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Exploring innate type B cells in an animal model for autoimmune arthritis

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Abstract
B cells have a central role in the pathogenesis of collagen-induced arthritis (CIA), an animal model of the autoimmune disease rheumatoid arthritis. In this report, a specific subset of an innate type of B cells, B-1 B cells, have been studied for the involvement in CIA. The B-1 B cells were shown to produce small amounts of collagen-specific antibodies upon stimulation in vitro, suggesting that they play a minor role in the development of CIA. This report also includes how marginal zone B cells, another innate type of B cells with natural collagen-reactivity, can be identified in the medullary sinuses of lymph nodes of collagen-immunized mice, implying involvement in auto antigen trapping.

Keywords
Rheumatoid arthritis, collagen-induced arthritis, B-1 B cells, MZB, MZBL, antibodies, polyreactive antibodies, collagen-specific antibodies, MACS, FACS, ELISA, ELISPOT, IHC.

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Exploring innate type of B cells in an animal model of collagen-induced arthritis

Maya Salomonsson

Populärvetenskaplig sammanfattning

Reumatoid artrit (RA) är en reumatisk autoimmunsjukdom som orsakar ledinflammation och som orsakas av att kroppens immunförsvar börjar attackera kroppsegen vävnad. Tidigare forskning har visat att immunförsvarets antikroppsproducerande B-celler spelar en viktig roll vid utvecklingen av sjukdomen.

Detta arbete beskriver hur man med hjälp av en djurmodell för RA, kollagen-inducerad artrit (KIA) i möss, kan undersöka om en viss typ av B-celler är involverad i utvecklingen av sjukdomen. Tidigare har man visat att en specifik population av B-celler, marginalzons B-celler, är naturligt självreaktiva mot kollagen och bidrar till det autoimmuna svaret mot kollagen i KIA. De B-celler som undersökts i detta arbete för möjlig roll i KIA är B-1 B-celler som är en del av det medfödda immunförsvaret och som producerar polyreaktiva antikroppar.

Analys av B-1 B-celler och deras antikroppsproduktion visade på en låg produktion av kollagenspecifika antikroppar vid *in vitro* stimulering av B-1 B-celler isolerade från mjälten från möss immuniserade för KIA. Dessa resultat tillsammans med tidigare resultat från gruppen, tyder på att marginalzons B-celler är den huvudsakliga producenten av kollagenreaktiva antikroppar i KIA och inte B-1 B-celler.

Arbetet inkluderar även en studie över hur marginalzons liknade B-celler med förmåga att presentera kollagen för T-celler är lokaliserade i lymfnoder. Detta undersöktes med hjälp av en immunohistokemisk analys som visade att cellerna finns lokaliserade i lymfnodens kapsel samt kring blod- och lymfkärl.

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Table of contents

Abbreviations

ACK	Ammonium chloride-potassium
AFC	Antibody forming cells
BCII	Bovine collagen type II
BCR	B cell receptor
BSA	Bovine albumin serum
CFA	Freund's complete adjuvant
CIA	Collagen-induced arthritis
CII	Collagen type II
CpG	Cytosine-phosphate-guanine
CPM	Counts per minute
DAB	3,3'-diaminobenzidine
dpi	days post immunization
ELISA	Enzyme-Linked Immunosorbent Assay
ELISPOT	Enzyme-Linked Immunosorbent Spot assay
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FOB	Follicular B cells
Ig	Immunoglobulin
IHC	Immunohistochemistry
ITIM	Immunoreceptor tyrosine-based inhibitor motif
LPS	Lipopolysaccharide
MACS	Magnetic-activated cell sorting
MZB	Marginal zone B cells
MZBL	Marginal zone like B cells
n.d	not determined
OD	Optic density
PBS	Phosphate buffered saline
RA	Rheumatoid arthritis
SEM	Standard error of mean
TBS	Tris-buffered saline
TLR	Toll like receptor
WT	Wilde type

1. Aim

The aim of this project has been to elucidate if innate type of B cells, specifically B-1 B cells, are important for the development of collagen-induced arthritis (CIA), an animal model for rheumatoid arthritis. Previous studies have shown that mice deficient in B cells or in immunoglobulin isotype M production are resistant to CIA [1-2] and therefore it is of interest to understand if particular innate type of B cells, which are major producers of IgM, are involved in the development of CIA. We were also interested in the inhibitory Fc receptor on B cells, FcγRIIb, and if it was involved in the regulation of B-1 B cells.

A second part of my project was to identify another innate type of B cells in the lymph nodes of mice immunized for CIA. Thus, a marginal zone B cell like subset with self-reactivity has previously been shown to expand in CIA, and my task was to identify their localization in the lymph nodes to understand their interacting environment.

2. Background

2.1 B-lymphocytes

B-lymphocytes are a type of lymphocytes in the immune system that produces antibodies (immunoglobulins). They can either be part of the innate immune system, which is the initial unspecific response, or part of the specific adaptive immune system. B cells in the adaptive immune system produce antigen-specific antibodies with the help from T-lymphocytes, whereas innate B cells produce polyreactive antibodies (towards more than one antigen), which also often are self-reactive.

The B cells can further be grouped into B-1 or B-2 B cells. The B-2 B cell subset can further be divided in to marginal zone B cells (MZB) and follicular B cells (FOB). Murine B-2 B cells are localized in the spleen, lymph nodes and recirculation in the blood. The B-1 B cells are also localized in the spleen, but are also present in the peritoneal cavity, in which they are in majority of all B cells [3-4]. A subset of B-2 B cell have recently been identified in the lymph nodes of mice and are referred to as marginal zone B cell like cells (MZBL) since they are very similar to MZB in the spleen [5]. MZB and B-1 B cells belong to the innate type of B cells.

The different B cell subsets differ in their function and are characterized by their expression of surface receptors. All B cells are B220 and CD19 positive, and the different B-1 and B-2 subsets are defined as $CD43^{+}$ or $CD43^{-}$, respectively [6-7]. MZB are characterized as $CD1d^{high}$ whereas FOB are $CD1d^{lo}$ [6, 8-10]. B-1 B cells and MZB are predominantly IgM^{high}. MZBL express similar phenotype as MZB [9].

2.1.1 Immunoglobulins and FcγRIIb

Immunoglobulins (Ig) can be divided into different isotypes; IgM, IgD, IgG, IgA and IgE. The most common Ig produced by B-1 B cells and MZB is IgM, which is a class of antibody often involved in the primary immune response. IgM has a pentameric shape where five monomeric subunits (figure 1 A) are held together with disulfide-bonds and a J-chain with their Fc regions pointing to the centre of the pentamer (figure 1 B) [11]. Antibodies of the IgM class are often polyreactive and can bind to more than one antigen at the time due to its pentameric shape [11].

IgG is the class of antibodies most abundant in serum and it is produced by B cells that have isotype switched, usually with the help of T-cells. The IgG antibodies are predominantly specific towards one specific antigen.

To recognize a specific antigen the B cell has a B cell receptor (BCR), which consists of Ig protein. The BCR signals through BCR accessory chains that contain immunoreceptor tyrosine-based activating motifs (ITAMs), and transduce the signal to activate the B cell. B cells also express inhibitory receptors that contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs), such as the Fc receptor for IgG class IIb (FcγRIIb). When IgG immune complexes bind to FcγRIIb it will down regulate the B cell response [12]. If the B cells lack this receptor they will fail to dampen the immune response and the B cell response will become stronger [13].

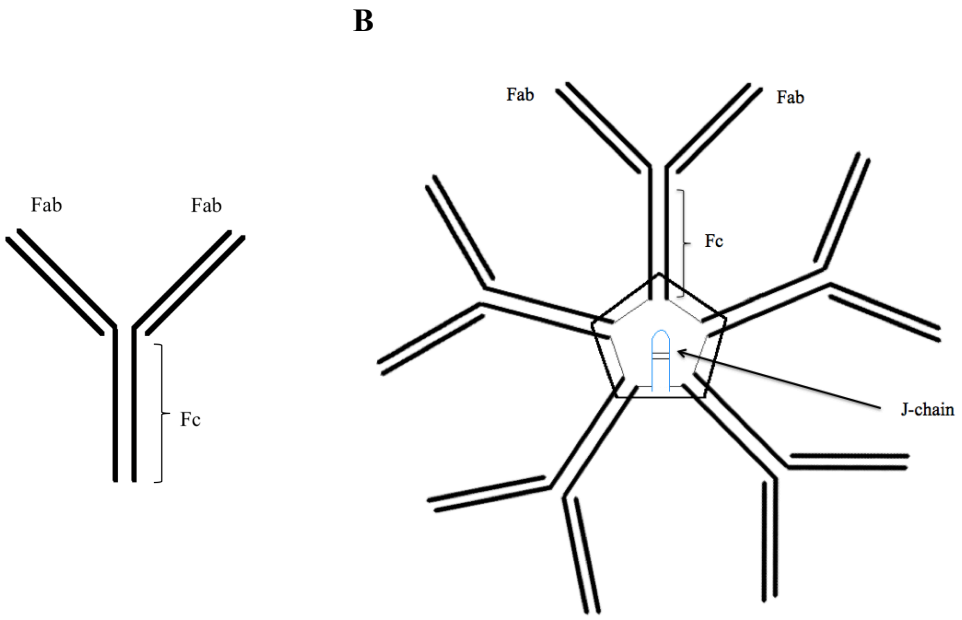

Figure 1. (A) Structure of an antibody (immunoglobulin), typically IgG. (B) IgM consists of five monomeric subunits with the Fc regions pointing into the centre of the pentameric shape. The J-chain holds the immunoglobulin subunits together. Fab is the site where the antibody binds the antigen and Fc is the part of the immunoglobulin that exerts the biological activity (by binding to Fc receptors or activating complement).

2.1.2 Toll like receptors and stimulation of B cells

All B cells have toll like receptors (TLRs) that are expressed on their cell surface or embedded in the cytoplasmic vesicles. The TLRs promote an immune response in the B cell, which starts to produce antibodies when stimulated. TLRs are highly conserved receptors and there are 9 different types in mice; TLR 1-9. They recognize conserved motifs on different pathogens, so called pathogen-associated molecular patterns (PAMPs), either on their own or by working together. TLR9 is a receptor that activates the immune response when interacting with unmethylated cytosine-phosphate-guanine (CpG) motifs that are common among pathogens such as viruses and bacteria. A synthetic version of CpG is a commonly used mitogen to stimulate B cell proliferation and has previously been shown to stimulate peritoneal B-1 B cells in vitro [14].

2.2 Rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune disease in humans that causes an autoimmune reaction towards the joints [15].The disease is characterized by rheumatoid factors, auto-antibodies which are specific towards the Fc portion of IgG, and inflammation of peripheral joints [16]. It is established that 15-21 % [17-18] of the risk of developing the disease is due to genetic factors and the main environmental risk is smoking. It is also known that women and elderly people have a higher risk for developing RA [15]. The illness is relatively common with 5-50 new cases per 100 000 every year [10]. It is recognized that B cells and their production of self-reactive antibodies play a major role in the development of the RA and that B-cell depleting therapy is an efficient method of treating the disease [19]. Therefore, it is of interest to study if particular B cell subsets contribute to the disease.

2.3 Collagen-induced arthritis

CIA is a commonly used animal model for RA [20]. The autoimmune response is induced by immunizing susceptible mice with bovine collagen (BCII) emulsified in Freund's complete adjuvant (CFA). This causes an immune response to the BCII, which eventually leads to an autoimmune response to endogenous collagen in the cartilage of the joints [20]. The advantage of this model is that the time from immunization to outbreak of the disease is relatively short, and the model is widely used among researchers to determine which type of cell is responsible for the disease [21]. Both MZB and MZBL have previously been shown to respond to collagen type II (CII) in the CIA model [6, 18].

2.4 Immunoassays

Immunoassays are different methods to detect specific proteins using specific antibodies. These methods are depending on the specificity between the protein and the antibodies as well as the specificity of the detection system. One of the most common immunoassays is the enzyme-linked immunosorbent assay (ELISA) which uses an antibody-conjugated enzyme that reacts with a chromogenic substrate and changes the colour of the sample [22] as shown in figure 2. This can be detected as a measurement of optic density (OD). The enzyme-linked immunosorbent spot assay (ELISPOT) is another immunoassay that uses the same principle to detect antibody forming cells as spots that can be counted in a microscope [19, 20]. Immunohistochemistry (IHC) is a method to detect proteins and antibodies in tissue sections. IHC uses the same principal with antibody-conjugated enzyme that reacts with a chromogenic substrate for detection. In addition, by using antibodies conjugated to biotin, which have four binding sites for avidin, (that is conjugated to an enzyme) this system can be used to amplify the IHC signal. Common enzymes used in all of these methods are horseradish peroxide and alkaline phosphatase [19, 20].

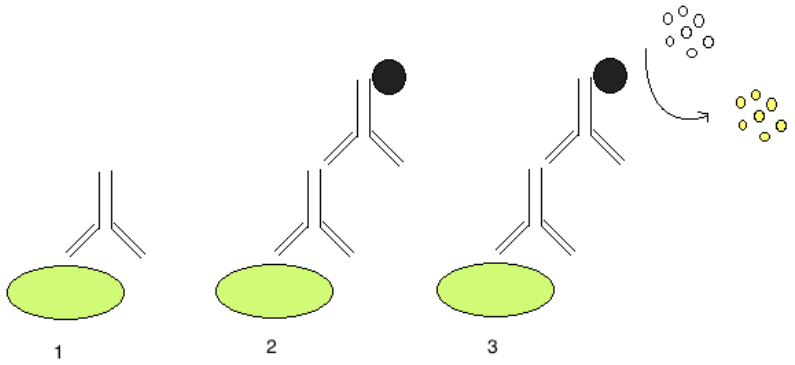

Figure 2. The principal behind an immunoassay. (1) Binding of primary antibodies to protein of interest. (2) Binding of secondary enzyme-conjugated antibody. (3) Addition of a chromogenic substrate, which change colour when cleaved by the enzyme conjugated to the secondary antibody. Depending on choice of immunoassay method, different detection methods are used for collecting the emitted signal.

3. Material and methods

3.1 Overview of experimental procedure for analysis of B-1 B cells

An overview of the different steps in the experimental procedure is shown in figure 3.

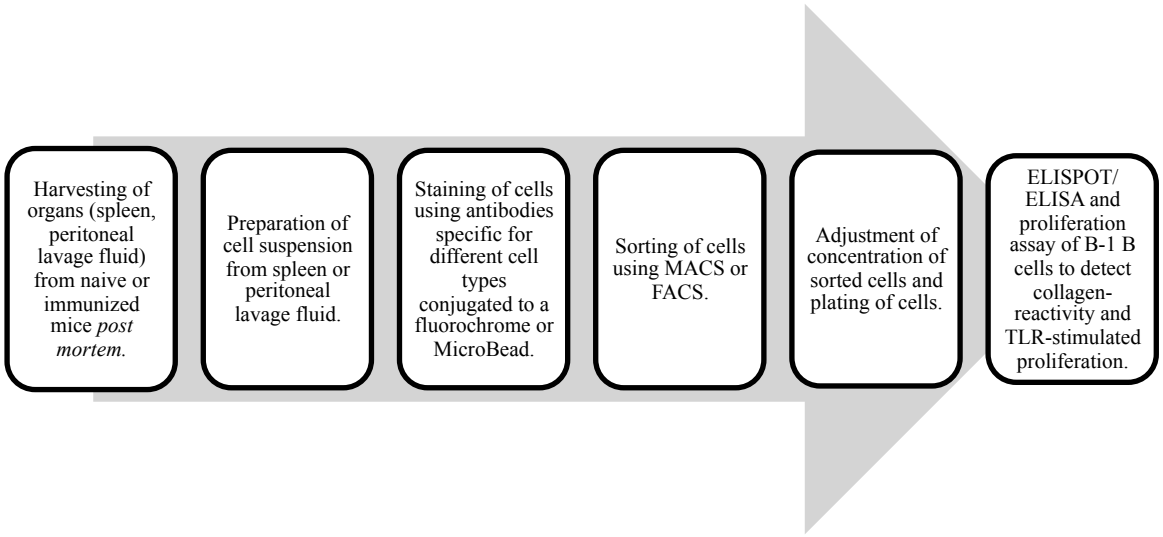

Figure 3. Overview of experimental procedure for detection of BCII-reactivity by B-1 B cells. Each step in the experimental setup is briefly described.

3.2 Mice

Mice used for the experiments were all on DBA/1 background and were originally obtained from Bommice, Bomholt Gaard Ltd (Ry, Denmark). The FcγRIIb-deficient mice [23] had been backcrossed to the DBA/1 background and expanded using interbreeding as described before [12]. The mice used in the experiments were 10-17 weeks old and were of both sexes. The mice were fed rodent chow and had constant access to food and water. All animals were bred and maintained at the animal facilities at the Biomedical Centre, Uppsala University in Uppsala. All experiments were approved under the ethical permit obtained by the group.

3.3 Immunization

BCII was dissolved in acetic acid at a concentration of 2 mg/ml and emulsified with CFA (Difco, Detroit, MI, USA) at a final concentration of 1 mg BCII per ml. The mice were immunized with an intradermal injection of 50 µl of the emulsion at the tail base for a final dose of 50 µg BCII [9].

3.4 Preparation of single cell suspensions

For all of the following cell preparations centrifugation (centrifuge 5702R; eppendorf AG, Hamburg, Germany) was carried out at 200 g at 4°C for 6 minutes after each washing.

3.4.1 Preparation of splenocytes

The spleen was removed from the mice *post mortem* by the PhD student of the group and stored in ice-cold phosphate-buffered saline (PBS) (137 mM NaCl (Sigma-Aldrich, Saint Louis, Missouri, USA), 2.68 mM KCl (Sigma-Aldrich), 8.09 mM $Na_2HPO_4 \bullet 2H_2O$ (Sigma-Aldrich), 1.47 mM KH_2PO_4 (Sigma-Aldrich), pH 7.4) until passed through an aluminium mesh and washed with PBS. Lysis of red blood cells was done with ammonium-chloride-potassium- (ACK) buffer (0.15 M NH_4Cl (Merck KGaA, Darmstadt, Germany), 0.1 mM EDTA (Merck KGaA) and 1.0 M $KHCO_3$ (Sigma-Aldrich)), and neutralization was done with addition of an equal amount of PBS. After being washed, the cells were suspended in magnetic-activated cell sorting- (MACS) buffer (0.5% bovine albumin serum (BSA, Merck KGaA) and 2 mM EDTA (Merck KGaA) in PBS) or fluorescence-activated cell sorting (FACS) buffer (1% BSA in PBS) depending on choice of sorting method. To count the cells they were diluted in trypan blue (Gibco Island, NY, USA) and applied on Bürker chambers to enable counting in a light microscope.

3.4.2 Preparation of peritoneal cells

Cells from peritoneal cavity where collected from the mice *post mortem* by rinsing the peritoneal cavity with Hank's balanced salt solution (HBSS, 0.056 M glucose, 4.4 mM KH_2PO_4 (Sigma-Aldrich), 0.01 M Na_2HPO_4 (Sigma-Aldrich), 0.013 M $CaCl_2 \bullet 2H_2O$, 0.053 M KCl (Sigma-Aldrich), 1.37 M NaCl (Sigma-Aldrich), 9.84 mM $MgCl_2 \bullet 6H_2O$ (Merck KGaA), 8.11 mM $MgSO_4 \bullet 7H_2O$ (Riedel-de Haën, Seelze, Germany)) with addition of 0.0002 % heparin (LEO Pharam A/S, Ballerup, Denmark). The peritoneal cells were diluted in MACS buffer for MACS sorting of B cells or diluted in 10 % fetal calf serum (FCS) complete DMEM (DMEM (National Veterinary Institute, Uppsala, Sweden) supplemented with 100 U/ml penicillin (Sigma), 100 µg/ml streptomycin (Sigma), 2 mM glutamine (Sigma) and 50 µM β-mercaptoethanol) for whole peritoneal cell suspension and spun down before counted in Bürker chambers as described above.

3.5 Sorting of cells

Sorting of cells was done using either MACS or FACS.

3.5.1 MACS

Cells from the spleen and the peritoneal cavity were prepared as described above and suspended in MACS buffer at a concentration of 10^7 cells per 90 µl buffer. Splenocytes were incubated with 10 µl of anti-CD43 MicroBeads (Miltenyi Biotec, Bergisch, Gladbach, Germany) per $2x10^7$ cells and peritoneal cells were incubated with 2 µg anti-CD19-PE (clone 1D3; BD Pharmigen™, San Jose, CA, USA) per $2x10^7$ cells on ice for 30 min. For the peritoneal cells an additional step was preformed where antibodies that did not bind were washed away using MACS buffer. The cells were thereafter spun down and the pellet was resuspended in MACS buffer at a concentration of 80 µl per 10^7 cells and incubated with 20 µl anti-PE MicroBeads (Miltenyi Biotec) per 10^7 cells on ice for 30 min. Both splenocytes and peritoneal cells were washed with MACS buffer and centrifuged before the cell pellet was suspended in 500 µl of MACS buffer. The cells were applied on an LS column (Miltenyi Biotec) attached to a magnet, allowing cells without a micro bead attached to pass through the column. The negative fraction was collected by washing of the column with 3x3 ml MACS buffer. The positive fraction (bound to the LS column through the micro bead conjugated anti-CD43 or anti-CD19 antibody) was collected by removing the column

from the magnet, adding 3 ml MACS buffer and using a plunger to apply pressure on the sample to eluate the fraction out of the column.

After the sorting the cells where suspended in complete DMEM medium with 10 % FCS.

3.5.2 FACS

Cells from the spleen were prepared as described above. Staining to distinguishing between B-1 and B-2 B cells was done in two steps: first anti-mouse-CD43-biotin (clone eBioR2/60; eBioscience, San Diego, CA, USA), followed by fluorophore-conjugated streptavidin (Biolegend, San Diego, CA, USA) along with fluorophore-conjugated anti-B220 antibody (clone RA3-6B2; Biolegend). Anti-CD43-biotin was used at a concentration of 0.5µg/1x10^7 cells and the cells where incubated on ice for 30 min. Excess antibodies were washed away twice with FACS-buffer (with centrifugation in between) before adding 1µg of streptavidin and 1µg of anti-B220 antibody per 1x10^7 cells. The cells were incubated on ice for 30 min and washed once before sorted using a BD FACSAriaIII and FACSDiva software v.5.1 (BD Biosciences, San Jose, CA, USA). The FACS sorting was performed by the PhD student of the group. Viability of the sorted cells was analysed using trypan blue and Bürker chambers. The cells were plated on a CII pre-coated 96-well MaxiSorp plate (NuncBrand Thermo Fischer Scientific, Roskilde, Denmark) at a concentration of 2.5x10^5 B-1 or B-2 B cells per well. For positive control B-2 B cells and un-sorted splenocytes were plated at a final concentration of 1x10^6 cells/well.

3.6 Purity analysis of sorted cells

Purity check of the MACS- or FACS-sorted cells was done using flow cytometry. Cells sorted by MACS were stained with fluorophore-conjugated anti-B220, anti-CD43-biotin and fluorophore-conjugated streptavidin as described above. Cells that were sorted with FACS was analysed using the same surface markers and gating as in the sorting step. Gating strategy and analysis was done using FACSDiva software v.5.1 (BD Biosciences) by the PhD student of the group.

3.7 Stimulation of sorted B cells and proliferation assay

Stimulation of B cells to induce proliferation and antibody secretion was done with CpG-B DNA oligodeoxyribonucleotide 2006 (CpG; Hycult Uden, The Netherlands). B-1 B cells were isolated from splenocytes by FACS sorting as described above with the addition of an anti-CD1d antibody (clone CD1.1, Ly-38; Biolegend) at a concentration of 1µg/1x10^7 cells to also distinguish and sort out CD1d positive MZB from the splenocytes. After sorting the B-1 B cells and MZB, the cells were diluted to a final concentration of 1x10^6 cells/ml in complete DMEM medium with 10% FCS in a volume of 100 µl/well. To each well 100 µl of complete DMEM medium with 10 % FCS and 3µg/ml of CpG was added. The plate was incubated at 5 % CO_2, 37°C for 44 h. After 44 h 100 µl supernatant was collected from each well (supernatant from triplicate wells were pooled) and stored at -20°C until analysis of BCII-specific antibodies with ELISA. For the proliferation assay 100 µl of 10 % FCS in complete DMEM was added together with ^{3}H-thymidine (Perkin Elmer, Waltham, MA, USA) at 1µCi/well and left to incubate at 5 % CO_2, 37°C for 18-22 h. The cells were rinsed and trapped on filter papers and the radioactivity of the filters were analysed in a liquid scintillation β-counter (Wallac 1450 MicroBeta Trilux, Perkin Elmer). Proliferation was measured as average of β decay per minute for each well.

3.8 ELISA

ELISA is a method used to detect soluble proteins in serum or cell supernatants. Coating of a 96-well MaxiSorp plate (NuncBrand Thermo Fischer Scientific) was done with BCII diluted in PBS at a concentration of 5 µg/well in a total volume of 50 µl/well and incubated at 4°C overnight. The protein that did not bind was washed away using PBS-Tween (0.05% Tween) (Sigma-Aldrich). Blocking was done with 1 % BSA in PBS in a total volume of 200 µl/well for 1 h. The blocking was washed away before applying the supernatants from sorted B-1 B cells and MZB or a positive control serum from a mouse 12 days post-immunization (diluted 1:25 or 1:125) and incubated at room temperature for 2 h. Samples were then washed away using PBS-Tween. Fifty µl/well of alkaline phosphatase conjugated goat-anti mouse IgM (Sigma-Aldrich) at 0.06 µg/well was added and the plate was incubated in room temperature for 2 h. Excess anti-IgM was washed away using PBS-Tween followed by a rinse in PBS, and the plate was subsequently developed with ρ -nitrophenylphosphate (Sigma-Aldrich) diluted in diethanoleamine buffer (1 M Diethanolamine 1 mM $MgCl_2 \bullet 6H_2O$, 1mg/ml). The plate was read with a spectrophotometer (VersaMax, Molecular devices) when the control serum at 1:25 had reached an OD_{405}=2.4 (approximately after 90-120 min).

3.9 ELISPOT

To determine if the B-1 B cells included BCII-reactive clones, the ELISPOT method was used.

3.9.1 Optimization of the ELISPOT method

To evaluate optimal incubation times for detection of spots, but without too high background an incubation time study was done. Three different protocols for ELISPOT were studied. One with incubation of cells at 5% CO_2, 37°C for 3 h and incubation of antibody overnight in 4°C, another with incubation of cells at 5% CO_2, 37°C overnight and incubation of antibody for 2 h in room temperature, and a third protocol with incubation of cells at 5% CO_2, 37°C overnight and incubation of antibody overnight at 4°C. Positive antibody forming cells per $1x10^6$ cells for BCII and BSA (negative control) was evaluated. Apart from the different incubation times the plates were treated the same throughout the ELISPOT protocol.

3.9.2 Optimized ELISPOT protocol

After optimization, a final ELISPOT protocol was established. Coating of a 96-well MaxiSorp plate (NuncBrand Thermo Fischer Scientific) was done with BCII or BSA (negative control) at a concentration of 10 µg/ well, in a total volume of 50 µl/well and incubated at 4°C over night. The protein that did not bind was washed away using PBS. The cells were loaded on the plate in complete DMEM medium with 10 % FCS at a final concentration of $1x10^6$ cells/ well in a total volume of 200 µl/well and incubate at 5 % CO_2, 37°C, for 3 h. The cells were washed away using 0.05% PBS-Tween. Alkaline phosphatase-conjugated anti-IgM, (Sigma-Aldrich) was added at 0.6µg/well in a total volume of 50 µl/well. The plate was incubated at 4°C overnight and excess anti-IgM was washed away using PBS-Tween and PBS the next day. Antibody spots were developed using BCIP/NTB Liquid Substrate System (Sigma-Aldrich) and 50 µl was added to each well and incubated in the dark for 60 min. Spots, distinguishing antibody forming cells, were manually counted using an inverted microscope.

3.10 Immunohistochemistry

To determine where in the lymph node MZBL are localized, the IHC method, analysed with light microscope, was used.

3.10.1 Optimization of IHC method

First I started to optimize an IHC protocol for double- and single-staining of surface markers on MZBL on frozen tissue sections of lymph nodes. I evaluated different substrates, buffers, antibody concentrations, thickness of tissue sections and incubations times for the different antibodies and substrates (table 1, 2).

Table 1. Different parameters for substrates evaluated to optimize an IHC protocol for localization of MZBL.

	Incubation time					Concentration			Buffer	
	3 min	**5 min**	**6 min**	**7 min**	**15 min**	**1:1***	**1:2***	**2:1***	**TBS**	**PBS**
DAB	x	-	+	-	-	+	x	x	DS	SS
BCIP/NTB	x	+	-	-	-	+	-	-	+	-
Fast Red	+	x	x	x	x	+	x	x	+	-

+ = Optimal signal
- = Non optimal signal
DS= Double-staining, SS= Single-staining.
*= Start concentrations, DAB=0.7 mg/ml; BCIP=0.15 mg/ml; Fast Red=1 mg/ml.
x = Not determined

Table 2. Different concentrations of antibodies (µg/section) and thickness of tissue sections evaluated for optimization of an IHC staining protocol for localization of MZBL.

7 µm		10 µm	
IgM	**CD1d**	**IgM**	**CD1d**
0.1*	0.1	0.1	0.1
0.167	0.167*	0.167*	0.167
0.5	0.5	0.5	0.5*

* = Optimal concentration of antibody.

3.10.2 Protocol

Following the optimization study, a final IHC protocol was established. Lymph nodes from mice 5-10 days post immunization (dpi) were snap frozen in liquid nitrogen and cut into 7-10 µm thick sections in a cryostat, and put on objective glass. The sections were thereafter left to dry in room temperature and stored in the freezer until fixated in 50 % acetone for 30 seconds and 100 % acetone for 5 min, and then again left to air dry for approximately 15 min. A liquid blocker pen (Super pap pen) was used to draw a circle around the section to prevent the antibodies on the same glass to mix with each other. Rehydration of tissue was done in two steps: first the sections were covered with buffer (PBS or tris-buffered saline (TBS) (0.15 M NaCl (Sigma-Aldrich), 0.05 M Tris base (Electran))) for 5 min followed by dipping the whole glass in a cuvette with buffer for 5 min. Choice of buffer was made depending on substrate; TBS was used for all sections with

alkaline phosphatase (Sigma-Aldrich) as enzyme, including double-staining sections, whereas PBS was used on sections single-stained with horseradish peroxidase as enzyme (Sigma-Aldrich). After rehydration quenching of endogenous peroxidase was done by covering sections with peroxide for 5 min. After that the peroxide was washed away, application of blocking buffer (PBS or TBS with 5 % goat serum) was added to block the sections for 30 min. After the sections had been blocked for 30 min the blocking-buffer was washed off before application of primary antibody. The primary antibody, biotinylated anti-CD1d (CD1.1 Ly-38, BD Pharmigen™), was diluted in blocking buffer, in a final concentration of 0.167 µg/section and left on the section for 1 h in room temperature. Washing of slides was done by covering the sections with buffer twice. After that, avidin-conjugated horseradish peroxidase diluted 1:100 (according to the manufacturer the stock concentration was 2-2.5 mg/ml) was added and the sections were left to incubate in room temperature in the dark for 1 h. Washing of abundant antibody was done with buffer before developing the staining with 3,3'-diaminobenzidine substrate tablet (DAB, 2 tablets (D4293), dissolved in 5 ml dH_2O, Sigma-Aldrich) for 6 min and then rinsed with tap water for 3 min.

For double-staining the sections were incubated with biotinylated anti-IgM (clone R6-60.2, BD Pharmigen™) diluted in blocking buffer at a concentration of 0.1 µg/section for 1 h at room temperature. The slides were washed with TBS to discard abundant antibodies. Next avidin-conjugated alkaline phosphatase diluted to 3 µg/section in blocking buffer was added and left to incubate in room temperature in the dark for 1 h. The staining was developed with Fast Red tablets (2 tablets (F4648), dissolved in 1 ml dH_2O, Sigma-Aldrich) for 3 min and additionally with tap water for 1 min. Counterstaining of cell nuclei for both single- and double-stained sections was done using hematoxylin (Mayers HTX, Histolab, Göteborg, Sweden) for 1.5 min and development of the colour was done with tap water for 5-10 min. Slides were mounted using Immu-mount (Shadon, Pittsburgh, PA, USA) and dried in the dark at room temperature before analysed using Olympus BX60 microscope light microscope at 10x, 40x and 100x magnification.

Isotype control slides were done according to the staining protocol mentioned above as single stainings. In short, anti-IgG2a-biotin (RTK2758, Biolegend) was used as isotype control for anti-IgM at a concentration of 0.1 µg/section and developed with avidin-conjugated alkaline phosphatase and Fast Red. IgG2b (A95-1, BD Pharmigen™) was used as isotype control for anti-CD1d at a concentration of 0.167 µg/section followed by biotinylated anti-rat-immunoglobulin (E0468 Dako, Glostrup, Denmark) at a concentration of 0.25 µg/section and finally developed with avidin-conjugated horseradish peroxidase and DAB.

3.11 Statistical analysis

For statistical analysis of data Prism 6.0 from GraphPad Software, Inc. (La Jolla, CA, USA) was used. Where two groups were compared to each other the statistical difference was determined with paired Student's *t*-test. For comparison between more than two groups, a paired One-way ANOVA method was used to determine statistical difference. All data in the graphs are presented as mean +SEM. P-values <0.05 were considered significant.

4. Results

4.1 Optimization of the ELISPOT protocol for BCII reactivity

To evaluate if there were any CII-reactive clones of B-1 B cells in the peritoneal lavage fluid or in the spleen the ELISPOT method was used. To determine which conditions were the most optimal for B-1 B cells an incubation time study was performed. The study showed that the optimal incubation times to get a positive signal (but as little background as possible) was 3 h of incubation of the cells at 37°C, and overnight incubation of the detection antibody at 4°C (Figure 4).

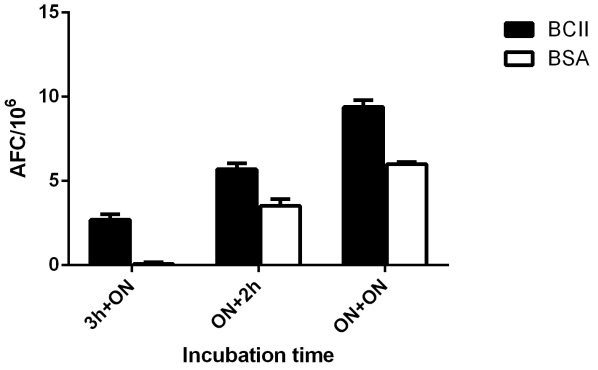

Figure 4. Incubation study for optimization of the ELISPOT protocol. Number of anti-BCII and anti-BSA (background control) positive spots (antibody-forming cells; AFC) per $1x10^6$ splenocytes detected with ELISPOT using three different protocols with combinations of different incubation times for cell cultivation (3h or overnight) and detection antibody (2h or overnight). All cells came from wild type (WT) DBA/1 mice (n=4) 10 dpi. ON= over night. Data presented as mean +SEM.

4.2 Antibody forming cells with BCII reactivity

To determine if there were any BCII reactive cells in the peritoneal cavity of WT DBA/1 mice 5 or 10 dpi the peritoneal lavage fluid was prepared and used directly, or was MACS-sorted on CD19 positivity to collect B cells. The cells were further analysed using the ELISPOT method, detecting B cells producing IgM anti-BCII (antibody-forming cells). Peritoneal cells from FcγRIIb-deficient mice were also analysed using the same sorting and analysing method. There was no positive signal detected in any of the peritoneal lavage fluid cells, either analysed as non-sorted cells or as CD19-sorted B cells, from BCII-immunized WT or FcγRIIb-deficient mice (table 3).

Table 3. Anti-BCII antibody forming cells in peritoneal lavage fluid. IgM antibody forming cells in non-sorted or MACS-sorted cell suspensions from peritoneal cavity of BCII-immunized mice.

	5 dpi		**10 dpi**	
	WT	FcγRIIb$^{-/-}$	WT	FcγRIIb$^{-/-}$
Non-sorted cell fraction	n.d	n.d	-	n.d
CD19$^+$ cells	-	-	-	n.d
CD19$^-$ cells (neg. control)	-	-	-	n.d

-= Not detected. (WT, n=1-3) (FcγRIIb$^{-/-}$, n=3)
n.d = Not determined

Next, we investigated the IgM anti-BCII response in the spleen of BCII-immunized mice by analysing the CD43-positive (including B-1 B cells) and the CD43-negative (excluding B-1 B cells) cell fractions. Both fractions showed positive signals with the highest IgM response at five days after BCII-immunization (figure 5 A). When comparing WT and FcγRIIb-deficient mice the FcγRIIb$^{-/-}$ mice showed a tendency towards higher anti-BCII response (figure 5 B). There was an overall tendency towards higher BCII-reactivity in the CD43-negative fraction at the different time points after immunization in both WT and FcγRIIb-deficient mice.

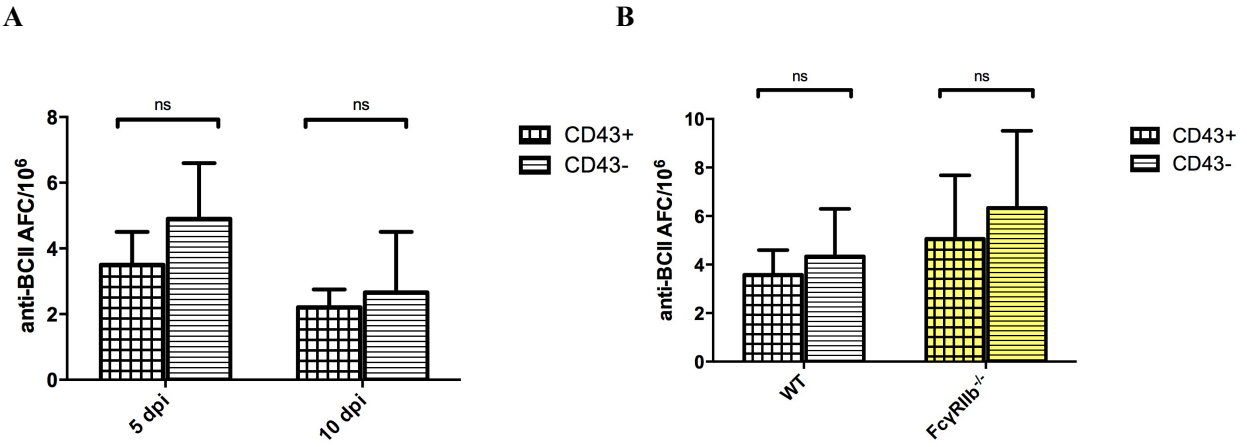

Figure 5. Number of anti-BCII antibody forming cells in the spleen of BCII-immunized mice. The ELISPOT method was used to detect antibody forming cells in MACS-sorted CD43-positive and CD43-negative cell fractions from the spleen of WT mice (n=1-4) at 5 and 10 dpi (A), or from FcγRIIb$^{-/-}$ mice (n=3) at 5 dpi (B). Data presented as mean +SEM and P-values <0.05 were considered significant.

To verify the B-1 B cell content in the CD43-positive fraction (and not in the CD43-negative fraction) purity analysis was done on the different fractions with flow cytometry. This inspection showed that the samples (in figure 5) were not clean enough and that there were both B-1 and B-2 B cells in both the CD43-positive and the CD43-negative cell fraction (table 4), which makes it difficult to determine if the B-1 B cell population gives rise to any of the anti-BCII positive signals (in figure 5).

Table 4. Percentage of B-1 B cells in the CD43-positive and negative cell fractions determined by flow cytometry. Purity check of MACS-sorted splenocytes.

	Frequency of B-1 B cells (%)	
	CD43$^+$ fraction	**CD43$^-$ fraction**
# 1	23.2	3.94
# 2	23.2	2.77

WT (BCII-immunized, 10 dpi); n=3.

To determine which B cell population in the spleen that gave the positive anti-BCII signal in the CD43-positive cell fraction we sorted the splenocytes by FACS instead of MACS. The FACS sorting was done by the PhD student of the group. The sorted B-1 B cells (B220$^+$, CD43$^+$) did not show any reactivity towards BCII but neither did the B-2 B cells (B220$^+$, CD43$^-$) (as shown in table 5), although previous studies have shown that there are CII-reactive clones among B-2 B cells such as the MZB [20].

Table 5. Anti-BCII antibody forming cells in splenocytes from WT mice. ELISPOT was used to detect number anti-BCII antibody forming cells in FACS-sorted single cell suspensions from spleens of BCII-immunized mice.

	5 dpi	10 dpi
$B220^+$, $CD43^+$ cells (B-1 B cells)	-	-
$B220^+$, $CD43^-$ cells (B-2 B cells)	-	-
Splenocytes (positive control)	n.d	3

- = Not detected. (WT, n=1-3)
n.d= Not determined

4.3 Stimulation of B-cells

Since we could not detect any CII-reactivity using the ELISPOT method it was decided that an ELISA method should be tested instead (detecting secreted antibodies specific towards BCII). To increase the concentration of secreted antibodies in culture supernatants the B cells were stimulated. A test ELISA, performed on supernatants from *in vitro*-stimulated MZB, showed a tendency of increased production of specific antibodies when the MZB were stimulated with CpG compared to stimulation with lipopolysaccharide (LPS) (figure 6). In contrast, stimulation of the B cell receptor did not show any increased production of specific antibodies (figure 6). Stimulation of sorted B-1 B cells, MZB or un-sorted peritoneal cells from naive WT DBA/1 mice was therefore done with CpG.

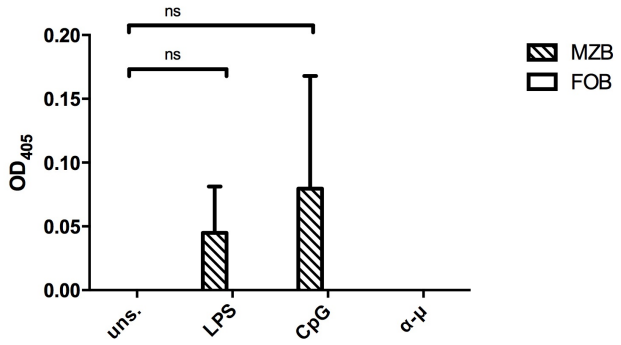

Figure 6. BCII-specific IgM antibodies produced by MZB after different types of stimulation. FACS-sorted MZB were cultivated for 48 h without any stimuli (unstimulated (uns.)) or stimulated with LPS, CpG or anti-BCR antibody (α-μ). Secreted antibodies were analysed by ELISA, n=2. Data are presented as mean +SEM and P-values <0.05 were considered significant.

When B-1 B cells and MZB from naïve and BCII-immunized mice were stimulated with CpG the cells proliferated (figure 7 A) and secreted antibodies (figure 7 B). There was no significant difference between the naïve and the immunized mice (in both the MZB and the B-1 B cell population). However, the MZB proliferated much better than the B-1 B cells and also produced more antibodies to BCII. B-1 B cells did secrete BCII-specific antibodies, but this was only seen in BCII-immunized mice (12 dpi) and not in naïve animals (figure 7 B). Since there previously had been a problem with the purity of B-1 B cells in the CD43-positive and negative cell fractions, a purity check was performed for each experiment. It showed that the CD43-positive samples contained 85.5% - 94.4 % B-1 B cells and the CD43-negative samples contained 82.1% - 94.2% MZB (used as positive control) (table 6) which was a satisfying result.

Table 6. Purity check post CD43 cell sorting using FACS. Determination of amount (%) of different cell types in the different cell fraction post sorting.

	$B220^+$, $CD43^+$		$B220^+$, $CD43^-$, $CD1d^{high}$	
	B-1 B (%)	**B-2 B (%)**	**MZB (%)**	**Other B cells (%)**
# 1	87.7	12	82.1	17.9
# 2	94.4	5.6	85.6	14.4
# 3	85.6	8.3	94.2	5.8
# 4	91.6	4.9	89.6	10.4

WT mice (BCII-immunized12 dpi); n=4.

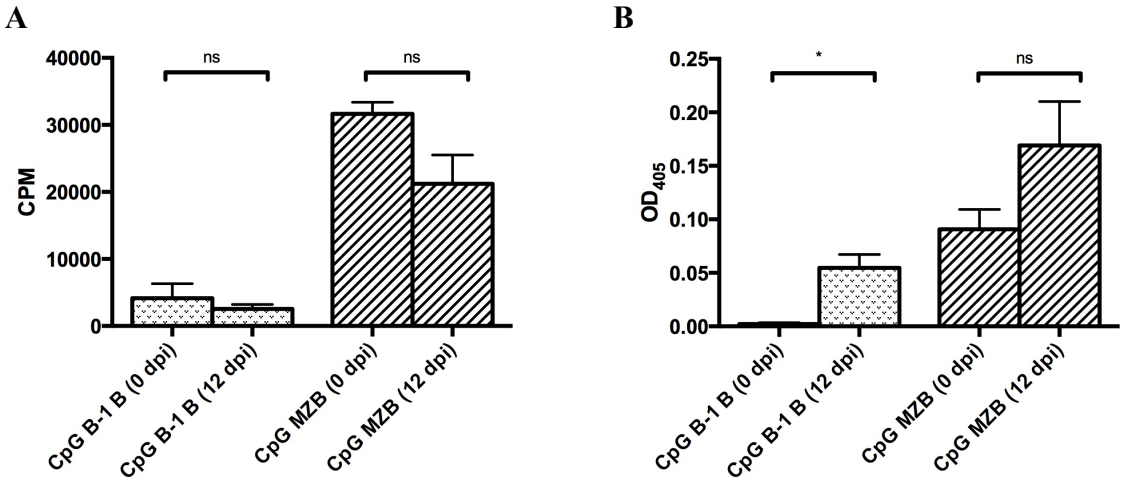

Figure 7. B1-B cells show low proliferation in response to CpG and only B1-B cells from BCII-immunized mice secrete BCII-specific IgM. (A) Proliferation was analysed by ^{3}H-thymidine incorporation and counting the radioactive decay per minute. Measurements were done on FACS-sorted CD43-positive (B-1 B cells) and CD43-negative, CD1d-positive cells (MZB), used as positive control, from the spleen of naïve (n=4) and BCII-immunized (12 dpi) WT DBA/1 mice (n=4). CPM = counts per minute. (B) ELISA measurements were done on FACS-sorted CD43-positive (B-1 B cells) and CD43-negative, CD1d-positive (MZB) cells from the spleen of naïve and BCII-immunized (12 dpi) WT DBA/1 mice (n=4). *= significance. Data are presented as mean +SEM and P-values <0.05 were considered significant.

4.4 Localization of marginal zone like B cells

4.4.1. Optimization of IHC protocol

The localization of MZBL in lymph nodes was determined using IHC as described in material and methods. Since this method has not been used for this specific cell type an optimization of the IHC protocol was necessary. Different parameters for the staining process were evaluated, such as thickness of tissue sections, different buffers, concentration of antibodies, concentration of enzyme-linked antibodies and incubation times. The optimal conditions for this type of staining was 7 µm thick sections, TBS buffer for double-staining, 0.167 µg anti-CD1d and 0.1 µg anti-IgM antibody per section, and 1 h incubation time for all antibodies.

Evaluation of which substrate to combine with the different enzymes for the double staining was also done. Horseradish peroxidase-conjugated antibodies and DAB worked for both anti-IgM and anti-CD1d as shown in figure 8 A, B. Alkaline phosphatase-conjugated antibodies and BCIP/NTB worked for anti-IgM, but gave unspecific staining of blood vessels (figure 8 C). In an

effort to quench the unspecific signal, levamisole (in different concentrations) was added to the substrate solution, but this did not reduce the unspecific staining around blood vessels. Instead another developing agent was tested, Fast Red, which already had levamisole for quenching of endogenous alkaline phosphatase added. This substrate did not give the unspecific staining around blood vessels as shown in figure 8 D. After titration of antibodies for the Fast Red substrate, an optimized protocol was determined as described in material and methods with 7 µm thick sections and substrate combination DAB and Fast Red. For the best result the anti-CD1d antibody should be developed for 6 min with DAB and the anti-IgM antibody with Fast Red for 3 min. To achieve as bright staining as possible a TBS buffer was used.

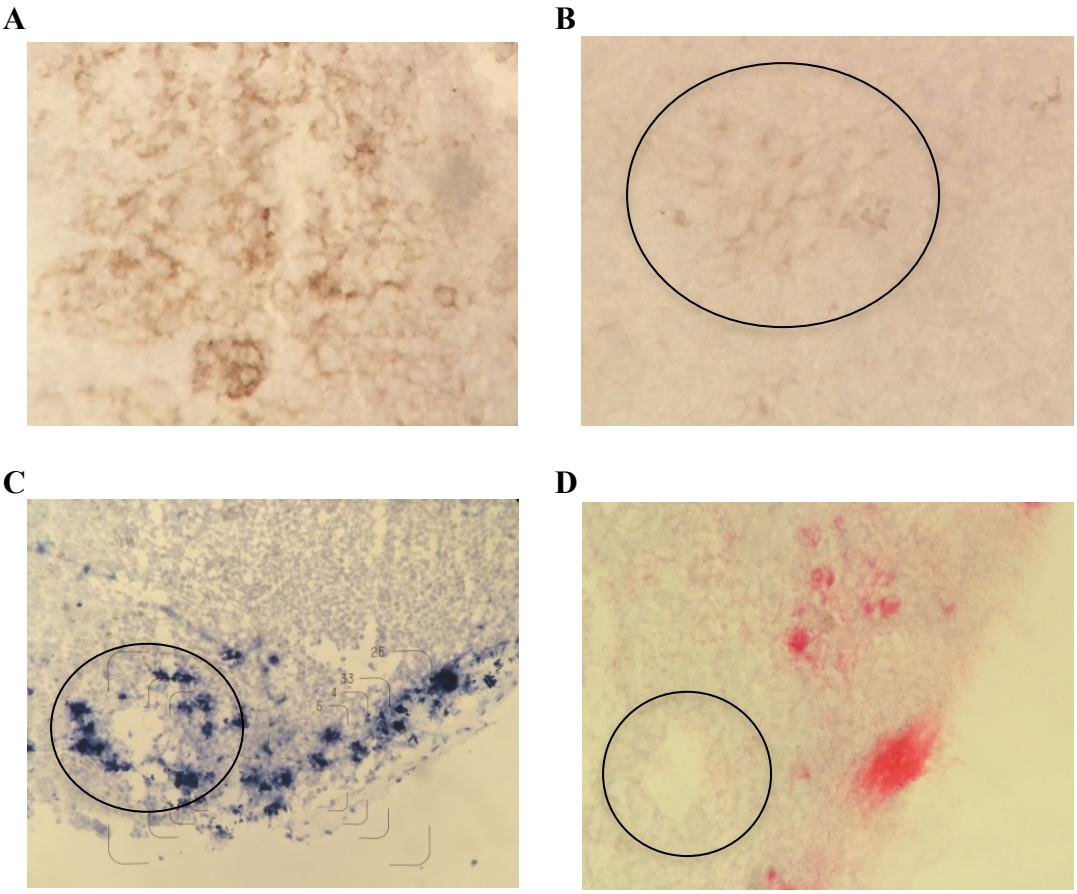

Figure 8. IgM and CD1d staining of lymph nodes from BCII-immunized mice. Single-staining of 10 µm thick sections from lymph nodes from BCII-immunized (10 dpi) WT DBA/1 mice. (A) IgM positive cells (brown) in the middle of the section (40 x magnification). (B) CD1d positive cells (brown) in marked area (40 x magnification). (C) Unspecific staining around blood vessels developed with BCIP/NTB® in the marked area (40 x magnification). (D) IgM positive cells in the sub capsular area without unspecific staining around blood vessel (circled in the picture) (40 x magnification).

4.4.2. Localization of MZBL

Double-staining of cells that expressed both CD1d and IgM was found in the capsule of the lymph node and in the medullary sinus close to the blood vessels of BCII-immunized (5 dpi) WT mouse (figure 9). There was no double-staining detected in the capsule of the lymph node from the naïve mouse but in an area rich of blood vessels in the medullary sinus, double positive cells could be detected (figure 10). To rule out the risk of unspecific staining, isotype controls for both anti-CD1d and anti-IgM antibodies was used, but no staining was detected for any of the isotype control sections.

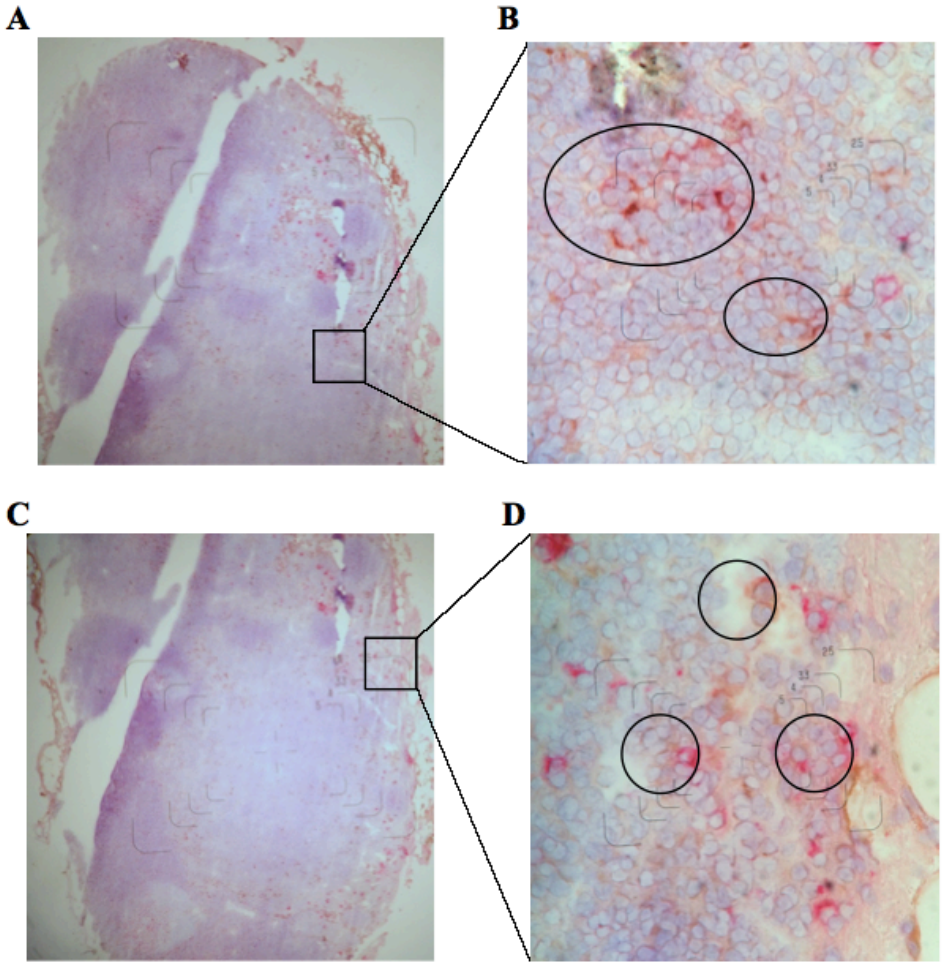

Figure 9. IgM and CD1d staining of lymph nodes from BCII-immunized mice. Single-staining of 7 µm thick acetone fixed sections from lymph nodes from BCII-immunized (5 dpi) WT DBA/1 mouse. (A) IgM positive cells (red) and CD1d positive cells (brown) close to a blood vessel (10 x magnification). (B) 100 x magnification of the marked area from A showing double-staining indicating MZBL. (C) IgM positive cells (red) and CD1d positive cells (brown) in the capsule of the lymph node (10 x magnification). (D) 100 x magnification of the marked area from C, double-staining indicating MZBL.

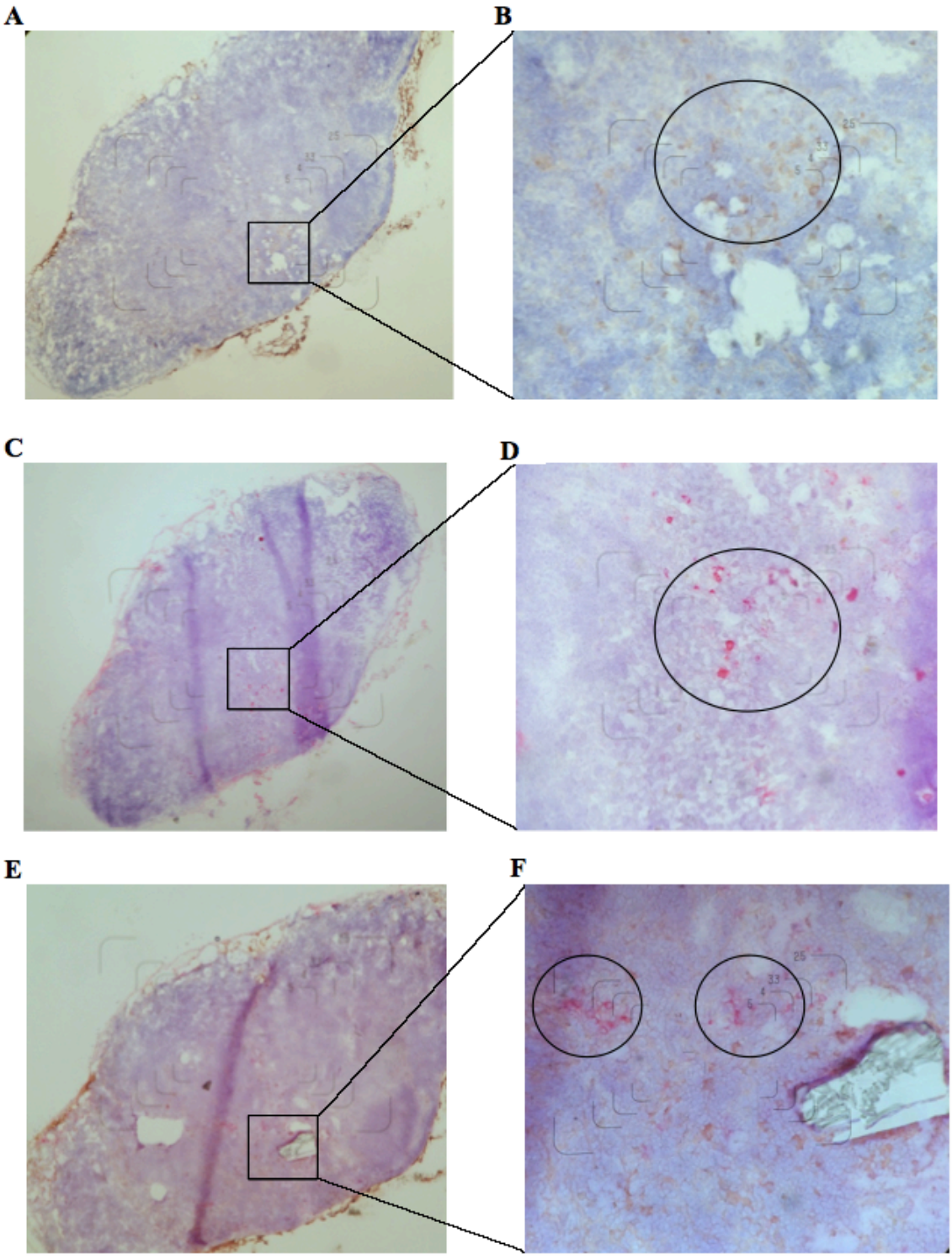

Figure 10. IgM and CD1d staining of lymph nodes from naïve mice. Single-staining of 7 µm thick acetone fixed sections from lymph nodes from naïve WT DBA/1 mouse. Overview pictures are taken using 10 x magnifications and close up using 40 x magnification. All pictures are taken of the same blood vessel rich area in the medullary sinus using consecutive sections. (A) CD1d positive cells (brown). (B) Close up of marked area from A. (C) IgM positive cells (red). (D) Close up of the marked area from C. (E) IgM (red) and CD1d (brown) double positive cells. (F) Close up of the marked area from E, double-staining indicating MZBL.

5. Discussion and conclusions

There was no reactivity to BCII in the peritoneal cells from BCII-immunized mice using ELISPOT. Neither were there any BCII-reactive clones when analysing sorted $CD43^+$ B cells from peritoneal cavity or when analysing the $CD43^+$ B cells after different time points after BCII immunization. Not even in the $Fc\gamma RIIb^{-/-}$ mice, which are more susceptible to CIA than WT mice, were there any $CD43^+$ B cells from peritoneal cavity that were reactive against BCII. Together these results suggest that there is no CII-specific reactivity among B-1 B cells in the peritoneal cavity in mice with CIA.

In the spleen there was CII-reactivity in both the CD43-positive and the CD43-negative cell fraction. This suggested that B-1 B cells in the CD43-positive fraction could be reactive towards BCII. When analysing the purity of the CD43-positive cells it showed that there were B-2 B cells, including MZB (which previously have been shown to be CII-reactive [9, 24]), in the sample which made it difficult to determine which cell population gave rise to the positive anti-BCII signal. To increase the purity of the sorted cells, FACS sorting was used instead of MACS since it has a higher accuracy. Unfortunately, the B220-positive, CD43-positive/negative sorted cell suspensions did not give rise to any positive antibody forming cells in the ELISPOT analysis for neither naive mice nor BCII-immunized mice. This suggests that there are no CII-specific B cell clones in the B-1 B cell population. The positive control for this setup were cells from whole spleen of immunized mice, which has previously been shown to produce antibody forming cells towards BCII [9] and even though the unsorted splenocytes gave a positive signal it did not give as strong signal as earlier demonstrated. Also the sensitivity of the ELISPOT method is dependent on having the exact amount of cells in each well and since the sorted B-1 B cells were fewer this might have affected the result. Therefore, a decision was made to change the setup of the experiments to analyse anti-CII antibodies in culture supernatants of B-1 B cells instead.

Thus, the new approach was to sort B cells from naive and BCII-immunized mice into B-1 B cells and MZB using FACS and stimulate the sorted cells with CpG and then analyse the supernatants from the cell cultures for CII-specific antibodies using ELISA. Stimulation of B cells was done with CpG since MZB showed a tendency to have increased anti-BCII antibody production when stimulated with CpG, and according to a pervious study [14]. CpG stimulation had a stimulating effect on B-1 B cell proliferation as well. The ELISA experiment confirmed the results from the ELISPOT analysis regarding the B-1 B cells from naïve mice (which did not show any positive antibody forming cells) and no anti-CII antibodies could be detected in the culture supernatants, even though the B-1 B cells responded to the CpG-stimulation with proliferation. However, B-1 B cells from mice 12 days after BCII-immunization proliferated in the same range as cells from naïve mice but gave a weak positive signal in the ELISA analysis. This suggests that there is a low production of anti-CII antibodies from B-1 B cells from BCII-immunized mice. It has previously been stated that 90 % of the antibodies B-1 B cells produced during an influenza infection is polyreactive IgM [3]. Therefore, a possible explanation to the low signal of antibodies detected in the ELISA for B-1 B cell cultures from immunized mice could be because the antibodies are polyreactive and bind with a low affinity to BCII. The new experimental setup also confirmed previous results from the group [9] that MZB from both naïve and immunized mice secrete anti-CII antibodies and that MZB from naïve mice have a proliferative response in the same range when stimulated with CpG compared to unstimulated

cells. Altogether these results verify that MZB are the major IgM anti-CII producing cells in the CIA model, whereas the B-1 B cell population only play a minor role in the secretion of IgM anti-CII antibodies.

The localization of MZBL in lymph nodes is still an on-going project, but after optimization of the IHC protocol we have been able to double-stain CD1d and IgM positive cells, which indicate MZBL cells. We also found that the 7 μm thick lymph node sections displayed the best morphology. Double positive cells were found around blood vessels in the medullary sinus of lymph nodes from both naïve and immunized mice and since there were no staining of sections incubated with the isotype control antibodies the staining seemed to be specific. Double positive cells were also found in the capsule of the lymph node from immunized mice. There are some indications of weak double-staining in the follicles of the sections from immunized animals as well, but to confirm this more sections need to be stained. There are also cells in the lymph nodes of both naïve and immunized mice that are very high in IgM, but due to the very bright red colour from the Fast Red substrate it is hard to determine if these cells are single or double positive. These results suggest that there are MZBL around and close to the blood vessels in the medullary sinus of lymph nodes in both naïve and BCII-immunized mice and there are indications that MZBL can be localized in the capsule and in the follicles of lymph nodes from BCII-immunized mice as well.

5.1 Future of the projects

For the B-1 B cell project it would be interesting to redo the experiment with FACS-sorted and CpG stimulated B-1 B cells from immunized mice at different time points to see when the antibody response to BCII develops. It would also be interesting to redo the ELISA for B-1 B cells and using BSA and BCII as coating side by side to determinate if the antibodies are polyreactive or specific to BCII. I also did some CpG stimulation experiments on cells from the peritoneal cavity of both immunized and naïve mice. This experiment gave a weak signal for CII-specific antibodies in the culture supernatants from both the immunized and the naïve animals, but due to the high variation between the different animals this experiment needs to be redone to confirm if there is a positive signal. The experiment was done on unsorted cells and it would also be interesting to redo the experiment on sorted B cells from the peritoneal cavity.

For localization of MZBL in the lymph nodes, more sections need to be analysed, both from naïve and immunized mice. Also, some fine-tuning of the method might be necessary to increase the staining of CD1d to be able to determinate if the IgM high cells are positive for CD1d as well or if they are single positive.

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7. References

1. Svensson, L., J. Jirholt, R. Holmdahl and L. Jansson, *B cell-dificient mice do not develop type II collagen-induced arthritis (CIA).* Clinical and experimental immunology 1998. **111**(521-526).

2. Zheng, B., X. Zhang, L. Guo and S. Han, *IgM plays an important role in induction of collagen-induced arthritis.* Clinical and experimental immunology, 2007. **149**: p. 579-585.

3. Baumgarth, N., *The double life of a B-1 cell: self-reactivity selects for protective effector functions.* Nature Reviews Immunology, 2011. **11**: p. 34-46.

4. Flavius, M., J. K. Kearney, *Marginal-zone B cells.* Nature Reviews Immunology, 2002. **2**: p. 323-335.

5. Mariño, E., M. Batten, J. Groom, S. Walters, D. Liuwantara, F. Mackay, and S. T. Grey, *Marginal-Zone B-Cells of Nonobese Diabetic Mice Expand With Diabetes Onset, Invade the Pancreatic Lymph Nodes, and Present Autoantigen to Diabetogenic T-Cells.* Diabetes, 2008. **57**: p. 395-404.

6. Duan, B., and L. Morel *Role of B-1a cells in autoimmunity.* Autoimmunity Reviews, 2006. **5**: p. 403-408.

7. Wells, S.M., A. B Kantor, and A. M Stall, *CD43 (S7) Expression Identifies Peripheral B Cell Subsets.* The Journal of Immunology, 1994. **153**: p. 5503-5515.

8. Cerutti, A., M. Cols, and I. Puga, *Marginal zone B cells: virtues of innate-like antibody-producing lymphocytes.* Nature Reviews Immunology, 2013. **13**: p. 118-132.

9. Palm, A.-K.E., H. C. Friedrich, A. Mezger, L. K. Myers, and S. Kleinau *Self-reactive marginal zone B cells present autoantigen to T cells and are regulated by CR1/2 and FCγRIIb.* Manucript in submission, 2014.

10. Tumang, J.R., W. D. Hastings, C. Bai, and T. L. Rothstein, *Peritoneal and splenic B-1 cells are separable by phenotypic, functional, and transcriptomic characteristics.* European Journal of Immunology, 2004. **34**: p. 2158-2167.

11. Boes, M., *Role of natural and immune IgM antibodies in immune responses.* Autoimmunity Reviews, 2000. **37**: p. 1141-1149.

12. Kleinau, S., P. Martinsson, and B. Heyman, *Induction and supression of collagen-induced arthritis is dependent on distinct fcgamma receptors.* The Journal of experimntal medicine, 2000. **191**: p. 1611-1616.

13. Abbas, A.K., A.H. Lichtman and S. Pillai, *Laboratory methods using antibodies* in *Cellular and Molecular Immunology*. p. 513-526.

14. Stoehr, A.D., C. T. Schoen, M. M .M. Mertes, S. Eigmeier, V. Holecska, A. K Lorenz, T. Schommartz, A-L. Schoen, C. Hess, A. Winkler, H. Wardemann, and M. Ehlers, *TLR9 in Peritoneal B-1b Cells Is Essential for Production of Protective Self-Reactive IgM To Control Th17 Cells and Severe Autoimmunity.* The Journal of Immunology, 2011. **187**: p. 2953-2965.

15. Scott, D.L., F. Wolfe, and T. W. J Huizinga, *Rheumatoid arthritis.* Lancet, 2010. **376**: p. 1094-1108.

16. Wegner, N., K. Lundberg, A. Kinloch, B. Fisher, V. Malmström, M. Feldmann, P. J. Venables, *Autoimmunity to specific citrullinated proteins gives the first clues to the etiology of rheumatoid arthritis.* Immunological reviews, 2010. **233**: p. 34-55.

17. Bellamy. N., D.L.D., P. Sambrook, R. R. C Buchanan, P. M. Brooks, H. Dunckley, S. C. Healey, S. Mason and N. G. Martin, *A methodological appraisal of the impact of different classification procedures used in three different phases of the autralian rheumatoid arthritis twin survey.* Inflammopharmacology, 1998. **6**: p. 81-89.

18. MacGregor J. A., H.S., A. S. Rigby, M. Koskenvuo, J. Kaprio, K. Aho and A. J. Silman, *Characterizing the quantitative genetic contribution to rheumatoid arthritis using data from twins.* Arthritis & rheumatism, 2000. **43**: p. 30-37.

19. Clark A. E., J.A.L., *How does B cell deplition therapy work, and how can it be improved?* Annals of the Rheumatic Diseases, 2005. **64**: p. 77-80.

20. Holmdahl, R., R. Bockerman, J. Bäcklund, and H. Yamada *The molecular pathogenesis of collagen-induced arthritis in mice - a model for rheumatoid arthritis.* Ageing Research Reviews, 2002. **1**: p. 135-147.

21. Brand, D.D., K. A. Latham and E. F. Rosloniec, *Collage-induced arthritis.* Nature Protocols, 2007. **2**(5): p. 1269-1275.

22. Kuby, J., T. J. Kindt, R. A. Goldsby and B. A. Osborne, *Antigen – Antibody Interactions: Principles and Applications*, in *Kuby Immunology*, Freeman.

23. Takai, T., M. Ono, M. Hikida, H. Ohmori, and J. V. Ravetch, *Augmented humoral and anaphylactic responses in FC gamma RII-deficient mice.* Nature, 1996. **379**: p. 346-349.

24. Carnrot, C., K. E. Prokopec, K. Råsbo, M. CI. Karlsson, and S. Kleinau, *Marginal zone B cells are naturally reactive to collagen type II and are involved in the initiation of the immune response in collagen-induced arthritis.* Cellular & Molecular Immunology, 2011. **8**: p. 296-304.