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Study of the autoimmune B cell response in complement receptor deficient mice

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Title (English) **Study of the autoimmune B cell response in complement receptor deficient mice**	
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Abstract Collagen-induced arthritis (CIA) is an animal model used to study the immune mechanisms behind the human autoimmune disease rheumatoid arthritis (RA). In this study wild type (WT) and complement receptor 1 and 2 (CR1/2)-deficient DBA/1 mice have been examined in order to investigate the role of complement receptors in the regulation of self-reactive B cells in CIA. The mice were immunized with collagen type II (CII) in adjuvant and the B cells from the spleen and the lymph nodes were analyzed using ELISPOT. Our results indicate that self-tolerance to CII in the spleen breaks earlier in CR1/2-deficient mice than in WT mice. Additionally, we have seen that naïve DBA/1 mice have CII-reactive B cells present in the spleen prior immunization.	
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Study of the autoimmune B cell response in complement receptor deficient mice

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Populärvetenskaplig sammanfattning

Autoimmuna sjukdomar uppstår när det egna immunförsvaret attackerar kroppsegna substanser, även kallade självantigen. Immunförsvaret är till för att skydda kroppen mot främmande substanser som till exempel mikroorganismer, så kallade antigen. Defekter på immunförsvaret kan leda till att toleransen mot självantigen bryts, vilket innebär att kroppen även attackerar självantigen.

B-celler är en typ av immunceller som har stor betydelse för immunsvaret. En viktig funktion hos dessa celler är att de producerar antikroppar, vars uppgift är att känna igen antigen. På B-cellernas yta finns komplementreceptorer som är viktiga komponenter för komplementsystemet, vilket är en del av immunsystem som bland annat startar inflammation.

Kollageninducerad artrit (KIA) är en sjukdomsmodell i möss som används för att studera den autoimmuna humana sjukdomen reumatoid artrit (RA). RA patienter utvecklar ledinflammation och producerar stora mängder antikroppar mot självantigen, bland annat mot kollagen. KIA induceras med en injektion av kollagen som framkallar kollagenreaktiva antikroppar och ledinflammation hos mössen. I den här studien har vi bland annat undersökt komplementreceptorers inverkan på B-cellssvaret. Två grupper av möss har studerats, vildtypsmöss och möss som saknar komplementreceptorer på B-celler. Resultaten visar att möss som saknar komplementreceptorer får ett kraftigare antikroppssvar mot kollagen, vilket visar att defekter i komplementsystemet leder till att toleransen mot självantigen bryts lättare.

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Abbreviations

AFC:	antibody forming cell
BCII:	bovine collagen type II
BSA:	bovine serum albumin
CIA:	collagen induced arthritis
CFA:	complete Freund's adjuvant
CR1:	complement receptor 1 (CD35)
CR2:	complement receptor 2 (CD21)
ELISA:	enzyme linked immunosorbent assay
ELISPOT:	enzyme linked immunospot assay
Ig:	immunoglobulin
PBS:	phosphate buffered saline
RA:	rheumatoid arthritis
RT:	room temperature
WT:	wild type

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune human disease causing polyarthritis and cartilage and bone destruction. The disease is more frequently occurring among women than men. The molecular mechanisms leading to RA are still not fully understood. Antibodies against collagen type II (CII) has been shown to be present in the sera of affected persons (Clague *et al.* 1980). B lymphocytes (B cells) as well as the complement system have been shown to play an important role in RA. RA patients have been shown to get reduced inflammation and less severe disease when treated with Rituximab, a drug used for depleting B cells, implicating the importance of B cells (Yanaba *et al.* 2008). Complement is involved in induction and progression of inflammation and several complement components can be found in synovial specimens of RA patients compared to patients with osteoarthritis, where only a few complement components are expressed (Neumann *et al.* 2002).

1.1 Collagen-induced arthritis

Collagen-induced arthritis (CIA) is an animal model of rheumatoid arthritis (RA). CIA can be initiated in susceptible mice, bearing the H-2^q haplotype, by an intra-dermal injection of heterologous collagen type II (CII) in complete Freund's adjuvant (CFA) (Wooley *et al.* 1981 and Trentham *et al.* 1977). CII is a major component of cartilage and antibodies against CII are induced in the mice immunized for CIA (Yanaba *et al.* 2008 and Wooley *et al.* 1981). Male mice are more susceptible to CIA than female mice (Holmdahl *et al.* 1985), which is unlike humans where women are more frequently affected by RA than men. B cells have been shown to play a crucial role in CIA, e. g. B cell deficient mice do not develop arthritis when immunized with CII (Svensson *et al.* 1998). Further, arthritis can be passively transferred to mice with serum containing antibodies against CII (Stuart and Dixon 1983). This strongly indicates the importance of B cells and anti-CII antibodies in the mechanisms leading to CIA.

1.2 B cells and antibodies

B cells can be divided into three subcategories, B-1 B cells, follicular (FO) B cells and marginal zone (MZ) B cells. MZ B cells are located in the marginal zone of the spleen (Allman *et al.* 2008) and are stationary in the spleen without any recirculation abilities (Cinamon *et al.* 2008). MZ B cells are characterized by early participation in immune responses (Martin *et al.* 2002). FO B cells are circulating cells, gaining their circulating ability in the spleen. They migrate via blood and lymph to lymphoid organs such as the lymph nodes and the spleen (Allman *et al.* 2008).

Antibodies or immunoglobulins (Ig) are produced by B cells and play an important role in the immune defense against pathogens such as bacteria and virus. These antibodies recognize the foreign substances and trigger other immunological systems, for example the complement system, to participate in the clearing of the substance. When it comes to the antibody response to blood-borne antigens the spleen is an important site for induction of these responses (Cinamon *et al.* 2008). Antibodies are divided into five different classes; IgA, IgD, IgE, IgG and IgM. IgG can be subcategorized into IgG1, IgG2a, IgG2b and IgG3 in the mouse. To prevent development of B cells producing antibodies that attack substances of the own body, named autoantibodies, all B cells go through a negative selection. Thus, B cells are exposed to self-antigen and those cells that produce autoantibodies are sent to apoptosis. This phenomenon that B cells do not produce autoantibodies is called self tolerance. When the negative selection fails autoimmunity can be the result. This is a classic view of how B cells can contribute to the pathogenesis of autoimmunity (Yanaba *et al.* 2008).

1.3 Complement and autoimmunity

The complement system is a cascade of events mediated by numerous proteins and enzymes that protects organisms from pathogens. Complement receptor (CR) type 1 and 2 (CR1/CD35 and CR2/CD21) are two complement receptors that mediate the biological activity of complement components. In mice CR1 and CR2 are products of one single gene generated upon alternative splicing (Kurtz *et al.* 1990). In humans on the other hand, CR1 and CR2 are products of two separate genes (Kurtz *et al.* 1990). Activation of

the complement system leads to generation of several complement components like C3b and C4b involved in opsonization of antibody antigen complex and formation of a cell lytic complex that causes cell death. C4b, C3b and iC3b, the cleaved product of C3b, bind to CR1 that act as an inhibitory receptor (Józsi *et al.* 2002). CR2 binds the cleaved products iC3b, C3dg and C3d and is believed to be able to lower the threshold of antigen-induced activation of B cells (Boackle 2003 and Isaák *et al.* 2006). Deficiency of complement components, e. g. CR1 and CR2 may lead to less effective antibody production and autoimmunity (Boackle 2003, Molina *et al.* 1996 and Chen *et al.* 2000). MZ B cells are characterized with high expression of CR1 and CR2 (Cinamon *et al.* 2008). CR1 and CR2 (CR1/2) deficient mice have been shown to be less susceptible to CIA than wild type (WT) mice (Kuhn *et al.* 2008). However, recent studies have shown that female mice deficient in CR1 and CR2 are more susceptible to CIA than WT mice if the disease is induced with a low dose of CII (Nilsson *et al.* 2009). Deficiency in CR1 and CR2 has also been shown to cause a more severe disease with earlier onset in a lupus-like autoimmune disease in mice (Isaák *et al.* 2006).

Antibodies against CII bind to collagen in articular cartilage. These antibody antigen complexes are probably initiating joint inflammation by triggering complement activation, which has been shown to be crucial for the progress of CIA (Kuhn *et al.* 2008 and Wang *et al.* 2000). Therefore, B cells and antibodies and their interaction with the complement system are interesting to investigate when studying the mechanisms of CIA.

1.4 Aim of the project

- To explore if CII-reactive B cells are present in naïve mice.

- To determine if immunization with a high or a low bovine CII (BCII) dose affects the number of CII-reactive B cells in the mice.

- To explore how CR1/2 deficiency on CII-reactive B cells affects the antibody response.

1.5 Previous findings and project description

Previously it has been shown in the Kleinau lab that unimmunized DBA/1 mice display IgM positive B cells reactive to BCII, even though they have not been exposed to the antigen previously (Nilsson and Carnrot *et al.* unpublished data). This study aims to determine whether or not this is unique for the DBA/1 strain. Therefore different mouse strains will be compared to see if they also express CII-reactive B cells. DBA/1 mice easily develop CIA in contrast to many other strains. During this project the hypothesis that DBA/1 mice have a tendency to develop CIA due to their naturally occurring anti-BCII IgM B cells will be tested.

An optimal dose to induce CIA is 50 µg BCII. However recent studies in the lab have been carried out using a lower dose of 20 µg BCII to better mimic the amount of self antigen that may be exposed in autoimmune diseases. Thus, to investigate if there are any dose related differences in B cell responses to CII we have investigated mice that have been immunized with 20 µg respectively 50 µg BCII and analyzed their B cell response.

Moreover, recent studies have shown that female CR1/2-deficient mice immunized with a low dose (20 µg) of CII in CFA develop an enhanced CIA and a prolonged IgM antibody response in serum compared to female WT mice (Nilsson *et al.* 2009). In order to investigate if the number of IgM positive CII-reactive B cells are increased in these mice we will analyze the B cell response to CII with ELISPOT at different time points after CII-immunization. CII-immunized wild type mice will serve as controls. Serum will also be studied at different time points by ELISA to see if the total IgM and IgG anti-CII response differ between female WT and female CR1/2-deficient mice.

2. Materials and methods

2.1 Mice

DBA/1 mice used in this study were bred and maintained at the animal facilities at the Biomedical Center, Uppsala University, Sweden. For this project WT DBA/1 female mice with H-2^q background and homozygous CR1/2-/- DBA/1 female mice on the 10th generation, also with H-2^q background were used. Mice used for immunization were between eight and twentythree weeks old when immunized. The mice WT and knock-out mice were aged matched in each experiment. All experiments were performed after approval by the local ethics committee (C60-8 and C59-8). BALB/c mice, NOD mice and C57/BL6 mice of both sexes were kindly provided by professor Birgitta Heyman, professor Stellan Sandler and associate professor Magnus Åbrink at Uppsala University. The number of mice used in each experiment is indicated in the figure legends and in the appendix.

2.2 Collagen-induced arthritis

BCII (2mg/ml) was emulsified with an equal volume (1:1) of CFA (Difco, Detroit, MI, USA) giving the final concentration of 1 mg BCII/ml. The adjuvant was added in portions of 150 µl – 250 µl during constant mixing and new portions were added with 2-5 minutes interval. When the emulsion was ready to use it was white and viscous. For immunization with 20 µg, BCII was first solubilized in 0.01 M acetic acid giving a concentration of 0.8 mg BCII/ml. Equal volumes of CFA and BCII was then mixed as described above giving the final concentration of 0.4 mg BCII/ml. Fifty µl of the emulsion was injected to each mouse by an intra-dermal injection at the base of the tail. This gives a dose of 50 µg CII or 20 µg CII respectively per mouse. All solutions containing BCII was kept on ice during the experiment.

2.3 Cell preparation

Mice were euthanized at different time points after immunization. Axillary, branchial and inguinal lymph nodes and spleen were removed from the mice and put into cold phosphate buffered saline (PBS). All lymph nodes taken from one mouse were pooled together to one sample. The Lymph nodes and the spleen were carefully pressed through a stainless steel mesh into single cell suspensions. The cell suspensions were transferred to Falcon tubes with 10 ml of PBS and were then centrifuged for 6 minutes at 1100 rpm. Then the supernatant were discarded and cells from the lymph nodes were resuspended in 900 µl DMEM culture medium (Sigma) with 100 U of penicillin (Sigma), 100 µg/ml of streptomycin (Sigma), 1% L-Glutamine (Sigma) and 10% fetal bovine serum (Sigma) referred to as complete DMEM medium, the cell suspensions were kept on ice until further analyzed. Splenocytes were resuspended in 5ml ACK (0.15 M NH_4Cl, 1.0 mM $KHCO_3$, 1.0 mM EDTA) lysis buffer in order to lyse erythrocytes. After 3.5 minutes the reaction was stopped with 5ml PBS then the tubes were centrifuged for additional 6 minutes at 1100 rpm. The supernatant was discarded and the splenocytes were resuspended in 1800 µl complete DMEM medium and kept on ice.

2.4 ELISPOT

The enzyme-linked immunosorbent spot (ELISPOT) assay was used to analyze the number of B cells secreting antigen-specific antibodies. Each spot in this assay represents an antibody forming cell (AFC). The ELISPOT was performed accordingly:

Ninetysix-well micro titer plates (MaxiSorp, NuncBrand Thermo Fischer Scientific, Roskilde, Denmark) were coated with BCII (0.2 mg/ml) or bovine serum albumin (BSA) (0.02 % in PBS). Each well was coated with 50 µl giving 10 µg BCII or BSA in each well. The plates were coated over night at 4^oC. BSA was used as a negative control. Ten µl of each cell suspension, lymph nodes and spleen, were diluted 1:100 in 0.04% bromothymol blue (BTB) and counted in a Bürker chamber. After washing with PBS 1 million cells diluted in 200 µl complete DMEM medium were added to the antigen-coated 96-well micro titer plates. The plates were then incubated at 37^oC in CO_2 for 22

hours.

All washing steps were performed with PBS if not otherwise stated. After incubation the plates were washed and secondary alkaline phosphatase coupled IgM or IgG antibody (Sigma) was added. The antibody was diluted 1:500 and 50 µl was added to each well. Plates were incubated at room temperature (RT) for two hours and then washed with 0.2M Tris-HCl. For measuring IgG subclass specific B cells biotin-conjugated secondary IgG1, IgG2a, IgG2b and IgG3 antibodies (initial concentration 0.5 mg/ml) (Southern Biotech, Birmingham, Alabama, AL, USA) were added to the plates after washing and incubated at 4°C over night or at RT for 2 hours. Plates were then washed and alkaline phosphatase-conjugated ExtrAvidin (Sigma) was added and incubated at 4°C over night or at RT for 2 hours. Plates were then washed with 0.2M Tris-HCl.

After washing the plates with Tris-HCl, 50 µl of 5-bromo-4-chloro-3-inodyl phosphate toluidine/nitro blue terazolium (BCIP/NBT) Liquid Substrate System (Sigma) was added to the plates. This substrate produces a blue-purple product when alkaline phosphatase is present in the well. The plates were incubated for about an hour in the dark, washed with distillated water to stop the reaction and left for drying. The dry plate can then be read using an inverted microscope to count the spots. Each spot represents an AFC. The spots can be diffuse or well defined and they can also differ in size. The size or intensity of the spots is however not taken into consideration, the number of spots is the only parameter considered in this study. A mean value of spots in BCII-coated wells was calculated.

2.4 Blood sampling and ELISA

At the same time as the lymphoid organs were taken an orbital sinus bleeding was performed to collect blood from the mice. These samples were left at RT over the day to coagulate. The blood was transferred to new eppendorf tubes leaving the coagulated blood. The blood was then centrifuged for seven minutes at 5 500 rpm. The supernatant was transferred to new tubes and stored at –20°C until further analysis. The serum samples were then analyzed with the enzyme-linked immunosorbent assay (ELISA) to

detect anti-CII levels of IgM and IgG in the sera. The ELISA was performed accordingly:

Ninetysix-wells micro titer plates were coated with BCII in the same way as described for ELISPOT. For all washing steps PBS-Tween (0.05%) was used unless otherwise stated. The coated plates were washed and 200µl BSA (1% in PBS) was added to each well in order to block non-specific binding sites to other proteins on the plate and incubated in RT for one hour. After washes the serum samples were added to the wells at an initial dilution of 1:50 for samples taken day 0, 7, 10 and 14, and 1:100 for samples taken day 28 and 56 after BCII-immunization. The samples were then 5-fold diluted twice down the plate. Samples were diluted in BSA (1% in PBS). A polyclonal IgG anti-CII standard was added on the plates detecting IgG. The initial dilution of the IgG standard was 1:66 and was then 2-fold diluted seven times down the plate. Plates were then incubated at RT for two hours. After additional washes alkaline phosphatase-conjugated anti-IgM or anti-IgG was added with 50 µl per well, diluted 1:3000 in BSA (1% in PBS). The plates were then incubated at RT for 2 hours. Additional washes were then performed and 50 µl of ρ-nitrophenyl phosphate substrate (Sigma) diluted in diethanolamine buffer (1 mg/ml) was added to each well. The plates were then read at 405 nm in an ELISA-spectrophotometer. IgG plates were read every five minutes during 40 minutes and the IgM plates every 20 minutes during 2 hours. IgM and IgG arbitrary units were calculated by multiplying the OD_{405} for each sample with the corresponding dilution factor.

2.5. Statistics

Statistical analyses were performed using the Student's t-test. P-values below 0.05 were considered significant.

3. Results

3.1 Unimmunized DBA/1 mice display more anti-BCII reactive B cells than C57BL/6 mice

Previously it has been shown that naïve DBA/1 mice that have not been exposed to BCII have BCII specific B cells in the spleen (Nilsson *et al.* unpublished data). In order to determine if this phenomenon is unique for the DBA/1 strain, splenocytes from DBA/1 mice were analyzed in comparison with splenocytes from three other mouse strains; NOD, BALB/c and C57BL/6. The splenocytes were analyzed with ELISPOT and CII-reactive IgM and IgG AFCs were counted. DBA/1 mice showed higher numbers of anti-CII IgM AFCs than NOD and BALB/c and significantly higher numbers ($p<0.05$) than C57BL/6 mice (fig. 1A). Thus, C57BL/6 mice showed almost no BCII-specific IgM AFCs at all in the spleen (fig 1A). DBA/1, NOD and BALB/c showed quite similar numbers of anti-BCII specific IgG AFCs and C57/BL6 had almost no IgG AFCs present in the spleen (fig. 1B).

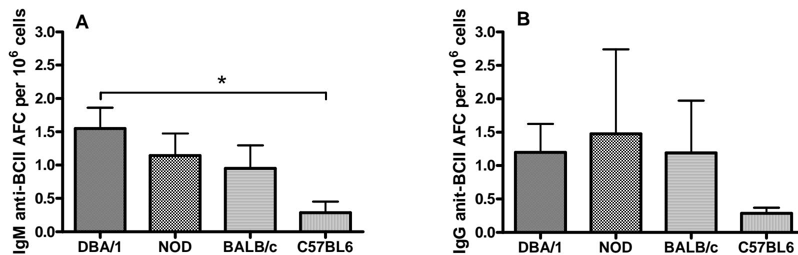

Figure 1. Three out of four naïve mouse strains show anti-BCII-specific IgM and IgG Ab forming cells (AFC) in the spleen. Mean anti-BCII AFC ± SEM per million splenocytes was determined with ELISPOT, IgM AFC **(A)** and IgG AFC **(B)** (n = 3-6 for each strain). *$p<0.05$ when comparing DBA/1 and C57BL6 (Student's t-test).

3.2 DBA/1 mice immunized with 20 µg BCII produce less IgG than mice immunized with 50 µg BCII

Former studies have given contradictory results regarding CR1/2-deficient mice and CIA. CR1/2-deficient mice have been shown to be less susceptible to CIA when using a high BCII dose (Kuhn *et al.* 2008). However, recent studies in the lab have shown the

opposite, but this was only seen in female mice and importantly a lower dose of BCII was used (Nilsson *et al.* 2009). Therefore a comparative study was performed to look at differences in CII-reactive antibody producing cells between female DBA/1 mice immunized with 20 µg or 50 µg of BCII. The analysis was carried out three weeks after immunization. Cells from the spleen and the lymph nodes were analyzed with ELISPOT detecting IgM and IgG AFCs and for splenic cells also IgG subclass specific AFCs were analyzed. Mice immunized with 20 µg of BCII showed significantly ($p<0.001$) lower numbers of BCII-specific IgG AFCs in the spleen than mice immunized with 50 µg of BCII (fig. 2A). No difference between the groups was observed for IgM AFCs in the spleen (fig. 2A). Distinct numbers of BCII-specific IgM and IgG AFCs in the lymph nodes were seen in mice immunized with the higher dose of BCII (fig. 2B). Splenocytes were further analyzed for differences in IgG subclass AFCs and mice immunized with the higher dose of BCII showed significantly ($p<0.01$) higher numbers of BCII-specific IgG2a AFC. A trend of higher numbers of IgG1 and IgG2b AFCs were also noted in the mice (fig. 2C). For IgG3 AFC no differences could be seen between the groups (fig. 2C).

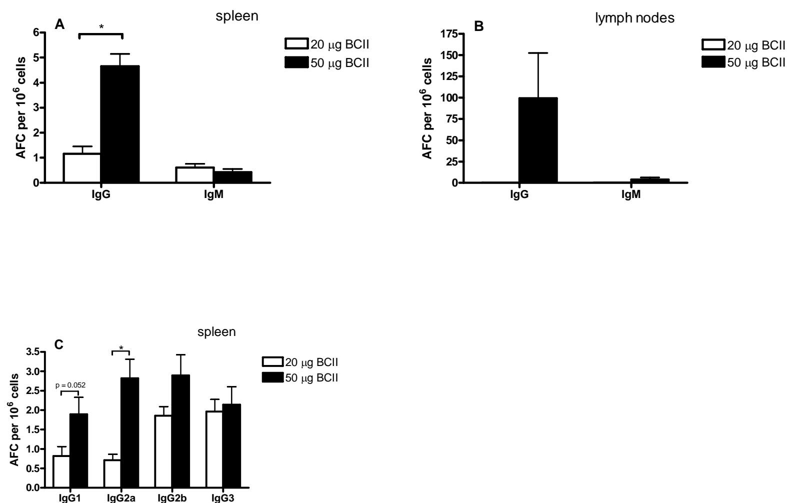

Figure 2. The amount of anti-BCII-specific IgM and IgG Ab forming cells (AFC) is dependent on the dose of BCII used for immunization. Mean number of BCII-specific IgM and IgG AFC ± SEM per million cells three weeks after immunization with 20 µg or 50 µg BCII was determined with ELISPOT. Cells are analyzed in the spleen **(A)** and lymph nodes **(B)**. Splenocytes were additionally analyzed for subclass IgG AFC (**C**). (n = 7 for both 20 µg and 50 µg BCII). *$p<0.01$ when comparing the different BCII doses (Student's t-test).

3.3 Complement receptor-deficient DBA/1 mice display more BCII-specific B cells following immunization

Female WT and CR1/2-deficient DBA/1 mice were immunized with 20 µg BCII and spleen and lymph nodes were obtained 7, 10, 14, 28 and 56 days after immunization for analysis with ELISPOT to detect anti-BCII specific IgG and IgM AFCs. IgG subclass AFCs were also analyzed but only for day 14, 28 and 56. Serum from each mouse was collected as well and analyzed for anti-BCII antibodies with ELISA. With day 0 means that unimmunized naïve mice were analyzed.

The numbers anti-BCII IgM AFCs in the spleen of WT mice were quite similar after immunization, but a possibly peak of anti-BCII IgM AFCs was noted at day 10 after immunization. Interestingly, the CR1/2-/- mice displayed significantly ($p<0.05$) more anti-BCII specific IgM AFCs in the spleen one week after immunization compared to WT mice (fig. 3). Generally the number of anti-BCII IgM B cells in the spleen seemed higher in the CR1/2-deficient mice compared with the WT mice at all time points, however, it was not significant different at day 10, 14,28 and 56.

Anti-BCII IgM AFCs were present in the lymph nodes of WT mice at day 10 up to day 28. The pattern looked the same for CR1/2-deficient mice and no differences could be seen between WT and CR1/2-deficient mice (fig. 3B).

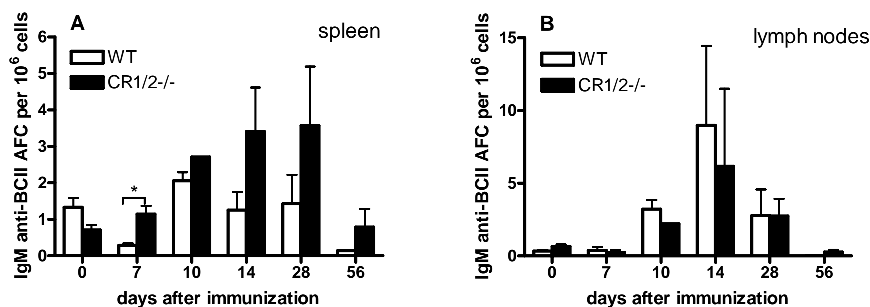

Figure 3. Complement receptor-deficient mice have more anti-BCII-specific IgM Ab forming cells (AFC) in the spleen following BCII immunization. Comparison between female CR1/2-deficient and wild type DBA/1 mice following immunization with 20 µg of BCII. The mean number of IgM AFC ± SEM per million cells at different time points in the spleen (**A**) and in the lymph nodes (**B**) is presented. The method used is ELISPOT. (n = 1-10) *$p<0.05$ when comparing wild type mice with CR1/2-deficient mice (Student's t-test).

Anti-BCII IgG AFCs in the spleen were increased at 10 days up to 28 days after BCII-immunization in the WT mice. At day 56 the number of IgG AFCs had declined. The pattern looked the same for CR1/2-deficient mice except that the numbers of IgG AFCs did not decline at day 56 (fig. 4A). However, this was not significant from WT mice.

WT mice exhibited anti-BCII specific IgG AFC at day 14 and 28 in the lymph nodes, whereas at day 56 there were no such cells present any longer. CR1/2-deficient mice displayed anti-BCII IgG AFCs in the lymph nodes only at day 14 and day 56 and the amount of AFCs were the same for these days (fig. 4B).

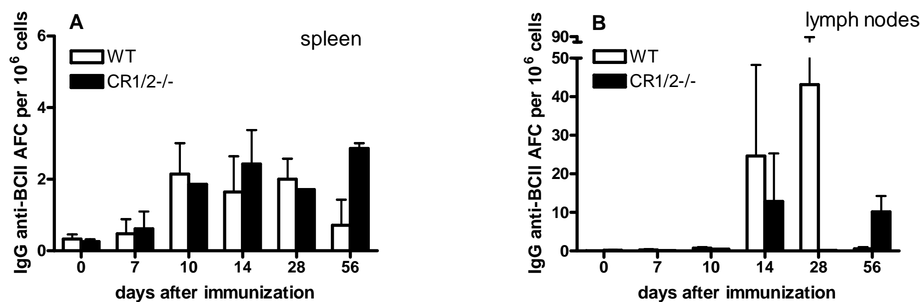

Figure 4. Complement receptor-deficient mice do not show any differences in anti-BCII-specific IgG Ab forming cells (AFC) in the spleen following BCII immunization. Comparison between female CR1/2 deficient and wild type DBA/1 mice following immunization with 20 µg BCII. The mean number of IgG AFC ± SEM per million cells at different time points in the spleen (A) and the lymph nodes (B). The method used is ELISPOT. (n = 1-8)

Concerning subclass specific AFCs WT had the same amount of anti-BCII specific IgG1, IgG2a, IgG2b and IgG3 AFCs in the spleen at day 14 after BCII-immunization. For CR1/2-deficient mice the pattern for the different subclass specific AFCs looked the same except for IgG2b AFCs, which were possibly increased in CR1/2-deficient mice (fig. 5A). No particular differences could be seen between the two groups regarding IgG-subclass specific AFCs at day 28 (fig. 5B). At day 56 the amount of IgG1 AFCs had increased about seven times and for IgG2a, IgG2b and IgG3 AFCs the numbers had increased about four times compared to day 14 and 28 in WT mice (fig. 5C). Interestingly, in CR1/2-deficient mice the amount of IgG1 AFCs had increased about ten times and for IgG2a, IgG2b and IgG3 the numbers had increased about eight times at day 56 compared to day 14 and 28. The numbers of IgG1 and IgG3 AFCs in CR1/2-deficient

mice at day 56 were significantly higher ($p<0.05$) than in WT mice.

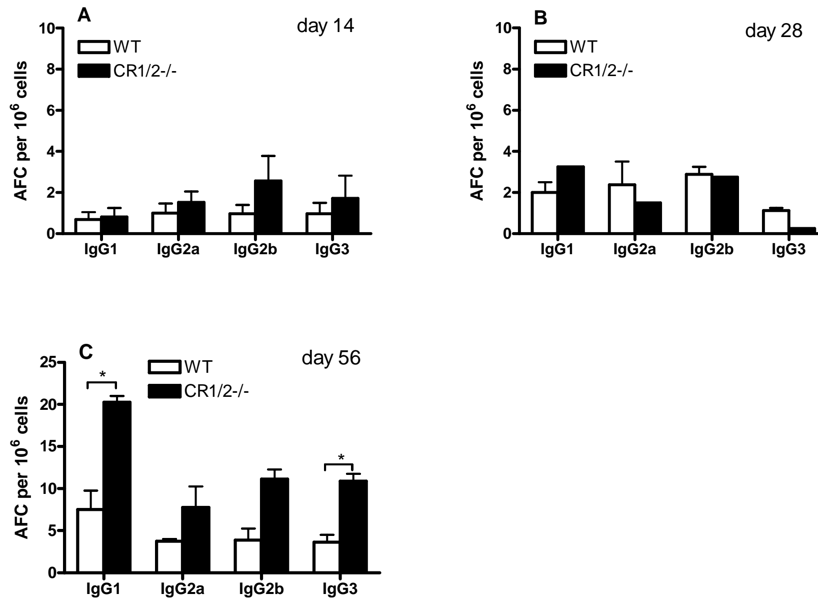

Figure 5. Complement receptor deficient mice display more BCII-reactive IgG subclass specific AFC than wild type mice eight weeks after BCII-immunization. The mean number of BCII-specific IgG1, IgG2a, IgG2b and IgG3 AFC ± SEM per million cells in the spleen at day 14 **(A)** 28 **(B)** and 56 **(C)**. The method used is ELISPOT. (n = 1-8). *$p<0.05$ when comparing wild type mice with CR1/2-deficient mice (Student's t-test).

3.4 The antibody titers are similar in CR1/2-deficient mice and WT mice

No anti-BCII IgM or IgG were present in serum before immunization, neither for WT or CR1/2-deficient mice. After BCII-immunization the IgM titer started to rise at day 10 to have a peak at day 28 and then declined. The pattern looked the same for CR1/2-deficient mice but the IgM titer declined a bit faster (fig. 6A). The anti-BCII IgG titer in WT mice serum started to rise between day 10 and 14 after BCII-immunization. The pattern looked the same for CR1/2- deficient mice but the increase of anti-BCII IgG between day 28 and day 56 was not as clear as for WT mice (fig.6B).

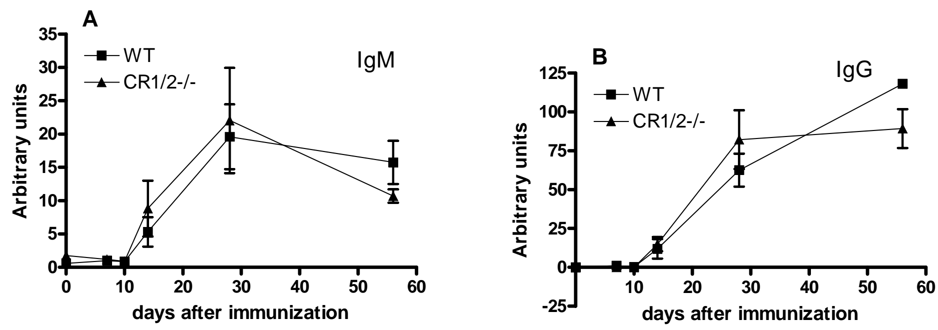

Figure 6. Antibody titers to BCII in serum eight weeks after BCII immunization. Antibody levels, meazured with ELISA, in sera from mice immunized with 20 µg BCII at different time points. The graphs show CII-specific IgM **(A)** and IgG **(B)** levels. The results are presented as mean ± SEM (n = 1-10). Arbitrary units = OD_{405} * dilution factor.

4. Discussion

4.1 Naturally occurring self-reactive B cells can trigger the immune response when mice are immunized with BCII

None of the strains NOD, BALB/c or C57/BL6 have the arthritis susceptible H-^{2q} haplotype. The NOD strain has a unique MHC class II called H-^{2g7}, BALB/c has H-2^{d} and C57/BL6 has H-2^{b} and none of them are considered susceptible to CIA. Still both BALB/c and NOD mice have self-reactive B cells producing anti-BCII IgM and IgG in resemblance with DBA/1 mice. The amount of IgM anti-CII AFC in the spleen of DBA/1, BALB/c and NOD are higher than for C57BL/6. Interestingly C57BL6/ mice are highly resistant to CIA (Seki *et al.* 1992). NOD mice, on the other hand, spontaneously develop autoimmune diabetes (Leiter 1997) and DBA/1 mice easily develop arthritis when immunized with CII. The fact that these autoimmune-prone mouse strains show a higher background of naturally occurring self-reactive B cells is very fascinating. This could be the explanation to why these mouse strains are more prone to develop autoimmune diseases. These self-reactive B cells present in the spleen can trigger the immune response when mice are exposed to an antigen like BCII and the self-tolerance breaks more easily than for mice without these self-reactive B cells. To further test this hypothesis it would be interesting to look for antibodies specific for another protein involved in some other autoimmune disease, like for exampel autoimmune diabetes. In this study only splenic cells were analyzed, but for DBA/1 mice there is data for the lymph nodes as well since unimmunized mice were analyzed for the third part of this degree project. We can in figure 3B and figure 4B see that no anti-BCII IgM or IgG AFCs are present in the lymph nodes prior to immunization. Since FO B cells circulate between the spleen and the lymph nodes and MZ B cells are stationary in the spleen it is likely that these self-reactive B cells are MZ B cells. If FO B cells were the ones producing anti-BCII specific IgM and IgG AFC in naïve mice this ought to be seen in the lymph nodes. On the basis of our observations it is clear that strains differ widely in how they behave due to their genetics and this is good to have in mind when comparing results from experiments with different strains.

4.2 The dose of BCII used for immunization affects the number of antibody producing cells

When it comes to the question whether or not the dose of BCII affects the B cell response our data reveals that there are considerable differences in anti-BCII specific B cells both in the spleen and the lymph nodes between mice immunized with 20 µg and 50 µg of BCII. Mice immunized with the higher dose of BCII produced more anti-BCII specific IgG AFCs both in the spleen and in the lymph nodes and also higher amount of anti-BCII specific IgM AFCs in the lymph nodes. The dose therefore seems to affect the production of IgG, but not the production of IgM in the spleen, as similar numbers of IgM AFCs were present in both groups of mice.

The amount of antigen does not seem to affect the cytokine environment, since the pattern of IgG subclasses produced looked the same regardless which BCII dose was used. The difference between the groups are in the amount of AFCs produced, not which ones that are produced. IgG2a is known to be efficient in activating complement (Zheng *et al.* 2007) and could therefore be important in CIA. Indeed, this is somewhat indicated in our study that mice given the high dose have significantly more IgG2a producing cells. For IgG1 AFCs the difference is almost as clear as for IgG2a AFCs, indicating the importance of IgG1 as well in the mechanisms of CIA. IgG3 AFCs seem to occur in the same amount in mice immunized with both low and high dose which is in line with that IgG3 is not thought to be of importance for CIA. Thus, IgM, IgG1 and IgG2a seem to play an essential role in CIA.

4.3 Self-tolerance breaks earlier if complement receptor 1 and 2 are lacking

The complement system is known to play a role in autoimmunity. Further, CR1 is thought to be an inhibitory receptor (Józsi *et al.* 2002) regulating complement activation and B cell activation. It has also been shown that female CR1/2-deficient mice induced with low dose of BCII exhibit an earlier onset of the disease and higher incidence than WT mice (Nilsson *et al.* 2009). This is in line with what we have seen in this study that CR1/2-deficient mice produce possibly more anti-BCII IgM B cells than WT upon

immunization with low BCII dose. Complement receptors in WT mice can control the B cell response and keep a satisfactory level of BCII specific B cells. The CR1/2 deficient mice however, do not have this control and therefore they can not regulate the activation of B cells. Since IgM can trigger the complement system, too high levels of IgM will induce too much complement activation which will eventually lead to disease. These thoughts are strengthened by the fact that the development of CIA can be inhibited and anti-CII antibody levels can be reduced, if the mice are treated with truncated soluble CR1 (Dreja *et al.* 2000). Since the anti-BCII response is initially higher in the spleen than in the lymph nodes MZ B cells ought to be the ones initiating the early immune response against BCII. This response then triggers the FO B cells, indicated by the rise of antibody producing cells in the lymph nodes.

On the basis of our observations we can see that IgM play a decisive role in CIA. We have shown that CR1/2-deficent mice display more anti-BCII IgM producing B cells in the spleen compared to WT mice upon immunization with a low dose of BCII. Additionally it has been shown earlier that these deficient mice are more susceptible to CIA than WT mice when immunized with a low dose of BCII. To continue this project it would be interesting to expand the groups and hopefully get even clearer results. It would also be of interest to collect data at additional time points after immunization.

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8. Appendix

Supplementary table 1. Description of the number of animal used in the different experiments presented in figure 3-6.

	WT						CR1/2-/-					
	IgM anti-BCII AFC		IgM	IgG anti-BCII AFC		IgG	IgM anti-BCII AFC		IgM	IgG anti-BCII AFC		IgG
days after immunization	spleen	lymph nodes	serum	spleen	lymph nodes	serum	spleen	lymph nodes	serum	spleen	lymph nodes	serum
0	3	3	6	3	3	6	4	4	4	4	4	4
7	4	4	4	3	3	4	4	4	4	3	3	4
10	2	2	2	2	2	2	1	1	1	1	1	1
14	10	10	10	8	8	10	10	10	10	8	8	10
28	4	4	4	2	2	4	3	3	3	1	1	3
56	2	2	2	2	2	2	2	2	2	2	2	2