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# Function and Regulation of B- cell Subsets in Experimental Autoimmune Arthritis

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### **Abstract**

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B lymphocytes play a significant role in autoimmune arthritis, with their function stretching beyond autoantibody production to cytokine secretion and presentation of autoantigen. However, the involvement and activation of different B-cell subset in the autoimmune response is not fully clear. The main focus of this thesis has been to understand the contribution of marginal zone (MZ) B cells in the induction of collagen-induced arthritis (CIA), a mouse model for rheumatoid arthritis (RA).

We show that MZ B cells in the spleen of naïve mice display a natural self-reactivity to collagen type II (CII), the autoantigen used for immunization of CIA. The CII-reactive MZ B cells expand rapidly following immunization with CII, and produce IgM and IgG antibodies to CII. They also very efficiently present CII to cognate T cells *in vitro* and *in vivo*. Moreover, absence of regulatory receptors such as CR1/2 or FcγRIIb on the MZ B cells increases their proliferation and cytokine production in response to toll-like receptor, but not B-cell receptor, activation. Further, FcγRIIb-deficient MZ B cells present CII to T cells more efficiently than wild-type MZ B cells. We additionally demonstrate for the first time the existence of a small population of nodal MZ B cells in mouse lymph nodes. Similar to splenic MZ B cells, the nodal MZ B cells expand after CIA induction, secrete IgM anti-CII antibodies and can present CII to cognate T cells. Finally, we show that mast cells, associated with ectopic B cell follicles in inflamed RA joints, in coculture with B cells promote their expansion, production of IgM and IgG antibodies as well as upregulation of CD19 and L-selectin. Coculture with mast cells further causes the B cells to upregulate costimulators and class II MHC, important molecules for antigen-presenting function.

In summary, my findings suggest that splenic and nodal self-reactive MZ B cells participate in breaking T-cell tolerance to CII in CIA. B-cell intrinsic regulation is needed to keep such autoreactive B cells quiescent. Mast cells can potentiate B-cell responses locally in the arthritic joint, thus feeding the autoimmune reaction.

*Keywords:* B cells, marginal zone, autoimmune arthritis, spleen, lymph node, antigen presentation, Fc gamma receptor IIb, complement receptors 1 and 2, mast cells

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*The answers are there.  
You just have to know where to look.*

The X-Files



# List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I Palm, A.-K. E., H. C. Friedrich, A. Mezger, M. Salomonsson, L. K. Myers, and S. Kleinau. 2015. Function and regulation of self-reactive marginal zone B cells in autoimmune arthritis. *Cellular and Molecular Immunology*, 12(4):493-504.
- II Palm, A.-K. E., H. C. Friedrich, and S. Kleinau. Nodal marginal zone B cells in mice: a virgin and memory subset with dormant self-reactivity. *Submitted manuscript*.
- III Palm, A.-K. E., G. Garcia Faroldi, M. Lundberg, G. Pejler, and S. Kleinau. Activated mast cells promote a pro-inflammatory phenotype of B cells. *Submitted manuscript*.

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# Abbreviations

|                  |  |
|------------------|--|
| AID              | activation-induced cytidine deaminase            |
| APRIL            | a proliferation-inducing ligand                  |
| BAFF             | B-cell activation factor of the TNF-family       |
| BCR              | B-cell receptor                                  |
| CFA              | complete Freund's adjuvant                       |
| CFSE             | carboxy-fluorescein diacetate succinimidyl ester |
| CIA              | collagen-induced arthritis                       |
| CII              | collagen type II                                 |
| CR               | complement receptor                              |
| DAMP             | danger-associated molecular patterns             |
| DC               | dendritic cell                                   |
| dpi              | days post-immunization                           |
| ELISA            | enzyme-linked immunosorbent assay                |
| ELISPOT          | enzyme-linked immunospot assay                   |
| Fc $\gamma$ RIIb | Fc gamma receptor IIb                            |
| FDC              | follicular dendritic cell                        |
| FO               | follicular                                       |
| GC               | germinal centre                                  |
| IFA              | incomplete Freund's adjuvant                     |
| IFN $\gamma$     | interferon gamma                                 |
| Ig               | immunoglobulin                                   |
| IL               | interleukin                                      |
| iNKT             | invariant natural killer T cell                  |
| ITIM             | immunoreceptor tyrosin-based inhibition motif    |
| MHCII            | class II major histocompatibility complex        |
| MMM              | metallophilic macrophage                         |
| MZ               | marginal zone                                    |
| MZM              | marginal zone macrophage                         |
| NK               | natural killer                                   |
| nMZ              | nodal marginal zone                              |
| PAMP             | pathogen-associated molecular pattern            |
| PRR              | pattern-recognition receptor                     |
| RA               | rheumatoid arthritis                             |
| S1P              | sphingosin 1-phosphate                           |
| SLE              | systemic lupus erythematosus                     |
| TD               | thymus-dependent                                 |

|                |                                |
|----------------|--------------------------------|
| T <sub>H</sub> | CD4 <sup>+</sup> T helper cell |
| TI             | thymus-independent             |
| TLR            | toll-like receptor             |
| TNF $\alpha$   | tumor necrosis factor alpha    |
| WT             | wild type                      |

# Introduction

## The immune system

The immune system has evolved to deal with invading pathogens while at the same time not attacking host tissues. It is not confined to a single organ or tissue but rather composed of specific cells and soluble compounds, such as complement, cytokines, chemokines, and antibodies. Based on functional properties, the immune system can be divided into two parts: innate and adaptive immunity. Immune cells that function mainly in innate immunity include granulocytes, macrophages, dendritic cells (DCs), mast cells, and natural killer (NK) cells. These cells together with the physical barriers of our bodies constitute a first line of defence that responds quickly but with limited specificity; relying on recognition of pathogen- or danger-associated molecular patterns (PAMPs or DAMPs) on an antigen. Adaptive immune responses, on the other hand, take longer to establish but are highly specific. The principal cells of adaptive immunity are the B- and T lymphocytes. These cells express unique antigen receptors that recognize a specific part, or epitope, on an antigen. Once the lymphocytes have been activated by their specific antigen they differentiate into effector or memory cells.

Despite this dichotomy of innate vs. adaptive immunity there is substantial crosstalk between the two systems. This is mediated both by cell-cell interactions and soluble compounds, and is necessary in order to establish an efficient as well as specific immune response to the antigen in question.

## B lymphocytes

B lymphocytes, or B cells, represent the arm of the adaptive immune response termed humoral immunity. They are mainly derived from common lymphoid progenitors in the bone marrow, where they develop from pro-B cells to pre-B cells to immature B cells before they are let out into the periphery as transitional T1 cells (reviewed in (1, 2)). When the immature T1 B cells reach the spleen they go through transitional T2 and possibly T3 stages before taking on their final phenotype as fully mature B cells. After activation, mature B cells can produce cytokines and present antigen to T cells, but their hallmark feature and main function is production of antibodies, or immunoglobulin (Ig). These are soluble proteins able to specifically recognize

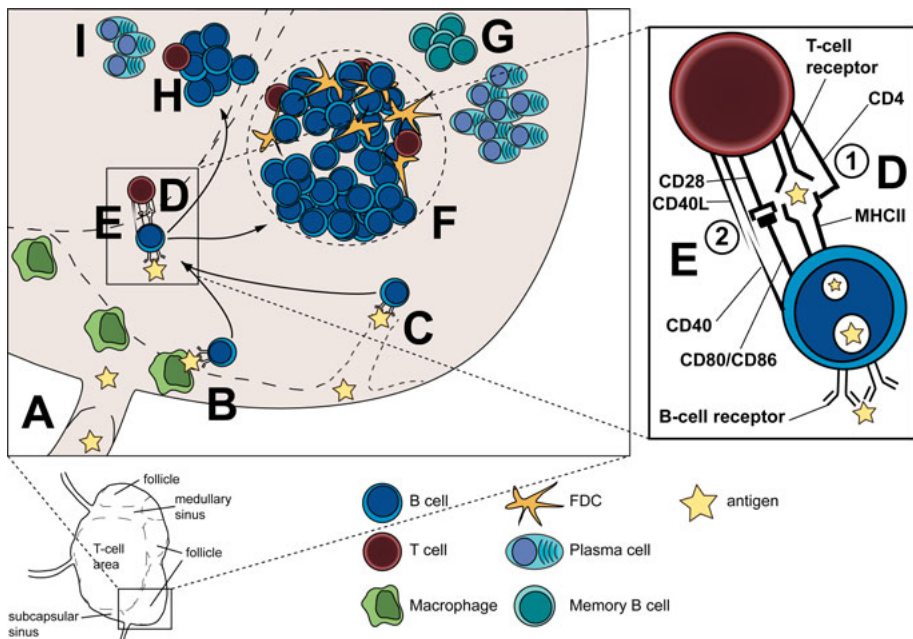
and opsonize antigens, marking them for clearance. There are five isotypes of antibodies, namely IgM, IgD, IgG, IgA, and IgE, each with unique effector functions. Mature naïve B cells express IgM and IgD, and after activation the B cells may go through a process of Ig class-switch recombination. Thus, depending on which type of immune response is needed, an activated B cell will respond to the cytokines produced by the adjacent cells by switching to the antibody isotype whose effector functions are most optimal for the given situation. The membrane-bound form of an antibody constitutes the antigen receptor on B cells, called the B-cell receptor (BCR). In addition to the BCR, B cells also express pattern-recognition receptors (PRRs) such as toll-like receptors (TLRs) (3). This makes them unique in the sense that they can respond not only in an antigen-specific manner by BCR crosslinking, but also in a more innate-like way by recognizing PAMPs or DAMPs on an antigen.

## The B-cell immune response

A classical B-cell response takes place in the follicles of secondary lymphoid organs, e.g. lymph nodes, and is initiated by thymus-dependent (TD) antigens. TD antigens are protein antigens that activate T cells, which in turn help the B cells to respond to the antigen. Typically, the antigen is delivered from the site of entry to the draining lymph node by the afferent lymph, which drains into the subcapsular sinus of the lymph node (figure 1A). Here the antigen can be captured by specialized CD169 (SIGLEC-1)<sup>+</sup> macrophages or, in the case of small soluble antigen, delivered via conduits directly to B cells in the follicle (follicular (FO) B cells) (figure 1B) (4, 5). The CD169<sup>+</sup> macrophages have limited phagocytic activity, which enables them to present intact antigen on their cell surface directly to B cells (6, 7). When the B cell has bound to its specific antigen, the crosslinking of the BCR will lead to signal transduction and activation. Additionally, the antigen:BCR complex is endocytosed, enabling processing of the protein antigen for presentation on class II major histocompatibility complex (MHCII). MHCII is expressed on antigen-presenting cells (APCs), including B cells, DCs and macrophages, and is essential for presentation of the antigen to CD4<sup>+</sup> T helper (T<sub>H</sub>) cells.

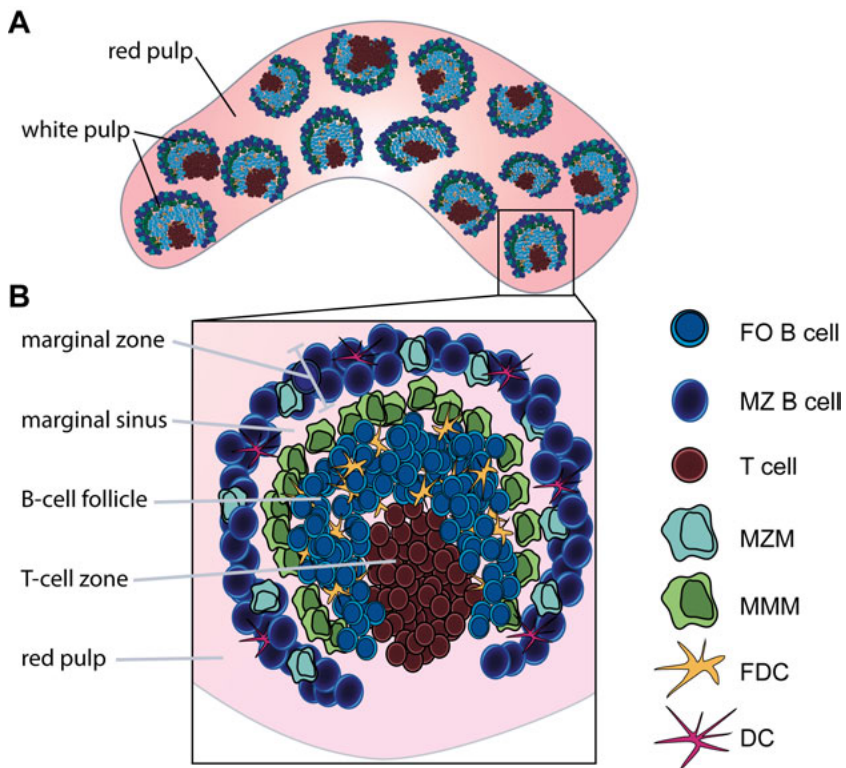
After the initial antigen-activation, the B cell migrates to the border between the B-cell follicle and the T-cell zone. Here it interacts with antigen activated T<sub>H</sub> cells that recognize the antigen peptide on the MHCII on the B cells (signal 1; figure 1D and insert). This contact will induce upregulation of the costimulatory B7-1 and B7-2 proteins (CD80 and CD86) and CD40 on the B cells. Antigen-activated T<sub>H</sub> cells express CD28 and CD40L, which bind to CD80/CD86 and CD40 respectively, thus providing the second activation signal after BCR engagement for the B cell (figure 1E and insert) (reviewed in (8, 9)). Moreover, the B cell secretes cytokines that will help

polarize the T-cell response. The signals received from these interactions will then cause the majority of the activated B cells to migrate back to the follicle and interact with T follicular helper cells and follicular dendritic cells (FDCs). A germinal centre (GC) reaction will be initiated, in which the B cells will go through clonal expansion, isotype switching, and somatic hypermutation (figure 1F) (reviewed in (10)). The GC reaction will result in the formation of long-lived antibody-secreting plasma cells and memory B cells with high affinity (figure 1G). Additionally, B cells initially displaying a relatively high affinity for the antigen may, instead of migrating to the follicle, form small extrafollicular foci at the T cell:B cell border (figure 1H) (11). Here isotype switching and affinity maturation is limited, and short-lived plasma cells are formed as a source of the initial antibody response (figure 1I).



*Figure 1. The B-cell immune response.* The antigen is delivered to the lymph node via the afferent lymph, which drains into the subcapsular sinus (A). Here the antigen is picked up by CD169<sup>+</sup> macrophages for delivery to B cells in the follicle (B), or it migrates via conduits into the follicle where it is directly picked up by B cells (C). Crosslinking of the B-cell receptor causes the B cell to migrate to the border between the follicle and the T-cell area where it meets a cognate T cell, to which it presents the antigen (D-E; insert). This interaction provides activation signals 1 (1; D) and 2 (2; E), causing the B cell to migrate back to the follicle. Here a germinal centre reaction is initiated (F), which eventually leads to the formation of isotype-switched high-affinity plasma cells and memory B cells (G). Some B cells will instead form extrafollicular foci at the T:B border (H), the outcome of which are short-lived plasma cells and a low-affinity initial antibody response (I).

In the case of blood-borne antigens, antigen capture and delivery to B cells take place in the spleen. This is a major site for filtering of the blood, and the cells residing here creates an organized structure optimal for this purpose (reviewed in (12)). The spleen consists of red and white pulp (figure 2A), the latter including the T-cell zone surrounded by B-cell follicles and the marginal zone (MZ) (figure 2B). Immediately encircling the B-cell follicles are the CD169<sup>+</sup> metallophilic macrophages (MMM), which are similar to the subcapsular macrophages in the lymph nodes (figure 2B). A special subset of B cells called the marginal zone (MZ) B cells is situated closest to the splenic red pulp. The MZ B cells are embedded in a network together with stromal reticular cells, MZ macrophages (MZM), which express c-type lectins and scavenger receptors, and DCs. This cellular network is termed the MZ and located between this and the MMM is the marginal sinus, which slows the blood flow and allows for efficient scavenging for pathogens.



*Figure 2. A longitudinal cross-section of the spleen. A* Overview of the organization of the splenic red and white pulp. *B* Close-up of the area marked in A. Surrounding the T-cell zone is the B-cell follicle, which contains follicular (FO) B cells and follicular dendritic cells (FDCs). Lining the outside of the follicle are the CD169<sup>+</sup> metallophilic macrophages (MMM), and bordering the outside of the follicle is the marginal zone (MZ). The MZ comprises MZ B cells embedded in a network of stromal cells, MZ macrophages (MZM) and dendritic cells (DCs). Between the MMM and the MZ B cells is the marginal sinus, into which arterial blood empties.

As in the lymph nodes, antigen not small enough for independent migration to the follicles is either delivered to FO B cells by the resident macrophages, or is picked up directly by the MZ B cells. In the latter scenario, the MZ B cells can shuttle the antigen into the follicle for delivery to FDCs or FO B cells, or for direct presentation to T<sub>H</sub> cells (13). Moreover, some antigens, i.e. thymus-independent (TI) antigens such as lipids, polysaccharides or certain protein structures, can activate B cells without the need for T-cell help. Many of these antigens display PAMPs, and are consequently able to simultaneously engage BCRs and TLRs (TI type I antigens). TI type II antigens have repetitive epitopes that can cross-link enough BCRs to trigger an activation signal (14, 15).

## B-cell subsets

Mature B cells can be subdivided into several subsets that are distinguishable in terms of their development, phenotype, function and anatomical localization. There are two fundamental B-cell compartments in mice: B-1 B cells, which can be subdivided into B-1a and B-1b subsets, and the B-2 cells, which are divided into MZ and FO B cells. Based on functional similarities to the rapid responses of innate immunity, the MZ and B-1 B cells are termed innate-like B cells. Additionally, several regulatory B-cell subsets are now recognized. These all rely on the production of interleukin (IL)-10, a cytokine that exert regulatory function on immune responses. Based on the differences in their surface expressions they are categorized into Breg, B10 and T2-MZ precursor subsets (16-18). The FO, MZ and B-1 B cells are the three major subsets that will be discussed within the scope of this thesis.

The largest B-cell subset comprises the FO B cells, which mainly reside in B-cell follicles in the white pulp of the spleen and in the cortex of lymph nodes. Additionally, FO B cells are found in other organized lymphoid tissue such as Peyer's patches and tonsils. They are recirculating cells that can also home to the bone marrow, where they occupy the perisinusoidal niche (19). The FO B cells can be distinguished from other B-cell subsets by their surface phenotype as B220<sup>+</sup>CD19<sup>+</sup>CD43<sup>-</sup>CD23<sup>hi</sup>CD1d<sup>lo</sup>IgM<sup>lo</sup>IgD<sup>hi</sup>. In the follicles, the FO B cells mainly mediate TD responses to protein antigens resulting in a GC reaction with subsequent differentiation into high-affinity and class-switched plasma or memory cells.

The B-1 and MZ B cells typically have semi-invariant BCRs with limited diversity, thus specifically recognizing conserved epitopes that are often shared between self-structures and pathogens (reviewed in (20-22)). Accordingly, these innate-like B cells use their antigen receptors similar to PRRs, giving each clone a multitude of potential targets for which there is low affinity but high avidity. This unique feature puts innate-like B cells on the first line of defence to infections, but can also be of importance for house-keeping functions such as clearance of apoptotic cells (reviewed in (23)). B-

1 B cells are mainly found in the peritoneal and pleural cavities, making up the majority of all B cells at these sites, and they comprise around 2-3% of the splenic B-cell population (20). B-1 B cells have a distinctive surface phenotype, being CD43<sup>+</sup> and expressing high levels of CD19 and IgM, and low levels of IgD, CD23 and B220 (20). The expression of CD5 distinguishes the two subsets B-1a (CD5<sup>+</sup>) and B-1b (CD5<sup>-</sup>) (24). The majority of B-1 B cells originate from foetal liver precursors and self-renew in adults, and even though the bone marrow is an alternative source of this subset both in the foetal state and in adults, B-1 B cells are considered to constitute an ontogenically distinct subset (20, 25, 26). The principal function of B-1 B cells is production of natural antibodies, i.e. low-affinity polyreactive antibodies secreted without prior antigen-activation (27, 28). These natural antibodies are of importance for clearance of apoptotic cells to maintain tissue homeostasis, but also for quickly neutralizing pathogens (20, 23). These functional properties, along with the strategic positioning of B-1 B cells, put this innate-like subset together with MZ B cells on the border between innate and adaptive immunity. Accordingly, MZ B cells mount immune responses to type I and type II TI antigens, but can also be activated in a TD manner when T-cell help is available ((22, 29-34) and reviewed in (35, 36). In mice the MZ B cells are confined to the spleen, where they are retained in the MZ by interactions between the integrins  $\alpha$ L $\beta$ 2 and  $\alpha$ 4 $\beta$ 1, as well as by the sphingosin 1-phosphate (S1P):S1P receptor 1 interaction (37, 38). Human MZ B cells, on the other hand, are recirculating and are in addition to the spleen also located in the subcapsular sinus of lymph nodes, the epithelium of tonsillar crypts and in the subepithelial area of mucosa-associated lymphoid tissues (39-41). Murine MZ B cells are identified as CD19<sup>hi</sup>CD23<sup>lo</sup>IgM<sup>hi</sup>IgD<sup>lo</sup> and express high levels of complement receptor (CR) 1 (CD35) and 2 (CD21), Fc gamma receptor IIb (Fc $\gamma$ RIIb), MHCII, CD80, CD86, and the non-classical class I MHC molecule CD1d (42-44). The strategic positioning in the MZ puts the MZ B cells on the first line of defence against blood-borne pathogens (12, 31, 32), and the high level of CR1/2 allows the MZ B cells to readily capture immune complexes directly from the blood (45). Furthermore, resident macrophages and DCs in the MZ can, after antigen capture, expose unprocessed antigen or immune complexes directly to the MZ B cells (46-48). After picking up lipid antigens, the MZ B cells can present those on CD1d, thus admitting interaction with CD1d-restricted invariant NK T (iNKT) cells, which facilitates a rapid extrafollicular response (49-51). Antigen-activated MZ B cells can then rapidly differentiate into plasma cells producing low-affinity IgM and IgG (33); antibodies that are important before subsequent production of high-affinity IgG antibodies by FO B cells. The MZ B cells can also shuttle antigen from the MZ into the follicles, where they can deposit it onto FDCs, thus facilitating the GC reaction (13). Additionally, the high surface expression of MHCII, CD80, and CD86 puts the MZ B cells in a pre-activated, or “natural



memory”, state with the ability to powerfully activate naïve T<sub>H</sub> cells directly after shuttling the antigen into the follicles ((33, 52, 53) and reviewed in (54)). Indeed, such interactions may include MZ B cells in a GC reaction, thus giving rise to long-lived plasma cells secreting high-affinity antibodies (34). Besides these important functions of antibody production, antigen delivery, and antigen presentation, MZ B cells are also efficient cytokine producers, especially after TLR-stimulation (3). Their cytokine profile is dominated by IL-6 and IL-10, thus suggesting both immunostimulatory and – suppressive functions.

## Regulatory receptors on B cells

B-cell intrinsic regulation is vital to limit normal B-cell responses as well as to avoid activation of self-reactive B cells in the periphery. It may also be of significance in the selection during lymphopoiesis, as BCR signalling strength determines the fate of immature and transitional B cells (reviewed in (55, 56)). CR1 (CD35) and 2 (CD21) are cell surface receptors expressed on B cells and involved in B cell immune responses and regulation. By binding complement fragments attached to pathogens or immune complexes, these receptors facilitate the clearance and transportation of the antigen (reviewed in (57)). In mice CR1 and CR2 are encoded by the same gene (*Cr2*) but are differently expressed through alternative splicing (58). CR1 has the same amino acid sequence and structure as CR2, but with an additional piece on its N-terminal region (reviewed in (59)). Thus, while both CR1 and CR2 bind to complement fragments iC3b, C3dg and C3d, CR1 has additional binding sites for C3b and C4b. In addition, CR1 can act as a cofactor for cleavage of C3b and C4b, and it can inhibit the complement activation pathway by promoting dissociation of the C3 and C5 convertases (60, 61). To study the function of CR1 and CR2, *Cr2*<sup>-/-</sup> (CR1/2-deficient) mice have been used, but as these mice lack both of these receptors it has been difficult to distinguish the particular function of each receptor. However, it is known that CR2 (CD21) clusters together with CD19 and CD81 close to the BCR on the surface of B cells, functioning as a BCR co-receptor that lowers the activation threshold for BCR-signaling (62-64). Mice that lack CR1/2 fail to delete autoreactive B cells during the selection process, probably due to a lower signaling strength induced by self-antigen when the BCR co-receptor is dysfunctional (65). Moreover, CR1/2-deficient mice overexpress CD19, which results in hyper-responsive B cells and secretion of autoantibodies (66-68). In humans, CR1 can act as a potent negative regulator of B cells, particularly when antigen is scarce, by blocking BCR-induced proliferation and differentiation to plasmablasts as well as antibody secretion (69-71).

FcγRIIb is the only IgG Fc receptor expressed on B cells and can bind all subclasses of IgG. It carries an immunoreceptor tyrosine-based inhibition motif (ITIM) in its intracellular part. When an IgG-opsonized antigen cross-

links Fc $\gamma$ RIIb and the BCR simultaneously this will result in an inhibition of the downstream signalling, and thus regulation of the antibody production (reviewed in (72)). Fc $\gamma$ RIIb can also inhibit TLR-responses, although the exact mechanism behind this is not known (73)

## Autoimmunity

The remarkable ability of B- and T lymphocytes to distinguish self from foreign is recognized as immunological tolerance. Failure to maintain tolerance results in the immune system turning on its host, and autoimmunity ensues. The triggers of autoimmunity are yet to be identified, although there are some known risk factors including genetic predisposition and environmental factors such as smoking ((74) and reviewed in (75)). What is recognized, though, is that the breaking of both B- and T-cell tolerance to self-antigens is a critical step. During lymphocyte development, cells that express antigen-receptors with high affinity for self-antigens are not allowed out into the periphery. Instead, the autoreactive cells first try to modify their antigen receptor in order to make it less self-reactive, and if that fails the cells are deleted due to lack of survival signals (reviewed in (76)). Nevertheless, some autoreactive cells escape these mechanisms of central tolerance and must instead be kept quiescent by peripheral tolerance mechanisms, by which they are deleted, put in a state of anergy, or become excluded from follicles upon encountering their cognate self-antigen. Despite these tolerance mechanisms, some lymphocytes, including innate-like B and T cells, seem to be positively selected for a low level of self-reactivity and are thus frequently found in the periphery where they survive as responsive cells (21).

The presence of autoantibodies in many autoimmune diseases indicates a pathogenic role for B cells. However, B-cell function in autoimmunity is much more complex and diverse than merely being a source of autoantibodies. They are efficient cytokine-producers, and can present autoantigen to cognate T<sub>H</sub> cells. Indeed, B cells have been implicated as important APCs in the pathogenesis of murine models of several autoimmune diseases, including systemic lupus erythematosus (SLE) (77, 78), type I diabetes mellitus (79), and rheumatoid arthritis (RA) (80). In order to successfully break T-cell tolerance to any self-antigen, this must be presented in a very efficient way. B cells have the advantage over other APCs in their ability to concentrate very small amounts of antigen through selective uptake via the BCR. This will in turn lead to high concentration of self-peptides on MHCII, thus endowing the B cells with the potential to activate low-affinity autoreactive T cells that have escaped central tolerance mechanisms (81-84).

In order for a B cell to efficiently present antigen it needs to be fully activated, either by extensive BCR crosslinking, innate signals, or T-cell help, the latter being impossible before T-cell tolerance has been broken. Howev-

er, several self-antigens recognized as important targets in autoimmune disease have properties of TI type I antigens, e.g. nuclear antigens such as double-stranded DNA in SLE, or TI type II antigens, e.g. collagen type II (CII) in autoimmune arthritis. Innate-like B cells respond vigorously to both type I and type II TI antigens, and this in combination with their reported natural self-reactivity makes it plausible that tolerance to self-antigen is first overcome in these subsets. Once activated, a self-reactive B cell can in turn efficiently activate even low-affinity autoreactive T cells that have escaped tolerance mechanisms, thereby initiating the autoimmune reaction.

## Collagen-induced arthritis

The most common autoimmune disease in humans is RA, which affects around 1% of the world's population, with women outnumbering men three to one (85, 86). It is a systemic autoimmune disease, which manifests as chronic joint inflammation with large cellular infiltrates in the synovia of affected joints. Rheumatic synovial infiltrates consist largely of T and B cells, but there are also neutrophils, macrophages and mast cells along with proliferating synoviocytes. This inflammatory tissue is called a pannus. Eventually the synovitis will lead to cartilage destruction and bone erosion in the joint. B-cell depletion therapy has been very successful in RA, strongly indicating a pathogenic role for B cells (reviewed in (87)). Further supporting this notion is the presence of specific autoantibodies, which can be both pathogenic and symptomatic. The autoantibodies can be detected in the circulation several years before overt disease in RA (88), suggesting that breakage of B-cell tolerance happens early in the pathogenesis.

One of the most widely used animal models for RA is collagen-induced arthritis (CIA). It resembles RA both clinically, immuno- and histopathologically, and genetically, thus making this a valid model to study pathogenesis as well as clinical features of the human disease. CIA was originally developed in rats by immunizing the animals with CII in either complete or incomplete Freund's adjuvant (CFA or IFA) (89). However, more common today is the CIA model in mice, in which genetically susceptible mice, e.g. the DBA/1 strain, are immunized with heterologous CII emulsified in CFA (90). CII is the major structural component of articular cartilage and a CII-specific immune response is one of the characteristics of both RA and CIA (90-94). Interestingly, susceptibility to CIA is strongly linked to the H-2<sup>q</sup> haplotype expressed by DBA/1 mice (90). The H-2<sup>q</sup> is associated with MHCII I-A<sup>q</sup>, and thus related to good presentation of the immunodominant and arthritogenic CII epitope (95). Similarly, RA is strongly associated to the human MHCII haplotype HLA-DR ((95, 96) and reviewed in (97, 98)), emphasizing the significance of MHCII and antigen presentation also in RA. Furthermore, the histopathological findings in CIA are similar to those found

in RA and include the same cellular infiltrates, pannus formation, cartilage destruction, and bone erosion. Clinical disease manifests as polyarthritis starting in the distal joints, and at later stages the carpal, tarsal, and even knee joints may become affected. If left untreated the arthritis eventually leads to joint destruction and ankylosis. This clinical, or effector, phase of CIA develops around three to four weeks after immunization. The pre-clinical period, or induction phase, between immunization and overt disease is dominated by IgM and later IgG responses to CII. Around the time of clinical disease onset the serum concentration of IgG anti-CII antibodies peak, reflecting the breakage of tolerance to CII (44, 90). These high-affinity IgG anti-CII antibodies are arthritogenic, as has been demonstrated by transfer experiments where either serum from arthritic mice or purified IgG anti-CII antibodies cause arthritis in naïve recipients (99-102). Thus, the presence of pathogenic autoantibodies in CIA suggests an important role for B cells in the disease process. Indeed, B-cell deficient mice are protected from CIA (103, 104), and mice lacking IgM do not develop arthritis (105) indicating a function for B cells early in the induction phase. This notion is further supported by the finding that B cells are the principal autoantigen-presenting cells in CIA (80). This calls for a strict regulation of B cells to avoid autoimmune reactions. Indeed, CR1/2-deficient mice are more susceptible to CIA than wild type (WT) mice, and soluble CR1 can stall the progression of CIA (106, 107). Fc $\gamma$ RIIb knockout mice develop high levels of autoantibodies, spontaneous lupus and augmented CIA (108-110). In addition, Fc $\gamma$ RIIb regulates autoreactive plasma cells as well as GC responses to autoantigen (111-113). In humans, B cells from RA patients have a lower expression of CR1, CR2, and Fc $\gamma$ RIIb than healthy controls, which contributes to enhanced proliferation in response to IL-2 *in vitro* (69, 114). Furthermore, healthy women express less Fc $\gamma$ RIIb on their B cells as they age (114) which could be one possible explanation for increased prevalence of autoimmunity in older individuals.

## Mast cells in autoimmune arthritis

Mast cells are densely granulated cells derived from CD34<sup>+</sup> hematopoietic stem cells. They act as sentinels in places where the body meets the surface, such as mucus membranes and the skin, where they specialize in the early, rapid responses against invading parasites and bacteria (115, 116). Besides this protective function, mast cells are also involved in IgE-mediated allergic disease (reviewed in (117)), and are ascribed functions in adaptive immune responses including being pathogenic in several autoimmune and inflammatory conditions (reviewed in (118-120)). Activation of mast cells is typically achieved through crosslinking of the highly expressed Fc $\epsilon$ RI by IgE immune complexes. However, mast cells also express Fc $\gamma$ Rs enabling activation by

IgG immune complexes, CRs for activation by complement, and PRRs allowing activation by PAMPs and DAMPs. The effect of mast cell-activation can be largely attributed to the release of granular contents upon activation. The granules are packed with preformed mediators, e.g. biogenic amines, cytokines, growth factors, and proteases (reviewed in (121)). Degranulation thus rapidly causes local inflammation, vasodilation, and tissue swelling. In addition, mast cells can *de novo* synthesize and secrete cytokines and lipid mediators.

Both RA patients and mice with CIA show accumulation of mast cells in the inflamed synovium (122-128), and local mast cell degranulation in the arthritic joint is associated with disease severity in RA (129). Mast cells also accumulate in the draining lymph nodes after CII-immunization for CIA, where they may interact with self-reactive B- and T cells (130). Interestingly, mast cell-deficient mice show decreased disease severity but not susceptibility to CIA along with reduced numbers of T- and B cells in the draining lymph nodes (130).

Mast cells can contribute directly to synovial inflammation and cartilage degradation ((131, 132) and reviewed in (133, 134)) but they can also modulate B- and/or T-cell responses in the joint. Thus, mast cell-derived IL-17 and IL-6 can promote polarization of the T-cell response into T<sub>H</sub>17 with subsequent recruitment of neutrophils (135), and tumor necrosis factor alpha (TNF $\alpha$ )-secretion by synovial mast cells may activate resident T cells (136, 137). The effect of mast cells on B cells is less explored, although mast cells have been shown to promote B-cell proliferation, differentiation to plasma cells, and antibody secretion *in vitro* (138, 139). Thus, mast cells pose a potential source of B-cell activating factors in the arthritic synovia.

# Present investigation

## Aim

This work has aimed to investigate the function and regulation of different B-cell subsets during the induction phase of experimental autoimmune arthritis. Additionally, the direct effect of mast cells on B cells has been explored as a possible mechanism for enhancing B-cell responses locally in the arthritic joint.

## Results

### Paper I. Function and regulation of self-reactive marginal zone B cells in autoimmune arthritis

Previous studies of the B-cell response in CIA from our group have demonstrated that the first B cells to respond to bovine CII, the antigen used for immunization, are the MZ B cells in the spleen (44). They produced a rapid IgM response to heterologous CII, and, interestingly, even MZ B cells from naïve DBA/1 mice had a spontaneous reactivity to bovine CII. In this study we addressed whether the B cell response in CIA is true self-reactive, i.e. reactive to autologous CII (murine CII). We were particularly interested in investigating the relative contributions of different B-cell subsets and their function in the autoimmune response to CII. Additionally, we asked whether the self-reactivity to CII was regulated by CR1/2 and/or FcγRIIb on the B cells.

To compare the B-cell response to autologous versus heterologous CII in CIA we used ELISPOT. Thus, the number of splenic and lymph node B cells reactive to murine respective bovine CII in naïve mice and after CII-immunization were analysed. We also analysed serum samples from the same mice for the presence of IgM and IgG antibodies to autologous and heterologous CII using ELISA. We found that naïve mice harbour IgM<sup>+</sup> and IgG<sup>+</sup> splenic B-cells that are reactive to bovine but not murine CII. Consistent with this we could only detect IgM antibodies to bovine CII, but not to murine CII, in the sera of these mice. However, following immunization the B-cell response also included reactivity to murine CII that paralleled the response to bovine CII. This was also evident when analysing the sera of the

immunized mice. Thus, the serum levels of IgM and IgG to bovine and murine CII were quite similar. Notably, at three weeks after immunization, which is a timepoint when the first clinical signs of arthritis often are observed in the mice, the IgG response to autologous CII was significantly higher than the corresponding response to bovine CII. When comparing the B cell response to autologous CII in the spleen versus lymph nodes in the immunized mice it was clear that the autoreactive B cell response to CII was initiated earlier in the spleen than in the lymph nodes. The splenic response started 5 days post-immunization (dpi) whereas the response in the lymph nodes was not detectable until day 12 pi.

We next asked whether the early splenic B-cell response to autologous CII in immunized mice was derived from MZ B cells, as they have been shown to be naturally reactive to bovine CII (44). CII is a type II TI antigen, and when administered together with CFA we can therefore anticipate that innate-like B cells are the first lymphocytes to respond. Indeed, we could demonstrate that IgM<sup>+</sup>, and to a lesser extent IgG<sup>+</sup>, CII-reactive clones were derived from MZ B cells within the first two weeks after immunization. This quick response was not seen in FO B cells, which instead displayed a very strong IgG<sup>+</sup> autologous CII response about three weeks after immunization. We also addressed if additional innate-like B cells such as the B-1 B cells in the spleen were reactive to CII. Splenic B-1 B cells did not show any CII-reactive clones by ELISPOT. However, when we activated B-1 B cells from the spleens of immunized mice with TLR stimuli *in vitro* we could detect low levels of IgM anti-CII antibodies in the culture supernatants. Notably, this was not seen when B-1 B cells from naïve mice were used, indicating that B-1 B cells do not carry natural reactivity to CII as MZ B cells do. Together these data highlights MZ B cells, in contrast to FO B cells and B-1 B cells, as driving the early autoreactive CII-response in CIA.

Intrinsic regulation of B cells is very important to avoid over-activation and autoimmune responses. Previous studies in the group have shown that mice deficient in CR1/2 or FcγRIIb are more susceptible to CIA than WT mice (106, 110). This suggests that the autoimmune B-cell response may be controlled by these inhibitory receptors. Indeed, when we analysed the B cell response to CII in CII-immunized CR1/2 or FcγRIIb-deficient DBA/1 mice by ELISPOT we observed that the CR1/2-deficient mice had significantly more CII-reactive IgM<sup>+</sup> B cells than the WT mice. This led us to ask if MZ B cells, which are the initiators of the CII response in CIA, were regulated by CR1/2 or FcγRIIb. Thus, we set out to investigate how MZ B cells, in comparison with FO B cells, were affected by deficiency of CR1/2 or FcγRIIb. The results showed that MZ B cells lacking CR1/2 had increased proliferation, measured by <sup>3</sup>H-thymidine incorporation, compared to WT MZ B cells in unstimulated cultures and after TLR9-stimulation. FO B cells deficient in either CR1/2 or FcγRIIb showed increased proliferation compared to WT FO B cells after stimulation not only through TLR9 but also TLR4.

Proliferation after BCR crosslinking was not affected in either subset by loss of CR1/2 or Fc $\gamma$ RIIb. We also measured the cytokine secretion in the culture supernatants from the different subsets by a bead-based multiplex flow cytometry assay. We found that WT MZ B cells secreted IL-6, IL-10, and TNF $\alpha$  when stimulated through TLR4 or TLR9, but not via the BCR. This cytokine production was enhanced in CR1/2 and/or Fc $\gamma$ RIIb-deficient MZ B cells. WT FO B cells secreted very little or no cytokines after either stimuli, but the levels increased in the absence of CR1/2 and/or Fc $\gamma$ RIIb. These data indicate that CR1/2 and Fc $\gamma$ RIIb regulate both MZ B cells and FO B cells.

The revealed CII-reactivity of the MZ B cells and the known antigen-presenting capacity of these cells (52) led us to hypothesize that breakage of T-cell tolerance to CII could be achieved by CII-reactive MZ B cells. To explore this possibility, MZ B cells, and as a comparison FO B cells, from naïve and CII-immunized mice (5 dpi) were used as APCs in a T-cell proliferation assay. Accordingly, CII-specific V $\beta$ 8.3<sup>+</sup> T cells were purified from spleens of qCII24 mice; a transgenic DBA/1 mouse strain that express a V $\beta$ 8.3<sup>+</sup> TCR specific for a immunodominant epitope on CII (140). The V $\beta$ 8.3<sup>+</sup> T cells were labelled with carboxy-fluorescein diacetate succinimidyl ester (CFSE) and then cultured for three days alone or with either B-cell subset before analysis of antigen-specific proliferation using flow cytometry. We found that the MZ B cells were superior as APCs in comparison to FO B cells. Even naïve MZ B cells could induce proliferation of the T cells and the proliferation was improved if we added a CII(245-270) peptide to the cultures. When the MZ B cells were obtained from CII-immunized mice and thus primed with CII *in vivo*, they efficiently activated the CII-reactive T cells while the FO B cells from the same mice could only do so to a limited extent. Notably, the MZ B cells did not only induce more T cells to proliferate than did the FO B cells, but evoked more divisions in the responding cells. Moreover, *in vivo* CII-primed MZ B cells lacking Fc $\gamma$ RIIb produced more cell divisions in the CII-specific V $\beta$ 8.3<sup>+</sup> T cells compared to MZ B cells from WT mice.

Having demonstrated the superiority of the MZ B cells as presenters of CII *in vitro*, we next asked whether this was also evident *in vivo*. To address this question, we purified and transferred MZ B cells, or FO B cells as comparison, from CII-immunized WT mice (5 dpi) to either V $\beta$ 8.3<sup>+</sup> qCII24 mice or WT littermate controls. We took serum samples from the recipient mice prior to transfer and at 5, 12 and 21 days post-transfer to check for IgG anti-CII antibodies. Interestingly, the V $\beta$ 8.3<sup>+</sup> qCII24 mice receiving MZ B cells had significant levels of IgG anti-CII antibodies 5 days after transfer that peaked at day 12, and then declined again at day 21. In contrast, V $\beta$ 8.3<sup>+</sup> qCII24 mice that received FO B cells produced only base-line levels of IgG anti-CII as did WT mice transferred with either MZ or FO B cells. This indicates that MZ B cells are largely responsible for presentation of CII to T



cells *in vivo* and that this may be crucial for breaking T-cell tolerance to CII in CIA.

In conclusion, our data reveal MZ B cells as important players in the early autoimmune CII-response in CIA. The key functions here are antigen recognition and production of CII-specific IgM, along with cytokine secretion and antigen presentation. Furthermore, we demonstrate that CR1/2 and Fc $\gamma$ RIIb can regulate the self-reactive MZ B cells, thus highlighting the need for intrinsic B-cell regulation in autoimmunity.

## Paper II. Nodal marginal zone B cells in mice: a novel virgin and memory subset with dormant self-reactivity

In paper I, we demonstrated that the B-cell response to autologous CII in CIA is initiated in the spleen, driven by the MZ B cells. We also showed that a B-cell response to CII is present in the lymph nodes, but this appears later compared to the splenic response. This delayed lymph node response to CII led us to challenge the existing dogma that murine MZ B cells are confined to the spleen, hypothesizing that a minor population of MZ B cells may be present in lymph nodes, as has been demonstrated in humans.

Albeit present in the normal repertoire, self-reactive innate-like B cells are rare and consequently can be difficult to detect. Therefore, in order to recover sufficient number of B cells for analysis, we pooled cells suspensions from popliteal, inguinal, axillary and brachial lymph nodes and stained the cells with markers for MZ B-cells. Indeed, a small lymph node population of B220<sup>+</sup>CD1d<sup>hi</sup>CD23<sup>lo</sup>IgM<sup>hi</sup>IgD<sup>lo</sup> cells were identified by flow cytometry. This phenotype is corresponding to MZ B cells in the spleen, and to keep with the nomenclature in humans we decided to term this population nodal MZ (nMZ) B cells. Interestingly, we observed that the frequency of nMZ B cells was higher in aged mice ( $\geq 40$  weeks old), and that females in this cohort tended to have a larger population than male mice of the same age.

By immunohistochemical analysis of cryosections of inguinal lymph nodes from naïve DBA/1 mice, stained for IgM and CD1d, we observed that the nMZ B cells were not localized to the B-cell follicles but rather in the subcapsular and medullary sinuses. These areas resemble the splenic MZ both histologically and functionally, suggesting that the nMZ B cells serve a similar function here as conventional MZ B cells do in the spleen; i.e. macrophages and DCs located in the MZ can expose unprocessed antigen to the MZ B cells, which in turn transport the antigen into the follicles. Indeed, we did find the nMZ B cells strategically located in close proximity to CD169<sup>+</sup> macrophages in the subcapsular and medullary sinuses, implying that such an interaction does take place.

We then continued our phenotypical analysis of the nMZ B cells to further characterize and thus distinguish the nMZ B cells from other B-cell

populations. Similar to nMZ B cells, both B-1a and B10 B cells express high levels of CD1d, and they are also CD5<sup>+</sup>. Therefore, by staining for CD5 we wanted to exclude that we were dealing with either one of these populations. Indeed, less than 1% of the nMZ B cells were CD5<sup>+</sup>, demonstrating that this is a separate population of lymph node B cells. We could further characterize the nMZ B cells as being CD80<sup>hi</sup>CD86<sup>hi</sup>FcγRIIb<sup>hi</sup>TLR4<sup>hi</sup>, which corresponds well to the splenic MZ B-cell phenotype. The high expression of the costimulators CD80/CD86 indicates that the nMZ B cells are in a pre-activated state, poised for antigen presentation to T cells. That FcγRIIb is highly expressed on the nMZ B cells suggests that they need tight intrinsic regulation, which is in line with their pre-activated state and position in an antigen-rich environment. The high expression of TLR4 provides the nMZ B cells with means for efficient activation by simultaneous TLR and BCR engagement, similar to what we observed in paper I for MZ B cells in the spleen. Moreover and in contrast to splenic MZ B cells, the nMZ B cells expressed similar levels of CD21 (CR2) and CD35 (CR1) as FO B cells, and were MHCII<sup>lo</sup>. The low expression of MHCII can be a disadvantage in the context of antigen presentation, but this is probably compensated for by the high levels of CD80 and CD86.

The observed pre-activated state of the nMZ B cells is in agreement with the description of innate-like B cells as having “natural memory”. Indeed, human MZ B cells express the memory marker CD27 (141) and the murine splenic MZ harbours both memory and naïve B cells (142). Therefore, we next asked whether the nMZ B-cell compartment contains any memory cells. There is no single marker that defines mouse memory B cells, but by using the approach described by Shlomchik and colleagues (143) we were able to define a substantial fraction of CD80<sup>+</sup>PD-L2<sup>+</sup>CD73<sup>+</sup> memory B cells in the nMZ B-cell compartment, especially in the aged mice. Around one third of these memory nMZ B cells were IgM<sup>+</sup>. This was in sharp contrast to the few memory cells within the FO B-cell compartment, the vast majority of which had class-switched. IgM<sup>+</sup> memory cells may be derived through a GC-independent pathway (144), further underlining the differences between nMZ and FO B cells in terms of their activation pathways. Moreover, the increased frequency of nMZ B cells in aged mice may be a reflection of accumulation of long-lived IgM<sup>+</sup> memory cells in this niche (145).

Finally, we examined the nMZ B-cell population in the context of autoimmunity, i.e. in CIA. We found that the frequency of nMZ B cells increased with around 50% in mice 10-12 dpi with CII in CFA, but not after immunization with ovalbumin in CFA, or CFA alone. Interestingly, at 35 dpi the frequency of nMZ B cells was back to the same level as in naïve mice, indicating their involvement in the early events leading up to overt disease in this model. Moreover, the nMZ but not FO B cells analysed at 10 dpi had an increased surface expression of FcγRIIb, MHCII, and CD70 compared to naïve mice, indicating antigen-activation. We also found that naïve nMZ B

cells, but not FO B cells, were naturally CII-reactive in a similar manner as splenic MZ B cells. The nMZ B cells produced CII-specific IgM upon TLR activation *in vitro*, and this antibody production to CII was increased when the nMZ B cells were obtained from CII-immunized mice, reflecting the expansion of this population in immunized mice. We propose that this self-reactive IgM has low pathogenicity *per se*, but is instead important for potentiating subsequent B-cell responses. The pentameric conformation of IgM ascertains high avidity for the CII and this immune complex may engage Fc $\mu$ R<sub>s</sub> on B cells, stimulating survival and activation (146). Furthermore, IgM bound to antigen can also activate complement, thereby enhancing antigen uptake by complement receptors on APCs such as CD169<sup>+</sup> macrophages, thus facilitating antigen delivery to B cells. Self-reactive B cells such as MZ B cells can then in turn present the autoantigen to cognate T cells, as demonstrated in paper I. Thus, the similarities of the nMZ B cells to splenic MZ B cells in terms of phenotype, anatomical localization and evident self-reactivity led us to hypothesize that they could present CII to cognate T cells. We employed the same approach as in paper I, with nMZ or FO B cells from CII-immunized mice (12 dpi) being used as APCs in a coculture with CFSE-labelled CII-specific V $\beta$ 8.3<sup>+</sup> T cells from the qCII24 mice. Supporting our hypothesis, we could demonstrate that the nMZ B cells were superior in inducing antigen-specific T-cell proliferation compared to FO B cells from the same lymph nodes. Not only were more T cells activated, but the nMZ B cells also provided a stronger costimulatory signal as indicated by the higher proliferation index of the responding T cells. Notably, no CII was added to the cultures, indicating that CII-uptake in nMZ B cells occurs *in vivo*.

Innate-like B cells are prone to produce cytokines upon innate stimuli (3), and we showed in paper I that splenic MZ B cells can produce TNF $\alpha$ , a cytokine that has major impact on disease in autoimmune arthritis (147). Interestingly, immunization of rodents with CII induces TNF $\alpha$  in cells within the subcapsular sinus of the draining lymph nodes (reviewed in (148)), and we thus hypothesized that the nMZ B cells are also able to generate this cytokine. Indeed, we could demonstrate that nMZ B cells produce TNF $\alpha$  upon TLR4- and TLR9-stimulation, but not after BCR crosslinking. Likewise stimulated FO B cells could also generate TNF $\alpha$ , but less so than the nMZ B cells. TNF $\alpha$  is a very potent pro-inflammatory cytokine, and B-cell derived TNF $\alpha$  has been implicated to promote interferon gamma (IFN $\gamma$ )-production by T<sub>H</sub>1 cells (149). Moreover, TNF $\alpha$  produced by B cells is important for normal histological architecture of lymphoid organs (150). The ability of the nMZ B cells to rapidly produce cytokines upon innate stimuli thus implies that they serve a role as modulators of immune responses.

In summary, this paper describes nMZ B cells as a novel component of the mouse immune system. This population of innate-like B cells share many phenotypical and functional characteristics with splenic MZ B cells. The self-reactive nMZ B cells are potent, and given their phenotype, strategic

localization and behaviour upon activation, we reason that they participate in the early events leading to breakage of immunological tolerance and induction of pathological autoimmunity.

### Paper III. Activated mast cells promote a pro-inflammatory phenotype of B cells

Papers I and II mainly deal with innate-like B cells, which can readily exert their effector functions without help from cognate T cells. In contrast, the classical view is that activation of conventional B cells demands not only antigen recognition but also depends on T-cell help. However, evidence accumulating over recent years has challenged this view, demonstrating that B cells can receive help also from a number of innate immune cells, including iNKT cells, DCs, granulocytes, and mast cells (151-158). Mast cells accumulate close to ectopic B-cell follicles in the synovia of RA patients and in mice exposed to CIA. It is therefore plausible that there is crosstalk between mast cells and B cells locally in the arthritic joint. In this study we explored the hypothesis that mast cells can modulate B cells into a more pro-inflammatory phenotype in a way that may be of significance for their role in autoimmune arthritis.

To this end, we set up a coculture system where CFSE-labelled naïve or BCR-activated B cells were cultured together with bone marrow-derived resting, IgE-sensitized, or antigen-activated mast cells. Sensitization of the mast cells was made with antigen-specific IgE, and activation (degranulation) was completed by adding the specific antigen directly to the cocultures. To confirm full maturation of the mast cells, IgE-sensitization and antigen activation was performed and release of  $\beta$ -hexosaminidase was used as a measure for functional degranulation. The mast cells of different activation stages, and the B cells were cultured for three days. The B cells were thereafter analysed by flow cytometry for B-cell activation markers and proliferation. Additionally, the culture supernatants were analysed for secreted antibodies by ELISA. Our first findings were that the presence of mast cells, particularly if antigen-activated, promoted proliferation of both naïve and BCR-activated B cells. Presence of activated mast cells also increased blast formation of both naïve and BCR-activated B cells, as compared to B cells in medium alone. These initial findings confirmed data from other groups (138, 139), thus validating our experimental system. Next, we found that coculture with mast cells caused an upregulation of CD19 on B-cell blasts in cultures of both naïve and BCR-activated B cells, with the strongest effect seen after mast cell activation. Moreover, naïve B cells upregulated both MHCII and CD86 after coculture with activated mast cells, suggesting that mast cells can enhance the antigen-presenting capacity of B cells. As the observed increase of CD19, MHCII, and CD86 was mainly seen after anti-

gen-activation of the mast cells, this strongly suggested that the effect was partly mediated by soluble compounds. Therefore, we used a protein array system for a panel of immunological mediators to compare supernatants from cultures of B cells in medium only, BCR-activated B cells alone, antigen-activated mast cells alone, and coculture of BCR-activated B cells and antigen-activated mast cells. We found that L-selectin, CXCL16, MCP5 and MIP-1 $\alpha$  were elevated as a result of B cell:mast cell coculture. The most prominent of these was L-selectin, and the increased levels in the supernatant after coculture suggest that mast cells regulate L-selectin expression and/or shedding in B cells. Indeed, using flow cytometry we were able to confirm that mast cells promote L-selectin expression in B cells. Not only were the proportion of L-selectin<sup>+</sup> B cells higher after coculture, but also the expression on each cell was increased. These data imply that mast cells can promote B-cell homing to lymphoid tissue and to sites of inflammation (reviewed in (159)). Interestingly, L-selectin was only expressed on the CD19<sup>hi</sup> blasts. Unsurprisingly we also found that IL-6 was abundant in cultures where mast cells were present. Indeed, IL-6 has been implicated as one possible mechanism for mast cell-dependent B-cell activation (138), and it is plausible that this cytokine plays a role also in our system.

To further elucidate whether the impact of mast cells on B cells was depending on cell-cell contact, we used a system where the B cells and mast cells were separated by a membrane that allows passage of soluble mediators but not cells. Mast cell-driven proliferation of naïve B cells was contact-dependent, while BCR cross-linked B cells also responded when being separated from the mast cells in the transwell system. However, as mast cell-conditioned medium was not sufficient for an increased proliferative response after BCR crosslinking, this suggests that there is crosstalk between the two cell populations when the B cells are antigen-activated rather than a one-way communication from mast cells to B cells. Blast formation and upregulation of CD19 were both strictly contact-dependent regardless of the activation status of the B cells. Induction of MHCII in naïve B cells was similar in direct and transwell cocultures, but no increase was seen after addition of mast cell-conditioned medium. Mast cell-driven upregulation of MHCII in BCR-activated B cells, as well as increase of CD86 expression regardless of activation status of the B cells, was seen only after direct coculture. L-selectin was found to be partially regulated by soluble mediators, as the number of L-selectin<sup>+</sup> B cells increased only after cell-cell contact while any L-selectin<sup>+</sup> B cells in the transwell system increased their expression comparable to B cells in direct contact with mast cells. However, mast cell-conditioned medium was not sufficient to influence expression of L-selectin. After BCR-activation the B cells were more sensitive to induction of L-selectin, but direct cell-cell contact was still the most favourable culture condition.

When analysing the culture supernatants for secreted antibodies, we found that mast cells promoted secretion of both IgM and IgG, also when only IgM<sup>+</sup> B cells were used in the coculture, indicating that a class-switch had taken place. This effect was most pronounced after activation of the mast cells and, although not significant, there was a strong trend for this to be contact-independent.

Finally, to determine whether mast cells had the same effect on different B-cell subsets, we repeated these experiments using MZ and FO B cells. Both of these subsets responded similar to coculture with mast cells, the only difference being a tendency for a stronger up-regulation of L-selectin and significantly higher antibody-secretion in the MZ B cells.

In conclusion, we show that, at least in an *in vitro*-setting, mast cells can drive B cells to take on a more pro-inflammatory phenotype. Consequently, in the context of autoimmune arthritis mast cells may provide activation factors for B cells in the inflamed synovium, thus feeding the autoimmune reaction. It is also possible that mast cells can promote B-cell homing to lymph nodes and/or inflamed tissue by promoting L-selectin on B cells.

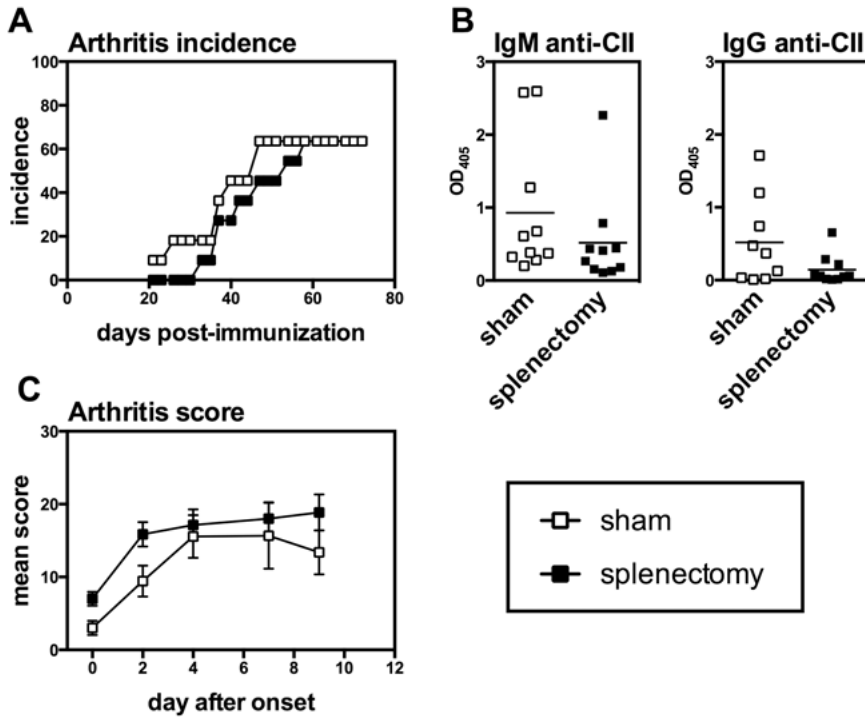
## General discussion

There is little doubt today that B cells are essential in the pathogenesis of autoimmune arthritis, both in RA and in models of this disease. However, there are still questions regarding the function of the different B-cell subsets besides production of pathogenic autoantibodies. The work presented in this thesis has been focused around the role of B cells in general, and MZ B cells in particular, as the APCs responsible for breaking immunological tolerance in T cells in CIA.

Successful antigen presentation is essential for breaking T-cell tolerance to any autoantigen. Autoreactive T cells that have escaped central tolerance mechanisms are only allowed to do so because they have very low affinity for self-antigen. In order to activate such T cells, an APC needs to display high concentration of antigen-derived peptide on its MHCII along with high expression of the costimulatory molecules CD80/CD86. Considering the self-reactivity that is recognized in MZ B cells along with their high expression of costimulators as well as MHCII, it is therefore reasonable to consider them likely candidates for the role of principal APCs for autoreactive T cells. MZ B cells can indeed activate cognate naïve T<sub>H</sub> cells in a powerful way (52), and there are indications that antigen presentation by MZ B cells is important in mouse models for type 1 diabetes mellitus and SLE. Thus, onset of diabetes in NOD mice is associated with MZ B cells colonizing the pancreatic lymph node (79). This colonization correlates with increased activation of diabetogenic T cells, indicating a role for the invading MZ B cells in antigen presentation. Furthermore, autoreactive MZ B cells in a lupus-prone mouse readily interact with T<sub>H</sub> cells in follicles, causing T-cell proliferation (78). In line with these studies, the data presented in paper I strongly suggest that splenic MZ B cells may be responsible for presenting autoantigen in CIA. These innate-like B cells show early CII-reactivity, express high levels of MHCII and costimulators, and we demonstrate that they, after being primed with CII *in vivo*, efficiently present CII to cognate T cells both *in vitro* and *in vivo*.

Consistent with the idea that the spleen is where T-cell tolerance is broken, the B-cell response to CII in the lymph nodes is undetectable until around 2 weeks after immunization. This delay suggests that the CII-response in lymph nodes is a direct consequence of that in the spleen. However, an alternative explanation is that the spleen and lymph nodes act in parallel rather than serial fashion and the reason for the observed delay is

insensitive methods rather than a non-existing early CII-reactivity in the lymph nodes. To try to elucidate this, we induced CIA in splenectomized or sham-operated mice and followed arthritis development and anti-CII autoantibody production (unpublished data; figure 3).



*Figure 3. Splenectomized mice are not protected from CIA.* DBA/1 mice were splenectomized or sham-operated 4 weeks prior to immunization with 50  $\mu$ g bovine CII in CFA. The mice were monitored for arthritis 3 days weekly from day 21 post-immunization. **A** Accumulated arthritis incidence as determined by visual inspection. **B** Serum levels of IgM and IgG anti-CII on days 12-13 post-immunization were determined using ELISA. **C** The mean  $\pm$ SEM total score for arthritic mice after disease onset. The data are derived from 2 independent experiments (n=10). CFA, complete Freund's adjuvant; CIA, collagen-induced arthritis; CII, collagen type II; OD, optical density

Somewhat to our surprise, there was no big difference between the two groups. The arthritis incidence was around 65% for both splenectomized and sham-operated mice (figure 3A), demonstrating that although splenic MZ B cells seem to drive the autoreactive response to CII, they are not indispensable for arthritis development. However, the disease onset was a little delayed in splenectomized animals compared to the sham-operated controls (figure 3A), and the levels of anti-CII autoantibodies were slightly decreased in the splenectomized group (figure 3B). While neither of these parameters was



statistically significant, these data do indicate that absence of a spleen, and hence of MZ B cells, might actually decrease disease susceptibility. Despite this delay in disease onset in splenectomized mice, disease severity was slightly exacerbated in this group (figure 3C). One possible explanation for this observation could be decreased levels of IL-10 after splenectomy. IL-10 is an important immunosuppressive cytokine, and mice with B cells unable to produce IL-10 display increased disease severity in CIA, including aggravated  $T_H1$  and  $T_H17$  responses in lymph nodes (160). Absence of a spleen not only eliminates the IL-10 producing splenic MZ B cells, but also causes reduction of another important source of IL-10: the peritoneal B-1a B cells (161). Thus, once breakage of tolerance is achieved in splenectomized mice, decreased numbers of IL-10 producing cells may result in increased joint inflammation evident as a higher arthritis score. Our CIA results in splenectomized mice are in agreement with a previous study (162), and show that without a spleen there must be other cells than the splenic MZ B cells that can present CII and break T-cell tolerance. In view of our findings in paper II, I believe that possible candidates are the novel nMZ B cells in the lymph nodes. I further believe that the reason for the slight delay in CIA onset in splenectomized mice is due to the relatively low frequency of nMZ B cells and consequently a somewhat reduced antigen-presenting capacity in the absence of a spleen.

The population of nMZ B cells is very small compared to their splenic counterpart. Thus, the fact that we can not detect any CII-reactive B cells in lymph nodes by ELISPOT until 12 dpi does not necessarily mean that there are none, but rather that our detection method is not sensitive enough. Indeed, when we isolate nMZ B cells from CII-immunized mice and stimulate them *in vitro* using TLR9-ligand CpG we can detect anti-CII autoantibodies in the culture supernatants. Moreover, we show that the nMZ B cells are superior to FO B cells when it comes to presenting the CII to cognate T cells after *in vivo*-priming, further demonstrating that they do have CII-reactivity. It would of course also be very interesting to find out whether the nMZ B cells also present the CII *in vivo*, as we demonstrated for splenic MZ B cells in paper I. Although a little difficult, it would certainly be possible to perform such an experiment, the main barrier being the small number of nMZ B cells recovered after sorting. I interpret these data as strongly indicative of the B-cell response to CII in the spleen and lymph nodes acting in parallel, and that we detect the splenic response much earlier than the lymph node response simply because the splenic MZ B cells are more abundant than the nMZ B cells.

The discovery of B cells with MZ B-cell characteristics in mouse lymph nodes breaks the central dogma that murine MZ B cells are solely confined to the spleen. The sinuses in the lymph nodes resemble the splenic MZ very much, both in terms of function and cellular composition. Therefore, I do not find it very surprising that a corresponding B-cell phenotype exists in the

lymph node sinuses. On the contrary, I find it curious that this population has not been described in the mouse before, except for the one finding of MZ B cells invading the pancreatic lymph node in NOD mice as they develop diabetes (79). When using flow cytometry to characterize different cell populations, it is not only essential to analyse a sufficient number of events, especially when studying a rare population, but it is also of vital importance to use the appropriate markers for the purpose. Knowing that CD21 (CR2) is down-regulated on recirculating human MZ B cells compared to when resident in the spleen (141), it is reasonable to think that this marker is probably of limited use to detect any MZ B cells in extrasplenic locations, also in the mouse. In our lab, we routinely use CD1d as a marker for MZ B cells, and I believe that this technical detail contributes to us identifying a B-cell population previously believed to be excluded from mouse lymph nodes.

One question that would be interesting to study is whether genetically modified mice with B cells unable to present antigen, and thus unable to assist in breaking T-cell tolerance, are protected from CIA. To investigate this, one approach could be to delete MHCII specifically in B cells. In a recent study from Mark Shlomchik's lab, it was shown that when MHCII was deleted in CD19<sup>+</sup> cells in lupus-prone MRL.Fas<sup>lpr</sup> mice, this resulted in reduced systemic autoimmunity (77). Importantly, by using this approach we would only answer the question whether bulk B cells are needed for CIA induction and not pinpoint specific B-cell subsets. Nevertheless, based on the data presented in this thesis, one could argue that if deletion of MHCII in B cells has an impact on CIA susceptibility and/or severity, it would be a strong indication that it is the antigen presentation by splenic or nodal MZ B cells that is of importance.

Another central question in this context is how depletion of MZ B cells would affect the outcome of CIA. I believe that if we could achieve successful and specific depletion of both splenic and nodal MZ B cells for a sustained period of time before and after CII-immunization, we would see decreased disease susceptibility due to lack of successful breaking of T-cell tolerance. This would indeed be a very interesting experiment to conduct, although it is not easily done. There are several ways described to target MZ B cells, including using monoclonal antibodies against the integrins  $\alpha$ L $\beta$ 2 and  $\alpha$ 4 $\beta$ 1 (38), or the S1P receptor antagonist FTY720 (163). An alternative approach could be using mice lacking Pyk-2 or CD19, or mice that specifically lack Notch2 or ADAM10 in B cells, all reported not to have any MZ B cells (31, 164-167). However, using the monoclonal antibody or the FTY720 approach would only cause a transient displacement of MZ B cells, and B cells from CD19-deficient mice are generally hyporesponsive, B cells lacking Pyk-2 have reduced motility, and ADAM10-deficient B cells have elevated CD23-expression but reduced CD21/CD35 (31, 67, 164), thus making it difficult to ascribe any observed phenotype specifically to the lack of MZ B cells.

Intrinsic regulation of B cells is essential for preventing autoimmunity. Especially innate-like B cells, which are constantly in a pre-activated state and poised for action, depend on regulatory proteins to be kept quiescent. Indeed, splenic MZ B cells express high levels of both CR1/2 and Fc $\gamma$ RIIb, thus emphasizing their need for tight regulation. Previous studies from our lab and others have established that mice lacking CR1/2 show increased arthritis susceptibility without altered IgG-response to CII, and that Fc $\gamma$ RIIb regulates disease susceptibility as well as disease severity and autoantibody production (106, 110, 168). The fact that not only disease severity but also susceptibility was affected when either of these regulatory receptors was lost suggests that the induction phase rather than the effector phase of CIA is affected in these strains. Thus, as presented in paper I, we explored how response to innate (through TLR4 and -9) and adaptive (BCR crosslinking) stimulation was affected by deficiency of either CR1/2 or Fc $\gamma$ RIIb in MZ and FO B cells. Both CR1/2 and Fc $\gamma$ RIIb are associated with the BCR, and we expected that BCR crosslinking in B cells from CR1/2- or Fc $\gamma$ RIIb-deficient mice would cause an augmented response compared to WT B cells. However, neither proliferation nor cytokine secretion was affected after BCR-stimulation, which was somewhat surprising. Responses to TLR-ligation, on the other hand, seemed to be regulated by CR1/2 and/or Fc $\gamma$ RIIb. Consequently, CR1/2- and Fc $\gamma$ RIIb-deficient B cells are likely to have a lower threshold for adjuvant activation in CIA, since the CFA used in the immunization protocol contains TLR-ligands. In agreement with this having an impact during the early B-cell priming in CIA, we show that CR1/2-deficient B cells have increased CII-reactivity compared to WT B cells. Importantly, the CR1/2 and the Fc $\gamma$ RIIb-deficient strain do not have altered TLR-expressions. Furthermore, the MZ B cells lacking Fc $\gamma$ RIIb could provide a stronger activation signal to cognate T cells in the antigen-presentation assay. Fc $\gamma$ RIIb can regulate BCR-mediated endocytosis in a dominant negative manner, consequently providing less CII-peptides for presentation on MHCII (169). B cells lacking this regulatory mechanism may endocytose more CII than WT B cells, which can in turn result in an increased loading of CII-peptides on MHCII with enhanced presentation to cognate T cells as a consequence.

Healthy women have lower Fc $\gamma$ RIIb expression on B cells than men, and decreased expression of this receptor correlates with age in women (114). This would suggest that elderly women have B cells that are less regulated. Indeed, RA debut is associated to middle-aged women, and B cells from RA patients have lower levels of CR1, CR2, and Fc $\gamma$ RIIb than healthy controls. This strongly suggests that a defective regulation of B cells contributes to disease (69, 114). Considering these observations together with our data showing that impaired intrinsic B-cell regulation causes augmented responses to TLR-stimulation, I believe it plausible that down-regulation of these receptors will have strong impact during the peri-articular phase of RA.

Thus, without this regulation, B cells are more likely to be activated by self-antigen, especially when TLR-ligands are also available. The consequences of this would be increased autoreactivity in B cells, and hence increased probability of antigen presentation to T cells followed by a breach in tolerance.

Although mast cells have been ascribed a pathogenic role in autoimmune arthritis, there is currently no conclusive evidence available describing their specific function. Our findings in paper III provide some new insights as to how mast cells might modulate the B-cell response and so be a part of arthritis pathogenesis. These data demonstrate that presence of activated synovial mast cells may, in addition to contributing to local inflammation, be a source of B-cell activating factors, consequently feeding not only the effector inflammatory response but also the underlying autoimmune reaction. The presence of ectopic B-cell follicles and GCs in rheumatoid synovium indicates that antigen presentation by B cells is not only important for the initial breakage of T-cell tolerance, but that it is an on-going process throughout the effector phase of the disease. In support of this hypothesis, it has been shown that T-cell activation in synovial lesions in autoimmune arthritis is B-cell dependent (170). Our data show that mast cells can influence B cells to up-regulate surface molecules that are involved in antigen presentation to cognate T cells. By accumulating in rheumatoid synovia, mast cells may thus contribute to disease processes locally in the joint.

In our *in vitro* cocultures, the presence of mast cells also influences the surface expression of CD19 and L-selectin on B cells. B cells that over-express CD19 become hyper-responsive due to a lowered activation threshold, and are consequently more prone to autoimmunity ((67, 68) and reviewed in (171)). L-selectin is an adhesion molecule that is crucial for the homing of B cells, and other leukocytes, to lymph nodes and sites of inflammation (159). These data suggest that mast cells can contribute to autoimmune arthritis by lowering the activation threshold and enhancing the antigen-presenting capacity of B cells while at the same time directing them to sites where this can take place.

One thing that we have not yet been able to elucidate is the exact mechanism by which the mast cells influence the B cells. The effects are only partially contact-dependent, suggesting that soluble compounds come into play. However, activated mast cells produce a multitude of mediators, and it is hard to pinpoint one or even a few that are responsible for the observed effects. Naturally, various cytokines are likely candidates, including IL-4 and IL-6, both of which are known to be secreted by mast cells and have a profound effect on B cells (reviewed in (172, 173)). However, previous studies have demonstrated contradictory results about the involvement of IL-6, indicating that this cytokine may not be the key component (138, 139). Furthermore, that the observed class-switch to IgG tended to be contact-independent was somewhat surprising, since B cells generally depend on cell-cell contact,

including CD40-ligation, for induction of activation-induced cytidine deaminase (AID) which is needed for class-switching (174-177). One possible explanation for this is that mast cell-derived exosomes expressing CD40L interact with the B cells (178, 179), thus providing signals usually acquired from cognate T cells in a GC reaction. It is also possible that mast cells secrete B-cell activating factor of the TNF-family (BAFF) and a proliferation-inducing ligand (APRIL), both of which are factors known to induce class-switching in B cells (155, 180). To identify the component(s) involved in mast cell-dependent B-cell activation we could use a number of different strategies, e.g. using mice deficient in IL-6, CD40L, or any other potential targets, as donor animals for the culturing of bone marrow-derived mast cells. There is also the possibility of using blocking antibodies or inhibitors of molecules in cytokine-receptor signalling pathways, e.g. JAK, to determine whether the observed effects are mediated by cytokines in general. Other questions that remain to be answered are whether B cells in the cocultures are in fact more efficient APCs than control B cells, and whether the antigen-presenting capacity is altered in B cells from mast cell-deficient mice. The first question could be answered by using B cells from CII-immunized mice in the coculture, and then use these as APCs in the CII-antigen presentation assay as described previously. The second questions should be fairly easy to answer in a similar way, with the limitation that mast cell-deficient mice are currently not available on the DBA/1 background, and consequently an alternative antigen-presentation assay would have to be used. By conducting these experiments, I believe we can gain further insight into the role of mast cells as mediators of B-cell responses in autoimmune arthritis.

## Concluding remarks

In summary, the data presented here provide some new insights into the function of different B-cell subsets and their regulation in the context of experimental autoimmune arthritis. As implied throughout this thesis, I believe that antigen presentation by innate-like B cells is a key event in the pathogenesis of autoimmune arthritis. I believe that these B cells are normally kept quiescent, but that under unfortunate circumstances their activation threshold is reached even in response to autoantigen. Plausible unfortunate circumstances include genetic predisposition in combination with environmental factors such as smoking, on-going infection where PAMPs are in abundance, and tissue injury with consequent release of autoantigen in combination with DAMPs. My data show that absence of the regulatory proteins CR1/2 and FcγRIIb make B cells particularly prone to activation by innate signals, which I think is of significance for the initial activation of autoreactive B cells. Lastly, my data show a plausible role for mast cells in potentiating antibody secretion as well as the antigen-presenting function of B cells, suggesting this as one mechanism for the pathogenicity of mast cells in arthritis.

Thus, the quest for the trigger of autoimmune arthritis continues, but I hope that the findings presented in this thesis can bring us a little closer to its completion.

## Summary in Swedish

### Hur olika typer av B-lymfocyter bidrar till sjukdom i en experimentell modell för ledgångsreumatism

Vårt immunförsvar är utvecklat för att skydda oss från infektioner utan att förstöra kroppens egen vävnad. För att uppnå detta måste cellerna i immunsystemet lära sig att skilja på vad som är kroppseget och vad som är främmande. Detta kallas för självtolerans och är särskilt viktigt för de immunceller som kallas lymfocyter (B- och T-celler). De mekanismer som ser till att självtoleransen upprätthålls är oftast mycket effektiva och de flesta självreaktiva lymfocyter förstörs redan innan de är färdigutvecklade. Ibland händer det dock att dessa toleransmekanismer brister vilket leder till att lymfocyterna börjar förstöra vår egen kropp. Detta kallas för autoimmunitet och kan resultera i autoimmun sjukdom. I min avhandling har jag studerat vilken roll olika typer av B-celler har för uppkomst av autoimmunitet samt hur B-cellerna kan regleras för att undvika autoimmuna reaktioner.

En av de vanligast förekommande autoimmuna sjukdomarna är reumatoid artrit (RA), även kallad ledgångsreumatism. RA drabbar ungefär 1 % av världens befolkning och är omkring 3 gånger vanligare förekommande hos kvinnor än hos män. Sjukdomen innebär att självtoleransen mot framförallt ledbrosk har brutits och immunförsvaret ser detta som något som ska förstöras. Detta leder till en kronisk inflammation i lederna (artrit), i synnerhet i händer och fötter. Inflammationen leder till att ledbrosket, och i senare stadier även det underliggande skelettet, bryts ned, vilket är mycket smärtsamt. För att studera RA används ofta djurmodellen kollagen-inducerad artrit (CIA). Här kan autoimmun artrit framkallas genom att möss injiceras med kollagen typ II (CII) från ko. CII är en viktig beståndsdel i ledbrosk, och CII från ko är strukturellt mycket likt musens eget CII. Därför kan det immunsvaret som utvecklats mot injicerat CII också reagera mot CII i musens eget ledbrosk, vilket slutligen resulterar i ledinflammation. Sjukdomsförloppet i både RA och CIA drivs till stor del av B-celler. I friska individer har dessa celler till uppgift att producera antikroppar, vilket är lösliga proteiner som kan neutralisera infektiösa ämnen. För att en B-cell ska bli en högeffektiv antikropsproducent måste den kommunicera med T-celler. Dessa är i sin tur beroende av aktiveringssignaler från andra immunceller, t.ex. B-celler. Denna tvåvägskommunikation mellan T- och B-celler är nödvändig för att skapa ett starkt och specifikt immunsvaret. I en autoimmun sjukdom som RA

eller CIA har både B- och T-celler förlorat sin självtolerans och kan därmed starta ett starkt och specifikt immunsvaret mot kroppsegna strukturer.

Vad är det då som gör att självtoleransen bryts och en autoimmun sjukdom uppstår? I min forskning har jag arbetat utifrån hypotesen att svagt självreaktiva B-celler på något sätt blir tillräckligt aktiverade för att i sin tur kunna trigga igång de mycket få självreaktiva T-celler som överlevt selektionsprocessen. Jag har framför allt studerat en viss B-cellstyp: marginalzons (MZ) B-celler. Dessa finns i mjältens marginalzon där de är viktiga för att skydda oss från infektionsämnen i blodet. De har också till uppgift att städa bort rester av våra egna döda celler, vilket gör att de måste kunna känna igen vissa kroppsegna strukturer. Normalt sett utgör detta inte ett problem, eftersom MZ B-cellerna är hårt reglerade. Denna reglering är bland annat beroende av aktiveringshämmande (regulatoriska) proteiner på cellytan. Viktiga regulatoriska proteiner på alla B-celler är komplementreceptor (CR) 1 och CR2 samt Fc-gamma-receptor IIb (FcγRIIb). Om svagt självreaktiva MZ B-celler trots denna reglering av någon anledning får en stark aktiveringssignal samtidigt som de har bundit till kroppseget protein kan självtoleransen brytas. För att en sjukdomsframkallande autoimmun reaktion ska uppstå behövs det dock att även T-cellernas självtolerans bryts. Detta kan ske om T-cellerna kommunicerar med de aktiverade självreaktiva B-cellerna. I enlighet med detta scenario har MZ B-celler visats sig spela en viktig roll i flera djurmodeller för autoimmunitet.

I den första delstudien såg vi att de första immunceller som reagerar på musens eget CII är naturligt självreaktiva MZ B-celler i mjälten. Dessa expanderar efter CII-injektionen och kan på ett mycket effektivt sätt aktivera CII-reaktiva T-celler. Detta tyder på att de spelar en viktig roll för att bryta självtoleransen mot CII i T-celler. Vidare fann vi att avsaknad av någon av de regulatoriska receptorerna CR1/2 eller FcγRIIb gör att B-celler är mycket mer lättaktiverade. Dessutom gör avsaknad av FcγRIIb att MZ B-celler är ännu bättre på att aktivera CII-reaktiva T-celler. Dessa fynd visar att reglering genom CR1/2 och FcγRIIb är viktig för att undgå aktivering av självreaktiva B-celler, vilka i sin tur skulle kunna aktivera självreaktiva T-celler. I nästa delstudie lyckades vi identifiera en liten population B-celler i lymfkörtlarna som är nästan identisk med MZ B-celler i mjälten. Dessa B-celler ligger i områden i lymfkörtlarna som liknar mjältens marginalzon, de har ett mycket snarlikt receptoruttryck på cellytan samt uppvisar liknande funktion som MZ B-celler i mjälten. Detta innebär att MZ B-celler i lymfkörtlarna skulle kunna fylla samma funktion som konventionella MZ B-celler i mjälten och att de på samma sätt kan bidra till att bryta självtoleransen mot CII. I inflammerade leder hos både RA-patienter och möss med CIA ses ansamling av inflammatoriska celler, bl.a. B-celler och mastceller. Mastceller är en annan typ av immuncell som bl.a. är kända för att kunna inverka på andra celltyper. I den sista delstudien undersökte vi därför hur B-celler påverkas av att odlas tillsammans med mastceller. Vi såg att mastcel-



ler hade förmågan att aktivera B-celler så att de delar på sig, producerar antikroppar samt förbereder sig för att kunna aktivera T-celler. Dessa fynd tyder på att mastceller kan bidra till ledinflammationen i RA och CIA genom att direkt aktivera och underhålla självreaktiva B-celler lokalt i leden.

Sammantaget visar jag i min avhandling att MZ B-celler, både i mjälten och lymfkörtlarna, kan aktivera självreaktiva T-celler och därmed bryta den immunologiska toleransen mot CII. De regulatoriska receptorerna CR1/2 och Fc $\gamma$ RIIb är viktiga för att hålla självreaktiva B-celler i schack och därmed förhindra att självtoleransen bryts. Slutligen har jag också visat att en viktig roll för mastceller i autoimmun artrit kan vara deras förmåga att aktivera B-celler och därigenom bidra till ökad T-cellsaktivering och antikroppsproduktion. Mina fynd belyser vilken roll de olika B-cellstyperna har i den initiala sjukdomsfasen i CIA och hur de kan regleras. Målet med min forskning är att öka förståelsen om B celler och hur de bidrar till autoimmun artrit.

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