to regulate disease.

5.6 Role of secretory IgM during EAE

In keeping with the notion that B cell intrinsic MyD88 signaling amplifies humoral immunity [509], we observed impaired natural IgM compartment, and delayed IgM responses in mice lacking MyD88 in B cells. Our finding that mice unable to secrete IgM (μS mice) displayed early and exacerbated EAE indicated another potential mechanism through which MyD88 expression in B cells might regulate disease. Absence of secreted IgM (sIgM) resulted in increased development of autoreactive Th1 and Th17 cells and their enhanced accumulation in the target organ (CNS), which correlated with earlier and more severe disease manifestation. Thus, reduced levels of sIgM could have at least partially accounted for the elevated T cells responses and exacerbated EAE observed in chimera mice with MyD88-deficient B cells. In view of our observation that short-term serum transfer was sufficient to restore wild-type disease course in mice lacking sIgM, it would be important to assess whether such treatment could ameliorate

disease in B-MyD88 chimera mice. However, it is unlikely that absence of sIgM would completely account for the phenotype of B-MyD88 mice. In contrast to lack of MyD88 expression in B cells, lack of sIgM did not prevent disease resolution, suggesting that IL-10-producing B cells -and perhaps other endogenous mechanisms- were still able to control disease in μS mice. In keeping with this, splenic B cells from μS mice were able to secrete IL-10 upon *in vitro* stimulation with LPS (our unpublished data).

Several possibilities could account for the aggravated EAE phenotype in mice lacking sIgM. These mice have expanded B1 and MZ B cell compartments, which are thought to harbor self-reactive B cells. Consistent with this, MOG-reactive IgM was detectable in sera of naive WT mice. Thus, considering that these B cell subsets could be the source of MOG-reactive IgM, increased MOG-reactive B cells could have led to enhanced antigen presentation to autoreactive T cells and consequently, to elevated T cell responses. Earlier studies demonstrated that exogenous WT serum could restore these B cells compartments to WT levels in naïve μS mice provided a two-week long administration [196]. Given that serum treatment in our experiments was much shorter, it is unlikely that it reversed the MZ and B1 subsets abnormalities, and hence, unlikely that this defect in B cell subsets could have promoted T cell responses in μS mice.

Another explanation may stem from the ability of IgM to facilitate antigen clearance. Natural IgM is polyreactive and, its pentameric structure allows high avidity binding of low affinity antigens. Therefore, circulating IgM may bind to MOG peptide, adjuvant-derived components, and in turn sequester them to the liver, thereby limiting their availability, so as to restrict early overt activation of the immune system. A way to test for this possibility would be to induce EAE in the absence of autoantigen/adjuvant in μS mice. This can be achieved by passive EAE that relies on the transfer of previously primed encephalitogenic T cells into naïve recipients, so that the latter develop disease in the absence of any exogenous administration of autoantigen and adjuvant, but pertussis toxin (PTX). Although IgM reactivity to PTX has not been reported in naïve pre-immune or EAE-induced mice, we cannot exclude that this might occur.

Several studies have suggested that in the steady state natural IgM can bind to early apoptotic cells, and by activating the classical complement cascade, facilitate their clearance by DC or macrophages [187-190]. Notably it was proposed that in the absence of sIgM, cells may undergo necrosis [187], which can have proinflammatory effects [187, 510-511]. These reports raised the possibility that lack of IgM might have led to an accumulation of apoptotic or necrotic

cells, which could in turn exacerbate inflammation upon EAE induction and accelerate disease onset. To address this, we measured expression of early apoptotic markers (annexin V binding and caspase-8) and co-stained with propidium iodide, which is taken up by dying cells. We found that in the absence of IgM, the extent of apoptosis and necrosis in dLN and spleen was unaltered both in the steady state and early after disease induction (data not shown). Therefore, it is unlikely that lack of this function of IgM could account for the EAE phenotype of μS mice.

Elevated T cell responses in μS mice could also be due to increased activation status of antigen presenting cells. Our preliminary data showed that in the steady state or after TLR activation *in vitro*, expression of MHC II and co-stimulatory molecules on DC from μS mice were indistinguishable from their control counterparts. However, it is still likely that such differences could have occurred *in vivo* upon disease induction. Support to this possibility lend our observations that before clinical disease onset (on day6), μS mice had higher numbers of activated T cells in dLN, and their total dLN cells proliferated more vigorously in response to auto-antigen, relative to WT mice. Thus, thorough investigation of the activation markers as well as cytokine production by APC *ex vivo* from early time points after disease induction is further required to address fully this mode of regulation by sIgM. A question that arises from this hypothesis is how sIgM would exert such effect. As mentioned earlier, a potential inhibitory effect of sIgM on APC activation could be indirect, by limiting antigen availability through antigen clearance. Alternatively, (or additionally), sIgM could interfere with B cell activation. sIgM has been recently shown to enhance BCR signaling and promote survival of splenic B cells [512]. BCR signaling has been suggested to promote the proliferative response of B cells to mitogens, like LPS [513]. It is therefore plausible to speculate that by crosslinking the BCR, sIgM might facilitate proliferation of TLR-stimulated B cells. It follows then, that absence of sIgM might have delayed optimal proliferation or expansion of IL-10-producing B cells primed by TLR. Our results indicated that during EAE, TLR-primed B cells may control autoreactive T cell responses by inhibiting DC activation through provision of IL-10. Thus, it is possible, that in μS mice, reduced TLR-primed B cells insufficiently inhibited DC, resulting in elevated Th1 and Th17 cells. This would be consistent with the observation that IL-10-producing B cells start to regulate T cell responses within the first ten days after disease induction [270]. Altogether, the aforementioned possibilities could be addressed by examining whether APC activation, B cell proliferation and IL-10 production are altered upon short-term serum transfer.

The ability of non-immune WT serum to rescue the phenotype of μS mice, suggested that natural IgM, rather than immunization-induced antigen specific IgM, was sufficient to mediate

the protective effect. Yet, considering that MOG-reactive IgM was detectable in the transferred serum, we cannot formally exclude a requirement for antigen specificity. Transferring serum previously rendered devoid of MOG-reactive IgM-for example by adherence to a MOG-coated column- would help clarify this issue.

Our data is first to indicate that sIgM can provide protection during CNS autoimmunity. Intriguingly, a protective role for sIgM has also been described in experimental models of SLE. Lupus-prone mice lacking sIgM exhibited accelerated and more severe nephritis [198], and transfer of IgM ameliorated renal pathology in recipients of another lupus-susceptible genetic background [201]. Therefore, apart from providing first line of defense against pathogens, sIgM may regulate immune responses and thereby confer protection in the context of autoimmunity. It should be noted though that the nature of disease induction in the lupus models and EAE is quite distinct. In the former, disease arises spontaneously, whereas in this model of EAE, disease ensues following immunization with the autoantigen mixed with adjuvant. Conceivably, increased T cell responses in μS mice might not be autoantigen-specific, so that immunization with another- foreign peptide would result in similarly enhanced T cell activation. The observation by Baumgarth and colleages that μS mice displayed normal antigen-specific CD4 T cell responses upon influenza virus infection [177], would argue against a generalized hyper-responsiveness of T cells to non self-antigens in μS mice and hence, also against a T cell-intrinsic defect in the absence of IgM. Nevertheless, assessing T cell activation in μS mice following the same mode of immunization as in EAE, but with a foreign peptide instead of MOG, would help determine if the protective role of IgM we observed is specific to CNS autoimmunity.

The role of sIgM in human autoimmune diseases is less clear and more difficult to assess. For example, myelin-reactive IgM+ oligoclonal bands have been described in the cerebrospinal fluid of MS patients but these do not associate with disease progression [514-516]. Primary IgM deficiency is very rare and as expected, patients suffer from recurrent infections, highlighting host-defense as the dominant function of IgM in this case [517-518]. On the other hand, secondary IgM deficiency –arising most likely from preferential class switching to IgG antibodies- seems to be more common and has been reported for subgroups of patients suffering from SLE, RA, Hashimotos thyroiditis, hemolytic anemia or impetigo [519-521], [522-523]. Despite a study showing that in certain SLE patients reduced nephritis correlated with increased IgM levels [200], similar correlations have not been reported in other autoimmune pathologies, and thus, a consensus suggesting a more general protective function for IgM in human autoimmune disease is currently lacking.

Collectively, our findings demonstrate a multifaceted regulation of immune responses by TLR-activated B cells involving cytokine production and antibody production. They further highlight the notion that different classes of antibodies may differ in their capacity to regulate or promote disease.

5.7 Why would TLR-activated B cells be suppressive?

Our data demonstrated that TLR signaling can endow B cells with immunosuppressive capacities that dampen immune responses *in vivo*. Considering that TLR mediate pathogen recognition and activation of innate and adaptive immune responses, we recently assessed whether this notion applies in the context of bacterial infection. Consistent with this study, we found that MyD88-signaling in B cells also suppressed immune responses to infection with the intracellular bacterium *Salmonella typhimurium* [524]. It thus appears that a major function of TLR-MyD88 signaling in B cells might be to control immunity originally activated by signals propagated by TLR (among others). This hypothesis is intriguing and at first appears paradoxical: why would such evolutionary conserved receptors that trigger immune responses to pathogens, also trigger suppression by B cells, and importantly, how could such suppression be beneficial to the host combating pathogens? We propose that TLR-activated B cells could be advantageous to the host at two levels: a) to limit exaggerated immune responses, potentially harmful to the host, and b) to facilitate robustness of the immune response.

Activation and regulation of immune responses are tightly associated processes, with activating signals often resulting in induction of inhibitory molecules that in turn regulate the magnitude of responses via inhibitory loops. For instance, upon TCR triggering T cells upregulate the co-stimulatory molecule CD28 whose interaction with B7 molecules on APC is critical for their efficient activation. Notably, shortly after activation T cells also begin to upregulate the receptor CTLA-4, which binds with higher affinity to B7 molecules to constraint excessive activation and promote T cell survival [525]. Similarly, several negative regulators of TLR signaling have been indentified in macrophages and DC, and are induced early after TLR stimulation [77, 526-534] to ensure appropriate strength and duration of these signals. Genetic ablation of any of these regulators often leads to the development of inflammatory diseases and impair host survival. We suggest that the suppression of TLR-activated DC by TLR-activated B cells constitutes an intercellular layer of suppression: on one hand TLR agonists activate DC

which orchestrate inflammatory responses, whilst at the same time stimulate B cells that counteract inflammation by restricting DC function. Interestingly, this mode of cellular interactions mirrors a particular pattern of negative feedforward loop refer to in the world of systems biology, as type 1 incoherent feedforward loop (I1-FFL). The I1-FFL motif consists of three elements -X, Y, Z- connected in a loop, whereby X stimulates expression of the target Z while it also induces a regulatory element Y that is an inhibitor of Z [535-536] (Fig. 5.1b). It is called “feedforward” loop because X in parallel induces Z and Y, whereas the term “incoherent” reflects the two opposite types of connections present in the loop, that is, stimulatory from X to Z and X to Y, and inhibitory from Y to Z. Such pattern of connectivity can be translated in our case as follows: TLR agonists X, activate DC-Z- and induces IL-10-producing B cells (Y) that subsequently inhibit DC (Z). How could this type of network of cellular interactions, help the host to survive the constant selection pressure posed by pathogens?

To this end, the host has to carry out a dual task: on one hand, it has to mount rapid and strong immune response to survive infection and at the same time must tightly control such response to prevent “self-damage” and return to steady state. Experiments and mathematical modeling have shown that the I1-FFL allows for a stronger and accelerated induction of the response, contrary to a linear connectivity motif, whereby X only stimulates Z. In a simple linear regulation, the kinetic of the response is determined by its final steady state (Fig. 5.1 a and c). In the I1-FFL model, the kinetic of induction of the response and the return to final steady state are controlled independently of each other, by respectively, activation of Z by X, and inhibition of Z by Y. This dissociation permits a transiently overshooting response that is superior to the final steady state. Thus, the input signal X induces an efficient response by Z, and in parallel triggers an inhibitory function by Y, which increases in a gradual manner, to allow both an overshooting response by Z to occur and to timely counteract it towards the steady state. All these features ofthe I1-FFL model are reflected in the regulation of immune response by TLR-activated B cells. In the absence of B cell-mediated regulation (Y) in B-MyD88 mice, TLR/MyD88 signals (X) still induced an overshooting response by DC (Z), because these mice developed. As TLR signals (X), failed to induce suppression by B cells lacking MyD88, disease remained uncontrolled and became chronic, and thus steady state was not reached and pathology ensued. The progressive inhibition of DC (Z) by TLR-activated B cells (Y) is evidenced by the findings that B.MyD88 mice developed EAE earlier than WT mice and displayed more severe symptoms. This is also consistent with the observation that IL-10-producing B cells regulated

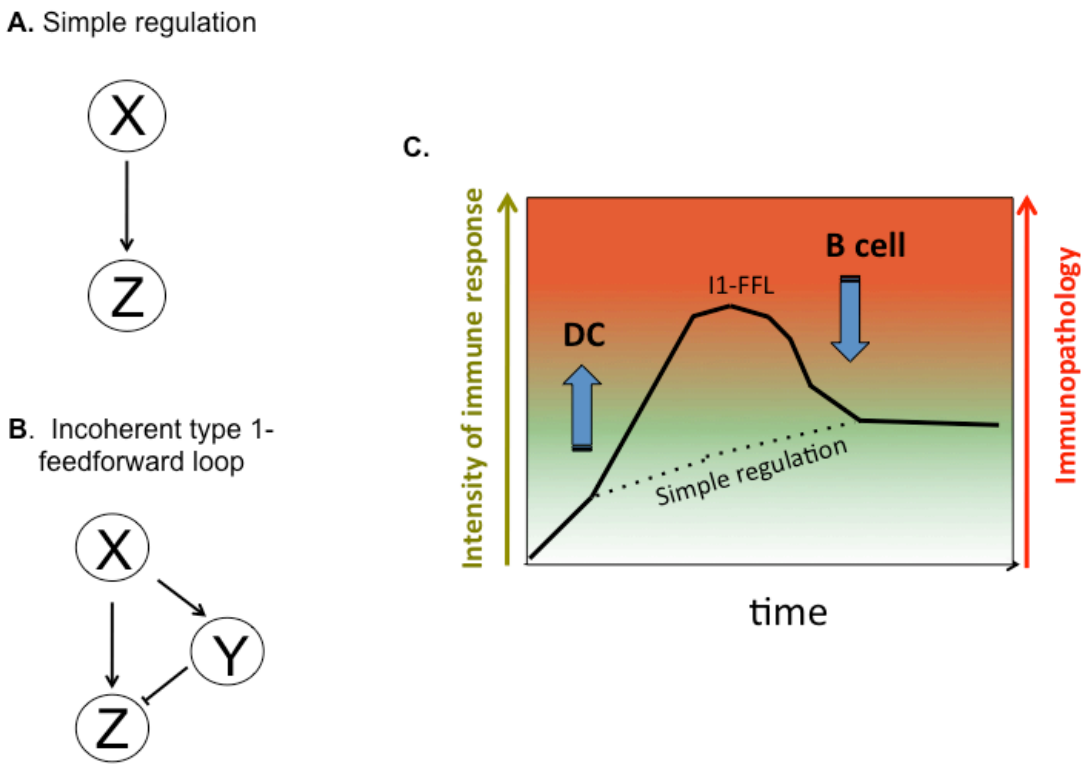

Figure 5.1 Scheme of a simple regulation and incoherent type 1 feedforward loop motifs and their predicted regulation of immune responses.

(A) In a simple regulation the input signal X stimulates directly the output Z. (B) In a I1-FFL connectivity, X stimulates Z but it also induces Y that subsequntly inhibits Z. In this way, I1-FFL allows an immune response to transiently overshoot (C; solid line) in response to TLR stimulation (X), thus ensuring efficient magnitude of immune response (mediated by Z, e.g. DC), which can then be controlled by the inhibitory action of B cells (Y; also induced by X), thereby avoiding immunopathology. In contrast, in a simple regulation, where an inhibotry arm is missing, magnitude of the response is determined by its steady state (dotted line), and it is therefore inferior to that allowed by I1-FFL.

immune responses on day 10, before symptoms appeared. Thus, TLR-activated B cells are part of a dynamic regulation of immune responses, that facilitate robust activation of other cells exposed to TLR signals whilst help protect host integrity from overt inflammation. It would be interesting to find out whether B cells regulate in a similar manner immune responses to other bacterial infections, as well as infections by fungi and viruses whose recognition is mediated by distinct pattern recognition receptors, including NLR, C-type lectins, RIG-like proteins.

5.8 Concluding remarks

Our observation that TLR recognition of microbial products endows B cells with the capacity to regulate autoimmune responses can be viewed as reminiscent of the so-called hygiene hypothesis, which postulates that repeated microbial exposure in early childhood may protect from autoimmune disease. This notion is suggested by epidemiological studies revealing an inverse correlation of infections and emergence of atopic and autoimmune disorders over the past century in Western countries, attributed to increased hygienic conditions, vaccination and use of antibiotics at early age [537-540]. The hygiene hypothesis has gained support from several studies both in mice and humans. Deliberate infections with parasites have showed considerable benefit as a treatment for inflammatory bowel disease and allergic rhinitis [541-542]. Infection with Mycobacterium bovis Calmette-Guerin (BCG) ameliorates EAE symptoms likely by limiting autoreactive T cell responses [543-544]. Consistently, adjuvant therapy with BCG vaccine resulted in reduced inflammatory brain lesions in MS patients, and, in fact, BCG vaccine is in clinical trials to treat type1 diabetes patients [545-546]; http://www.mgh.harvard.edu/about/pressrelease.aspx?id=1254). Furthermore, MS patients with asymptomatic helminth infection displayed reduced relapse rates, number of active lesions and disability compared to non-infected MS patients [547]. Improved disease in infected patients correlated with the presence of a B cell subset producing IL-10, brain-derived neurotrophic factors and nerve growth factors [547]. Therefore, B cells stimulated by microbial products during infections could help limit autoimmunity, by keeping autoreactive T cell responses at bay and possibly by promoting tissue repair. To avoid potential side effects resulting from live infections, the search has begun for microbial products with immunosuppressive capacities as a safer and more rational approach to treat patients suffering from autoimmune disease. Indeed, several microbe-derived compounds have shown immunomodulatory effects in animal models of inflammation and autoimmune disease [548]. Thus, exploiting pathogens to identify molecules that trigger regulatory activities in B cells, e.g. IL-10 secretion, could be of potential clinical application to treat autoimmunity.

An issue to consider when translating findings “from the bench” to the clinic is the differences that exist between the mouse and human immune systems. For instance, in relevance to the current study, naïve human B cells display a distinct expression pattern of TLR compared to mouse B cells, and are poorly responsive to mere TLR agonist stimulation [148], [149]. We identified TLR4 as a critical receptor triggering regulatory activities in mouse B cells. However, it has been long thought that naïve human B cells do not respond to LPS, as they

express low to undetectable levels of TLR4 [148-149]. Considering that human naïve B cells can make IL-10 in response to TLR9 agonist CpG [549-550], it is possible that distinct TLR may induce suppressive functions in human and mouse B cells. Interestingly, though, human B cells may also up-regulate TLR4 under inflammatory conditions [551-553]. Moreover, TLR4 expression has been reported in leukemic B cells from Multiple Myeloma (MM) patients [554-556], correlating in certain cases with poor prognosis. Consistent with this, *in vitro* stimulation of MM cells with TLR4 agonists inhibited cytotoxic T cell responses [557], implying that TLR4 triggering on leukemic B cells could mediate suppression of anti-tumor immunity. It might be important to assess TLR4 expression on B cells also in non-B cell tumors. Several studies have reported accumulation of B cells in the vicinity of certain solid tumors that secrete the B cell-attracting chemokine CXCL13, and growing evidence suggests that B cells may promote tumor growth or even metastasis [165, 263]. It is worth noting here, that growing tumors can secrete (endogenous) TLR4-binding agonists [558-559], implying that these agonists might be available to stimulate suppressive functions in nearby-residing B cells. Collectively, these observations indicate that although differing in their regulation of TLR expression, mouse and human B cells may share common TLR that trigger their suppressive functions and call for a systematic analysis of TLR profile on human B cells from different diseases. They also raise further caution now that TLR agonists, owing to their immunostimulatoty capacity, are being clinically evaluated as adjuvants for the treatment of cancer [560].

In this regard, it is conceivable that the efficacy of TLR agonists as adjuvants will be largely determined by the balance of immune responses elicited by these agonists in various cells types. The current study exemplified TLR /MyD88 as a signaling pathway with pleiotropic non-redundant functions depending on the cell type in which it is engaged. We found that microbial products induced distinct cytokine profiles by DC and B cells. The former produced mainly inflammatory mediators, whereas B cells secreted the anti-inflammatory mediator, IL-10 and shared only one inflammatory cytokine with DC, namely IL-6. Importantly, TLR activated B cells and TLR-activated DC had opposing functions both *in vitro* and *in vivo*. These distinct responses suggest that B cells and DC might differ in their TLR signaling machineries and/or might utilize different receptors to recognize a given microbial product. For example, recognition of LPS by myeloid cells involves, besides TLR4, an accessory integrin CD14 and the molecule MD2, which associates with TLR4 [561]. Notably, B cells do not express CD14 and, in addition to MD2, they express the related molecule MD1. Furthermore, engagement of the receptor RP105, which may also participate in LPS recognition [562], has contrasting effects in B cells

and DC. In the former, it is required for optimal response to LPS, whereas in DC it has an inhibitory effect [563-564]. How the different receptors and signaling components operate in various cell types to translate identical microbial stimuli into distinct responses remains elusive. This is especially true for B cells, as most mouse and human studies on TLR and other PRR signaling, have focused mainly on myeloid cells. Altogether, these and our findings highlight the need for further investigation of TLR signaling in B cells (mouse and human) compared to other cells types, in order to understand how TLR agonists affect immune responses.

The molecular understanding of the suppressive function of B cells triggered by TLR may aid the development of more effective vaccination strategies. In view of the immunosuppressive role of TLR/MyD88 signaling in B cells demonstrated by the current study and by Neves and colleagues, it is plausible that TLR-activated B cells suppress protective responses induced by conventional vaccines and thereby, compromise their efficacy. Therefore, it might be desirable to search for pharmacological inhibitors that would silence MyD88 signaling specifically in B cells, or to eliminate or alter vaccine components that trigger inhibitory functions in B cells. For example a derivative of LPS, monophosphoryl lipid A, preferentially triggers the TRIF- over the MyD88-dependent TLR4 signaling pathway [565], resulting in reduced toxicity compared to LPS, a feature that granted its approval as vaccine adjuvant in humans [560]. Along this line, synthetic TLR agonists that stimulate DC but are weak B cell activators could be used to enhance immunogenicity of vaccines and, as mentioned earlier, of cancer treatments. The exploding advances in high throughput screening technologies and in antibody engineering for cell-specific targeting of compounds are likely to aid the development of such interventions.

6 References

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7 Appendix

7.1 Individual contribution of TLR2 and TLR4 in B cell-mediated suppression of EAE.

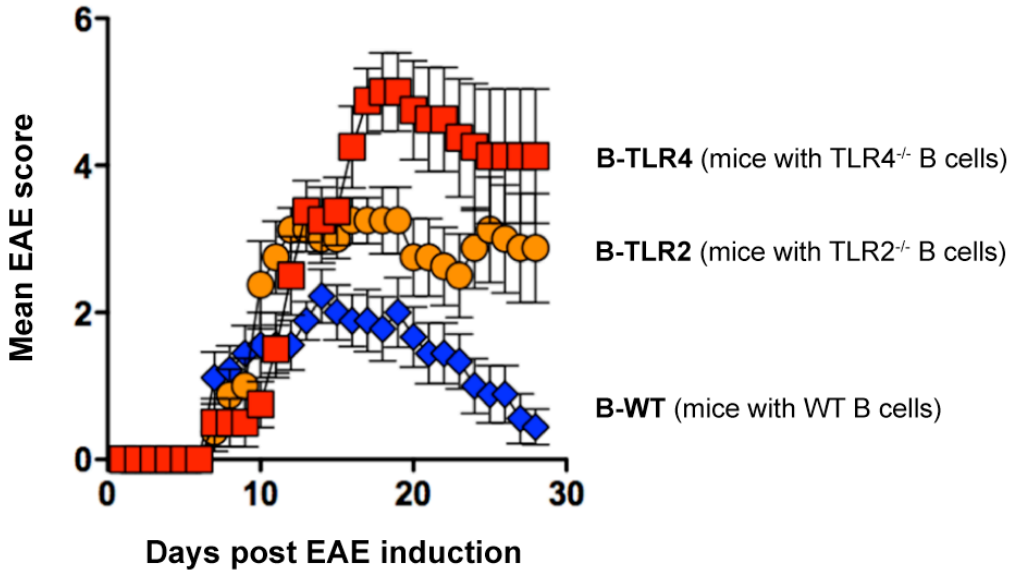

Figure 7.1 Individual contribution of TLR2 and TLR4 in B cell-mediated suppression during EAE
Chimera mice lacking TLR4 or TLR2 selctively in B cells, B-TLR4 and B-TLR2, or chimera mice with WT B cells, B-WT, were subjected to EAE induction. Diseasee symptoms were recoreded daily and are presented as mean EAE score. B-WT, n=5; B-TLR4, n=5; B-TLR2, n=6.

7.2 Abbreviations

Ab	Antibody
APC	Antigen-presenting cell
APC	Allophycocyanin
BCR	B cell receptor
Bio	Biotinylated
BM	Bone marrow
BSA	Bovine serum Albumin
CD	Cluster of differentiation
CFA	Complete Freund´s adjuvant
CIA	Collagen-induced arthritis
CNS	Central nervous system
CpG	Oligodeoxynucleotides carrying unmethylated CpG motifs
CXCL	Chemokine (C-X-C Motif) Ligand
Cy5	Cyanine 5
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DNP	2,4-Dinitrophenol
EAE	Experimental Autoimmune Encephalomyelitis
EAU	Experimental Autimmune Uveitis
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
Fc	Constant region of antibodies

FCS	Fetal Calf Serum
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box protein 3
FSC	Forward scatter
GF	Germ-Free
HLA	Histocompatibility leukocyte antigen
IBD	Inflammatory bowel disease
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
i.p.	Intraperitoneal
ITP	Idiopathic thrombocytopenia purpura
i.v.	Intravenous
IVIg	Intravenous immunoglobulin
KLH	Keyhole limpet hemocyanin
LN	Lymph node
dLN	Draining lymph node
LPS	Lipopolysaccharide
LT	Lymphotoxin
mAb	Monoclonal antibody
MACS	Magnetic-activated cell sorting
MBP	Myelin basic protein
MHC	Major Histocompatibility Complex
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
MyD88	Myeloid differentiation primary response gene 88

NK	Natural killer
NKT	Natural killer T cell
NOD	Non-obese diabetic
OD	Optical density
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PE	Phycoerythrin
PeC	Peritoneal cavity
Pen-Strep	Penicilline-Streptomycine
PerCP	Peridinin-chlorophyll-protein complex
PI	Propidium iodide
PLP	Proteolipid protein
PNA	Peanut agglutinin
PRR	Pattern-recognition receptor
PTX	Pertussis toxin
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
rm	Recombinant murine
RPMI	Roswell Park Memorial Institute 1640 medium
rpm	Rotation per minute
RRMS	Relapsing-remitting multiple sclerosis
RT	Room temperature
SA	Streptavidin
SEM	Standard errors of the mean
sIgM	Secreted IgM

SLE	Systemic Lupus Erythromatosus
SPF	Specific-pathogen free
SSC	Sideward scatter
T1D	Type-1 Diabetes
TCR	T cell antigen receptor
TIR	Toll/Interleukin 1 receptor
TGF	Transforming growth factor
Th	T helper
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Treg	T regulatory cell
TRIF	TIR-domain-containing adapter-inducing interferon-β
UC	Ulcerative colitis
(w/v)	Weight per volume;
WT	Wild-type

7.3 List of Figures

7.4 List of Tables

8 Acknowledgements

My supervisor, Dr Simon Fillatreau, is the first receiver of my gratitude for this work. It has been truly a multifaceted training: multiple experimental methods, intellectually stimulating discussions, constructive criticism and advice, a continuous exercise on critical and analytical thinking, endless enthusiasm for meaningful research, perserverence, team-work and extramural collaborations. These qualities enriched my practical experience, acted as a catalyst to my intellectual growth and further fed my enthusiasm for research. Importantly, they led to a fruitful PhD project. Dear Simon, thank you for this opportunity.

I would like to thank Prof Lauster, my “doctor-father”, for agreeing to evaluate this thesis and for his invaluable academic support and advice, especially as I am an international student.

I next thank Prof. Radbruch, the director of DRFZ, for his scientific feedback, and for providing us with the opportunity to participate in regular intramural seminars and lectures by world-wide renowned scientists; all important aspects of a good PhD training.

Im grateful to Dr Steven Anderton from the University of Edinburgh for collaborating with us for the EAE experiments performed with chimera mice.

Several colleagues have contributed in various ways in this work:

From a chronological perspective, I thank my first colleagues Kai Höhlig and Toralf Roch. I thank you both, both for a literally excellent team-work and for your help with the “hurdles” of the german language, especially in the first years when my german was non-existing.

It is impossible to dismiss the support and the contribution of Patricia Neves, a dear friend and former colleague. Patricia, it has been a complete pleasure to work with you and to be surrounded by your endless positive energy and resourcefulness.

Another important contributor to this pleasant environment, as well as a scientific stimulator has been my colleague Elisabeth Calderón-Gómez. Noteworthy, all three of us, together with an external colleague, Helena Radbruch, we formed the so-called Kitchen Club: a series of fruitful scientific brainstorming sessions accompanied by delicious dinners!

I also thank Ping Shen and Ulrik Stervbo, who readily joined the group´s workforce and team-working spirit as this PhD project was progressing. Further thankful I am to my colleagues and friends Sascha Rutz, Andrey Kruglov for their challenging scientific discussions and advice on the thesis.

In addition to all aforementioned friends, I have to highlight the support of my friends in other parts of the world, like Greece, Italy and Scotland and express my gratitude to all for sharing with me all the joyful and stressful moments that came with this work… and for their patience.

Lastly, I will be no exception in concluding this section by acknowledging the unique contribution of my family. Life-long companions, my parents and my three siblings, even though outside the world of research, just knew how to be there for me and keep the fun going during this phd work. You are already aware of my eternal gratitude!