UPTEC X07 021
Examensarbete 20 p
Mars 2007

The antibody response in gene-targeted mice, producing IgM unable to activate complement

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UPTEC X	**Date of issue 2007-03**

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Title (English)

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Title (Swedish)

Abstract

Comprehension about the immune system and how it is regulated is of major importance for fundamental immunology. The complement and immune complexes play an important role in autoimmune diseases. There are data showing that natural IgM is needed to mount a normal antibody response and it is suggested this lies in its ability to activate complement. Therefore, a knock-in mouse with a point mutation, making it unable to activate complement via IgM, was constructed. Characterization of this mutant mouse was done with several immunological methods and antibody responses were compared with normal mice.

Keywords

IgM, complement activation, IgG response, B-cells

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Project name	Sponsors
Language **English**	Security
ISSN 1401-2138	Classification
Supplementary bibliographical information	Pages **28**

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Sammanfattning

Antikroppar är en viktig komponent i vårt immunsvar men de kan också orsaka allergier och autoimmuna sjukdomar. De produceras av immunsystemets B-lymfocyter (B-celler) då dessa har kommit i kontakt med ett kroppsfrämmande ämne, ett s.k. antigen (bakterier, giftiga substanser eller pollen). Antikroppar kan i komplex med dessa antigen aktivera komplementsystemet, som är en samling proteiner i blodet. Detta kallas ”klassisk komplementaktivering” och är en betydelsefull del av immunförsvaret, nödvändig för initiering av normal antikroppsproduktion.

Vi tror att naturligt IgM som alltid finns i blodet kan binda till det främmande antigenet trots sin låga affinitet, med hjälp av sina fem armar. Detta antigen-antikropp-komplex aktiverar komplementet, vilket möjliggör en ko-ligering mellan B-cellsreceptor och CR2/CD19 på B-cellens yta. Tröskeln för aktivering av B-cellen blir därmed tillräckligt låg för att B-cellen ska stimuleras till att producera antigen-specifikt IgM som binder starkare till antigenet. Komplementet aktiveras igen och leder till ytterligare förstärkning av B-cellsaktiveringen.

För att kunna studera vår hypotes har möss med en mutation i IgM molekylen framställts. Denna mutation innebär att komplement inte kan aktiveras via den klassiska vägen, men att IgM molekylen i övrigt ska fungera normalt. De muterade mössens fenotyp karakteriserades och resultaten tyder på att IgM från dessa möss inte kan aktivera komplement samt att mössen har ett lägre antikroppssvar än normala möss.

Examensarbete 20 p i Molekylär bioteknikprogrammet

Uppsala universitet Mars 2007

1. ABBREVIATIONS

BCIP/NBT	5-Bromo-4-Chloro-3' Indolylphosphate p-Toluidine Salt and Nitro Blue Tetrazololium. Substrate for the enzyme alkaline-phosphatase.
BSS	Hank's Balanced Salt Solution
C57BL/6	Control mouse strain
CBA/J	Mouse strain whose serum was used for IgM purification
Cmu13	Mouse bearing complement activation deficient IgM
CR1, 2	Complement receptor 1, 2
CD21	CR2
CD19	Co-receptor for CR2
CVF	Cobra Venom Factor
DMEM	Dulbecco/Vogt Modified Eagle's Minimal Essential Medium
ELISA	Enzyme-Linked ImmunoSorbent Assay
ELISPOT	Enzyme-Linked ImmunoSpot Assay
IC	Immune complex
IgG, M…	Immunoglobulin G, M…
IgM-IC	Antigen-specific IgM in complex with that antigen
KLH	Keyhole Limpet Hemocyanin
Knock-in technology	Replacement of endogenous gene with a mutated form of that gene
mAb	Monoclonal antibody
Natural antibody	Antibody existing without prior immunization
PBS	Phosphate buffered saline
PFC	Plaque Forming Cell Assay or Plaque Forming Count
PLL	Poly-L-lysine
SRBC	Sheep Red Blood Cells

2. INTRODUCTION

2.1 The immune system

All living entities need protection against the harmful environment they live in. Even predators at the top of the food chains have enemies on which their predatory qualities do not apply. Such a threat is in the form of microorganisms, e.g. bacteria, worms and viruses. Therefore, an immune system has been evolved in order to protect the organism. Different organisms have different immune systems adapted to their life style, size etc. The immune system has two global functions; recognition and response. Recognition is dependent on high specificity, the ability of making a distinction between different foreign structures, and the important distinction between self and non-self structures [1]. Following this recognition is a specific, appropriate response.

Jawed vertebrates have two branches of the immune system; the innate and the adaptive immunity. The innate comprises anatomical barriers such as skin and mucosal surfaces, complement system, macrophages and natural killer cells. It is characterized by being relatively unspecific, fast in response, broad reactivity, having no memory cells and is present in the body before infection. On the other hand, adaptive immunity has a slow response but may show very high specificity. It has antigenic specificity and can distinguish proteins with preciseness of a single amino acid difference [1]. By generating billions of recognition molecules, the adaptive immune system displays a great diversity. It consists of two parts; the humoral part, i.e. antibody production by B-cells, and the cell-mediated part that activates T-cells into cytotoxic T-cells or inflammatory T-helper cells. It is also equipped with a memory which generates a faster and stronger response the second time of exposure. This means that there are always some cells left that bear antibodies against the antigen after an infection. When exposed to the same antigen again, the response will be faster and stronger, because the immune system has "remembered" the antigen. Another important factor of the adaptive immunity is the distinction between self and non-self structures. Without this control, autoimmunity would easily take over and the consequences would be fatal.

Antibody production by B-cells is essential for the function of the adaptive immunity. Together with other components of the immune system such as T-cells, cytokines and the complement system, B-cells produce antigen-specific antibodies as an aid in the fight against foreign substances.

2.1.1 Antibodies

Antibodies are present on B-cell membranes but are also produced and secreted in high amounts following an infection that has activated the immune system. The subunits of all antibody classes (IgA, IgD, IgE, IgG and IgM) are built up in the same way, as depicted in Figure 1 [2]. The antibodies have a variable region at the amino terminal composed of complementarity-determining regions (CDRs). This is the site that binds the antigen and it is where antibodies differ in sequence structure.

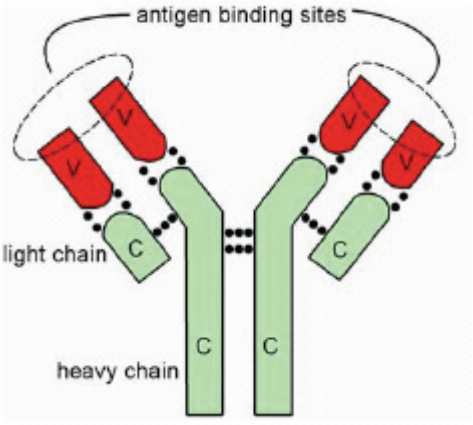


FIGURE 1. Antibody structure showing placement of antigen binding sites, chains and characteristic regions. *Illustration used with permission from Jan Troeng.*

The rest of the structure is the so called constant region that displays a limited variation and rather makes up the classes and subclasses. An IgG antibody has a molecular weight of about 150,000 Da. It is composed of two biologically different regions; the CDRs that bind antigen and the Fc fragment that binds to Fc-receptors on cells of the immune system and provokes a cell response, such as release of inflammatory histamines [3]. IgA antibodies exist as dimers and IgMs are preferably assorted as pentamers.

2.1.2 B-cell maturation

B-cell development and antibody production are tightly linked together. The B-cells start their development in the bone marrow and they derived from hematopoietic stem cells that can regenerate indefinitely. Within the bone marrow its maturation is antigen independent and the first progenitor B-cell will differentiate to a mature B-cell by rearrangement of its immunoglobulin genes [4]. This means that the actual genetic sequence in the B-cells bearing the information of the immunoglobulin is changed. The germline gene segments are part of multiple gene families which are located on different chromosomes and brought together forming functional immunoglobulin genes. Every chain has a variable (V), constant (C) and a junctional (J) gene segment. The heavy-chain has an additional diversity gene segment (D). All these segments are the basis for the enormous amount of diversity that can be achieved among antibodies. Later on in the peripheral lymphoid organs, mature B-cells are exposed to antigen. This leads to the differentiation and activation of the B-cell and the secretion of first wave of IgM antibodies, specific for the antigen in question.

2.1.3 The complement system

In the 1890's at the Institut Pasteur, Jules Bordet was working on experiments regarding antibodies directed against bacteria [5]. He heated the antibody-containing serum, making it unable to lyse bacteria and found that when adding fresh serum without any antibodies directed against the bacteria, the ability to lyse bacteria was restored. Similarly, Paul Erlich in Berlin independently conducted tests showing the same phenomenon and described it as "the activity of blood serum that completes the action of antibody" [6]. Thus, it seemed as there were two different systems in the fight against bacteria, both bacteria-specific antibodies and a component with lytic activity. This component was named complement. This system is a potent mediator of both innate and acquired immunity.

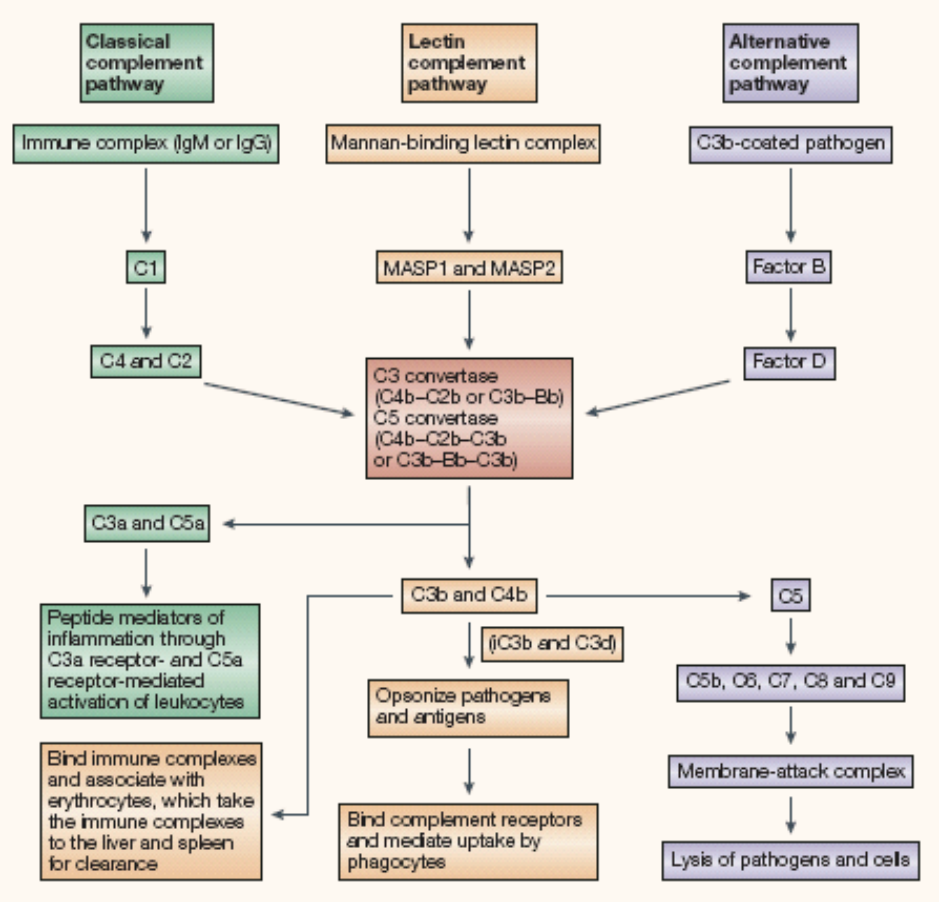


FIGURE 2. Overview of the complement activation pathways. Most of the components in the complement pathways exist as pro-enzymes and are activated when needed in a cascade-like fashion by each other. *Illustration used with permission from Michael Carroll.*

The complement system consists of several large and complex proteins, both serum and cell surface proteins, that help the immune system in its defense against harmful, foreign substances. This is done by cell lysis (e.g. bacterial cells, viruses), opsonisation, clearance of immune complexes and co-binding to cell surface complement receptors (CRs) that trigger

various immunologic mechanisms. There are three different ways in which complement is activated as shown in Figure 2; the classical, the lectin and the alternative pathways [7]. An antigen-antibody complex, most frequently IgM and its antigen, activates the complement via the classical pathway. The mannose-binding lectin (MBL) binds mannose on foreign surfaces, which in turn is recognized by so called associated proteases that can activate the complement. Finally, the alternative pathway is the result of slow but spontaneous cleavage of complement component C3 into C3b. Normally C3b is quickly inactivated by hydrolysis, but may be deposited onto microbial surfaces where it will be stabilized by binding of factor B. After cleavage of factor B into Bb by factor D, Bb still bound to C3 constitute the C3bBb complex, or C3 convertase. This enzyme will rapidly amplify the amount of C3b which in time will bind to the convertase itself and form C3bBbC3b, a C5 convertase. The component C3 is common for all three pathways, leading further down the pathway to the formation of C5b necessary for the lytic machinery. This machinery is a multimolecular structure called membrane attack complex (MAC) and it causes small perforations in cell membranes. The holes enable ions and small electrolytes to diffuse freely and the cell is killed by loss of electrolytes.

2.2 Project background

2.2.1 IgM mediated enhancement of antibody responses

Antibodies can feedback regulate the production of antibodies, being either enhancing or suppressing depending on antibody subclass involved. This regulation is of outmost importance because of the destructive nature of the immune system. Among the first experiments showing the regulating effects of antibodies were done in 1968 by Claudia Henry and Niels Jerne. Here, IgM was administered in mice prior to immunization with SRBCs. The result was an enhancement of the primary antibody response when suboptimal doses of SRBC were used [8]. Later on, it was shown that the enhancement by IgM was dependent on antigen-specific T-cells [9]. When SRBC-specific IgM was given together with SRBC, without adjuvant, enhancement of the primary antibody response also stimulated the development of memory B-cells required to obtain a secondary response, which also suggests the involvement of T-helper cells [10]. In addition to SRBC, IgM-mediated enhancement has been seen with other particulate or large antigens such as KLH [11] and malaria parasites [12].

However, the question of how IgM enhances immune responses is still unanswered and needs to be investigated. Starting with the hybridoma cell line PC7, Shulman et al. could in 1982 generate mutant cell lines, producing IgM molecules that did not activate complement. Mutant #13 was described as one of two mutants having pentameric secretory IgM but with complement activation defects. The mutant was generated by mutagenesis and suicide selection was done in agarose medium containing complement. Thus, cells secreting normal IgM were lysed because of the ability of their IgM molecules to activate complement, whereas IgM-defective mutants survived [13]. DNA of mutant cell lines were isolated and digested to obtain the fragment containing the site of mutation. This fragment was ligated into the pR-SP6 plasmid, substituting the wildtype DNA segment that encodes the Cμ region. This plasmid was transferred to igm-10 cell line [14]. By extensive cloning and sequencing, the group could narrow this mutation down to a single point mutation in the third constant region of the μ heavy chain where a substitution C → T resulted in the amino acid change from proline to serine at position 436. This change made in the IgM molecule made complement component C1q unable to bind and resulted in a 50-fold suppression of cytolytic activity [14].

Using IgM produced from this mutant cell line, it was shown that IgM which had lost its capability to activate complement also lost its ability to enhance antibody responses in mice [18]. In analogy, IgM in the form of monomers, that do not activate complement, does neither promote the deposition of antigen onto follicular dendritic cells nor enhance antibody responses [15, 16].

2.2.2 The importance of complement for an antibody response

A sufficient complement system is necessary for the generation of a normal antibody response [7]. The depletion of complement component C3 in mice by using cobra venom factor (CVF) resulted in suppression in antibody responses [17]. There is evidence showing that guinea pigs [19], dogs [20] and humans [21] have weakened antibody responses due to hereditary deficiencies in complement components C2, C3 or C4. Further evidence of the importance of the complement came with the knock-out systems in mice, where the lack of C1q [22], C3 [23] and C4 [24] proteins resulted in antibody response similar to that of mice suffered from genetical deficiencies in complement factors [19-21]. Neither complement factor B nor C5 knock-out mice exhibit significant changes in antibody responses to T-dependent antigens, suggesting that it is the classical pathway that is required [27, 28]. As the complement system comprises not only circulating proteins, but a number of cell surface receptors, Heyman et al.

in 1990 conducted experiments where monoclonal antibodies (mAbs) were used to block the activity of mouse complement receptors CR1 and CR2. They found that CR2 has an important role in antibody response and that blocking of this receptor suppressed more than 99% of the thymus-dependent primary antibody response *in vivo* [25]. Similar results were achieved in mice lacking CR1 and CR2 on B-cells. Without these receptors the mice lost their ability to generate a T-dependent antibody response [26].

It is now clear that specific IgM can enhance an antibody response, but that this phenomenon is dependent on the ability of IgM to activate complement. Ehrenstein et al. found that antibody responses were diminished in mice lacking natural secretory IgM and that there was a delay in the maturation of response to T-cell dependent antigens [29]. When these mice were "treated" with normal serum IgM (see Figure 3), the antibody response was restored to normal levels.

The results by Ehrenstein are strong evidences that natural IgM, i.e. antibodies circulating prior antigen challenge are needed for a normal antibody response. This is however not an obvious reasoning; how can an antibody with relatively low affinity for any antigen, solely cause such an effect on the antibody response? The answer to that question probably lies in the structure of the IgM molecule.

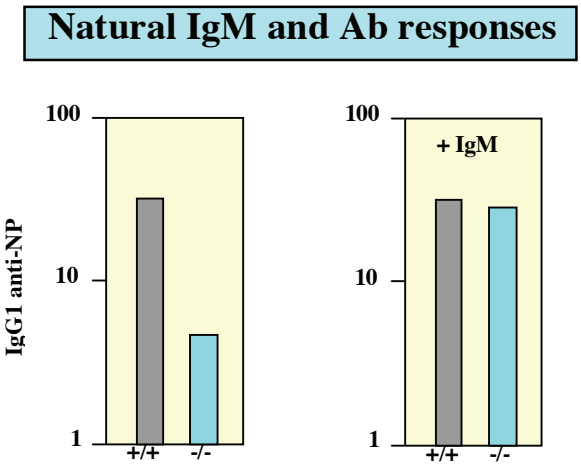


FIGURE 3. Antibody response restored after addition of normal IgM. *Illustration used with permission from Michael Ehrenstein.*

In serum form, the molecule exists as a planar star-shaped pentamer (five monomers of IgM are bound together to form a 950 kDa heavy molecule) [30]. The affinity for interaction between this natural IgM and an antigen is in this way increased. When binding to a large, particulate antigen such as SRBC takes place, the IgM molecule undergoes a conformational change that reveals the binding site for complement component C1q [31]. Since enhancement of immune responses by IgM in complex with smaller, soluble antigens is not seen, a theory is that with smaller antigen, the conformational change needed to activate complement cannot take place.

These findings are concluded in a theory of how antibody response is regulated by IgM. Natural IgM can despite its low affinity and specificity bind to antigen. This is done because of the unique pentameric structure of IgM compared to other antibodies. The interaction induces a conformational change in the IgM molecule, exposing the site for complement factor C1q to bind. When C1q has bound to the IgM-antigen complex it will become activated, inducing fragmentation of C3 into C3d which will bind to the IC. This large complex of antibody, antigen and complement components co-crosslink the B-cell receptor with CR2 (CD21)/CD19, inducing a signal within the B-cell to lower its threshold for activation. Once the specific B-cell is activated it starts to produce IgM with specificity for the antigen in question and further reinforcement of the B-cell activation is enabled. Finally, after about 6-7 days the B-cell will undergo an isotype switch initiating the production of the IgG antibodies.

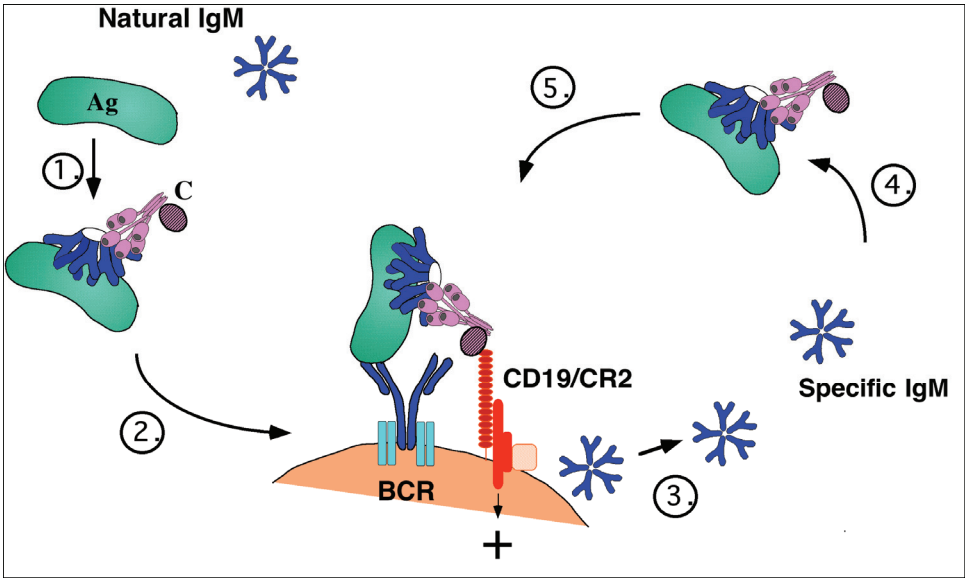


FIGURE 4. The theory behind IgM mediated activation of complement and how this result in enhancement of early antibody response via CR 2. **1)** Natural, non-specific IgM bind to antigen, which induces a conformational change. This activates a complement cascade, and complement components C1 and C3d bind to the antigen-antibody-complex. **2)** Activation of the B-cell occurs easier when this multimolecular complex bind to BCR and CR2. **3)** When the B-cell is activated it starts to produce IgM with specificity for the antigen, which in turn can bind more antigens. **4)** More complement can now be activated and a feedback loop has been generated **5)**.

2.3 Project description

2.3.1 Aim

In this project, the interaction between natural IgM and the complement system and their ability to enhance antibody production will be investigated. Therefore, in collaboration with Michael Carroll (Harvard University), a knock-in mouse has been constructed (not yet published) that has a mutation in position 436 in the constant domain of the μ heavy chain as described by Shulman [14]. The mutant mouse strain has the same variable gene repertoire and constant regions for all isotypes, except IgM, as in wildtype mice. The mutant mouse should be normal except in the ability of recruiting and activating complement via IgM.

Up until now, experiments done have been with normal and mutated IgM molecules produced by B-cell hybridomas, or natural with IgM in normal mice. This project enables studies on mutated IgM produced directly in the mouse, achieving a natural biological environment. In this study, I will try to elucidate the following questions:

- Can IgM from Cmu13 mice activate complement or not?
- Can IgM from Cmu13 mice enhance an antibody response?
- Is the antibody response lower in mutant than in wildtype mice? This would be expected if the capacity of natural IgM to mount a normal antibody response lies in its ability to activate complement.

2.3.2 Significance

Comprehension about the immune system and how it is regulated is of major importance for fundamental immunology. The complement and immune complexes play an important role in autoimmune diseases such as systemic lupus erythematosus (SLE). In this yet today incurable disease, one cause is that natural IgM binds many of the SLE auto-antigens (i.e. DNA and nuclear proteins). Through complement activation an inflammatory response against these self antigens is established [33].

3. MATERIALS AND METHODS

3.1 Antigen

Sheep Red Blood Cells (SRBC), a thymus dependent antigen, were purchased from the National Veterinary Institute (Uppsala, Sweden) and stored in sterile Alsever's solution at 4°C. Before use, erythrocytes were washed by centrifugation, 2500 rpm for 5 min, three times in PBS.

3.2 Mice

Male and female wildtype (see below), Cmu13 and Cmu13Z aged 8-12 wks and sex matched, were held at the National Veterinary Institute (Uppsala, Sweden). Cmu13 was made by Michael Carroll and Birgitta Heyman at Harvard University (data not published) and two breeding pairs were sent to us. The complement activation deficient mutant cell line was chosen by using the information provided by Shulman et al. [13, 14]. IgM gene segment was isolated from the mutant cell line #13 and ligated into the pR13μEi vector. This vector was transfected into a mouse embryonic stem cell from line 129/SV, using knock-in technology. Cmu13 mice were then created by backcross five times of 129/SV mice onto C57BL/6 mice and subsequently intercrossed. The created mouse strain Cmu13 was now homozygous for the mutation and the IgM allotype was IgM^a. In Uppsala, one additional backcrossing of Cmu13 onto C57BL/6 resulted in a F1 generation of heterozygotes which were mated with each other to generate F2s. F2 mice, homozygous for C57BL/6 IgM, were used as founders for the control mouse (hereafter wildtype) and F2s homozygous for Cmu13 IgM were used as founders for the Cmu13Z knock-in strain. The Cmu13Z strain was created because of the poor reproduction and defective jaws of the Cmu13 strain. Genotyping was done by PCR.

3.3 Immunizations and blood sampling

Mice were immunized intravenously in the tail vein with different doses of SRBC diluted in PBS ranging from $5x10^5$ cells per mouse up to 10^8 cells per mouse. Cells were counted in a Bürker chamber and exact dilutions were made from those calculations. Mice were bled from their tail veins either once every week during four weeks or on day five post immunization, when the IgM response is at its maximum [34].

3.4 ELISA

This technique allows for the detection and quantification of specific proteins, e.g. antibodies or antigens, in a sample. First, ELISA plates are coated with an antigen of choice and the sample is then added and incubated. Antibodies, specific for the particular antigen, bind to it and excess are washed away. A secondary enzyme-conjugated antibody which is specific for the primary antibody is added. After incubation, excess enzyme is washed away and the substrate for the enzyme is added [35]. The amount of change in color can be measured with a spectrophotometer and the OD-value measured is proportional to the concentration of antigen-specific antibody in the sample [10].

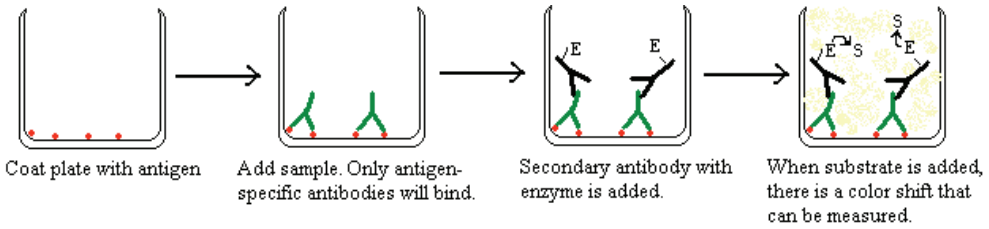


FIGURE 5. The ELISA experimental procedure step by step.

This technique was applied in the experiments in this project. Blood was collected from tail veins pre immunization, then every seventh day post immunization. Sera were extracted and tested using IgG anti-SRBC specific ELISA. Plates (Immunolon® 2HB, Thermo) were coated with SRBC by first adding 50µl (all volumes per well) of poly-L-lysine (25µg/ml in H_2O) and incubated in 37°C for 45 minutes. Plates were then briefly washed in desalted water. 100µl of washed erythrocytes (0.25% in PBS) were added and incubated in room temperature for one hour. To fix SRBCs, plates were put in glutaraldehyde (0.25% in PBS) for 8-10 minutes and thereafter washed three times in PBS. Sera were diluted 1:5 to 1:1250 in PBS + 0.05% Tween. Then 50µl of diluted sera were added in duplicates to the ELISA plates and incubated over night at 4°C. The detergent Tween, added to PBS, was used to prevent unspecific binding. Excess sera were washed away three times with PBS and 50µl of the secondary antibody, goat anti-mouse IgG-alkaline phosphatase (Jackson ImmunoResearch Laboratories, West Grove, PA), diluted 1:1000 in PBS + 0.05% Tween, was added and incubated three hours at room temperature in a humid chamber. In the next step, the excess secondary antibody was washed away four times in PBS and 100µl of enzyme substrate; tablets dissolved in ELISA buffer to a concentration of 1mg/ml (phosphatase substrate, Sigma-Aldrich, Inc. St. Louis, MO) was added. After incubation in room temperature for 60 minutes the ELISA plates were read at 405 nm and analyzed with a computer program (Softmax; Molecular Devices, Menlo Park, CA). The collecting of blood during four weeks enabled a

visualization of antibody responses during this period of time. Since no standard has been used, ELISA data should only be compared within the same experiment. OD values of blank samples (PBS + 0.05% Tween instead of serum) were subtracted from serum values. Statistical differences were determined by Student's t test. P values are presented as: not significant, ns; $P < 0.05$, *; $P < 0.01$, **; $P < 0.001$, ***.

3.5 ELISPOT

Through a modification of the ELISA set up, it is possible to measure the amount of cells producing antibodies specific for a certain molecule. Coating procedure and plates are the same as in ELISA. The samples added however, contain living cells in a culture medium (DMEM with 5 % FCS). After excess is washed away, an antibody specific for the molecule of interest, secreted by the cells, is added. This secondary antibody has an enzyme coupled to it, as in ELISA, and in the last step a substrate for the enzyme is added. After incubation it is washed away and the number of "spots" can be counted.

In this project cells to be counted were B-cells taken from mouse spleens. These mice were immunized with SRBC (dose 10^8) and spleens were taken five days after immunization. Spleens were mashed with a piston from a syringe in PBS to retrieve the B-cells. This cell solution was filtered through cloth and spun down in 1200 rpm for 10 minutes. The pellet was resuspended in 1 ml of DMEM. After appropriate dilution of this cell suspension (here 1:200 to 1:1600), 100µl of DMEM was first added to the plates and then 100µl of the samples were added. When added to the plates and incubated at 37°C in CO_2 for 2-3 hours, the cells will settle on the surface of the plate and secrete IgM specific for SRBC. These IgM molecules bind to SRBCs around each cell that is producing the specific IgM. After incubation plates were washed three times in PBS and 50µl of goat anti-mouse IgM-alkaline phosphatase (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:50 in PBS was added. After overnight incubation in 4°C plates were again washed three times in PBS. 50µl of a precipitating substrate BCIP/NBT Chromogen (R&D Systems, Inc. USA) was added for 30 minutes in room temperature. After that, plates were washed three times in PBS, once quickly in H_2O and left to dry. When developing the plates, antibodies produced by the single cells were visualized after 30 min as spots as shown in Figure 6. For every spot there is one B-cell producing IgM anti-SRBC. Duplicates and a number of dilutions were made. The number of spots was counted blindly under stereo microscope.

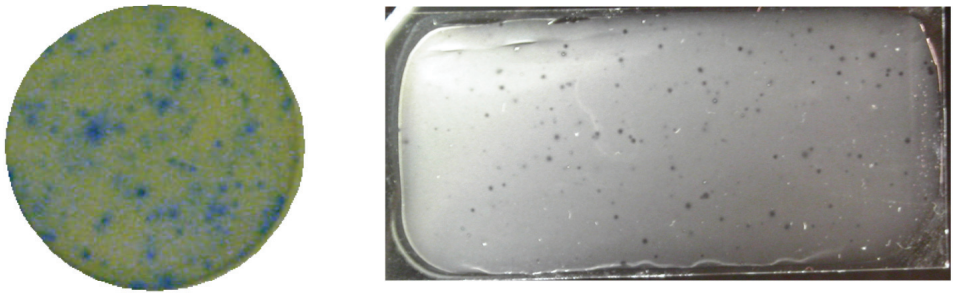


FIGURE 6. ELISPOT (left) and PFC (right) results. Spots and plaques are considered to be B-cells producing IgM able to activate complement.

3.6 Plaque Forming Cell Assay

The assay is developed for the detection and quantification of B-cells secreting antigen-specific IgM, not unlike the ELISPOT method. However, in this assay complement is added and therefore BSS must be used instead of PBS. This is because the complement proteins need the ions Na^+ and Mg^{2+} to function. After immunization with SRBC (doses $5x10^5$ and 10^8) spleens were taken on day five and mashed to single B-cells (same procedure as in ELISPOT) in 10 ml of BSS. 0.25g Low melting agarose (Seaplaque GTG) and 0.25g ordinary agarose ("USB ultrapure") were mixed with 100 ml of BSS, boiled until agarose is dissolved in microwave oven and set in water bath of temperature 45°C. SRBCs were washed in BSS and diluted to 10% volume/volume. Complement from guinea pig serum was thawed from -70°C and diluted 1:6 in BSS. 100µl (25µl) cell suspension, 25µl SRBC, 25µl complement-solution and 300µl agarose were mixed and immediately poured on glass slides. B-cells will during incubation (3 hours at 37°C) secrete SRBC-specific IgM that binds to the erythrocytes. In the presence of complement, the IgM will mediate lysis of the SRBCs and this reaction will appear as clear zones, or "plaques", in the agar matrix [36]. For every plaque there is one SRBC-specific IgM-producing B-cell and in this way, with proper dilutions, B-cells per spleen can actually be counted. The test was done with duplicates and in two dilutions (1:100 and 1:400 of whole spleen).

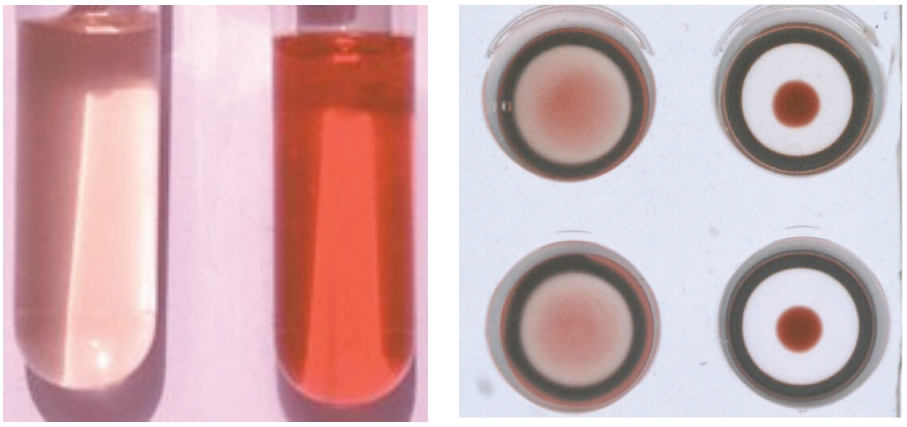
3.7 Hemolysis

This test is complement-dependent and therefore all dilutions must be made with BSS. Sera were collected from mice five days after immunization with SRBC (doses 10^6 and 10^8 cells/mouse). In V-shaped plates 50µl BSS and 50µl serum was added and 2-fold serial dilutions were made. Then 25µl 1% SRBC and 25µl of complement (final dilution 1:128) was added to each well. Incubation was carried out at 37°C for one hour. If the samples contain SRBC-specific IgM that have the ability to activate complement, the SRBCs will lyse. By

doing 2-fold serial dilutions starting with 1:4 volume of serum, the hemolytic titer can be determined.

3.8 Hemagglutination

This complement-independent test was used for the detection of SRBC-specific IgM molecules. The test is similar to the hemolytic test and was carried out in a similar manner. The major difference is that no complement is added. If the samples contain the SRBC-specific IgM, those antibodies will crosslink SRBCs which will then agglutinate. In negative samples, the SRBCs will fall down to the bottom of the wells forming a pellet. As with hemolysis 2-fold serial dilutions enables the titer of hemagglutination to be determined.



No hemolysis Hemolysis Hemagglutination No hemagglutination

FIGURE 7. Visual result of hemolysis test (occurrence of complement activation) and hemagglutination test (occurrence of IgM).

3.9 IgM purification

Two methods were used in attempts to purify IgM from mouse serum. Agarose with covalently attached goat IgG anti-mouse-IgM was used as the stationary phase in column chromatography (Sigma-Aldrich, Inc. St. Louis, MO). These polyclonal IgG antibodies specifically recognizes the μ-chain of mouse IgM molecules. Experiment setup was followed as recommended by Sigma-Aldrich protocol (see appendix for buffer ingredients) and serum used was from mouse strain CBA/J bled day five after immunization with SRBC. A two milliliter column of antibody-agarose was prepared using 4 ml of the antibody-agarose suspension. The column was equilibrated with a sodium phosphate buffer (PB, see appendix). Mouse serum, after having been centrifuged in 5000 rpm for 10 minutes, was applied slowly and the column was then once again equilibrated with PB. Serum was incubated in the column over night at 4°C. The column was then stripped by washing with elution buffer. Fractions containing the specific antibody were collected, brought to neutral pH by TRIS-HCl and measured for concentration by spectrophotometry at absorbance 280nm.

The other method for purification was dialysis against distilled water. In 1968 Claudia Henry and Niels Jerne showed that IgM and other euglobulines precipitate in distilled water (pH 5.5) [8]. An experiment was therefore setup according to a more recent protocol where it was shown that both IgG3 and IgM were purified through dialysis and precipitation over night [37]. Serum from CBA/J was dialysed over night at 4°C in 5 liters of demineralized water (pH 5.5). The precipitate was recovered and centrifuged at 22 000 x g for 30 minutes. The supernatant was decanted; the pellet was resuspended in 10 ml of demineralized water and then washed twice by centrifugation at 22 000 x g for 30 minutes. The pellet was resuspended in 1ml of PBS and spun down in 13,000 rpm for 1 minute. Supernatant was collected from this and adjusted to 1 ml with PBS. To check for IgM presence and activity, a hemagglutination test was done. Any IgG contamination was tested with IgG anti-SRBC ELISA.

4. RESULTS

4.1 Characterization of the Cmu13 knock-in mice

A total of 30 mice (all male) from groups Cmu13, Cmu13Z and WT mice were immunized intravenously with 0.2 ml of 10^6 or 10^8 SRBC/mouse. Spleens were taken on the fifth day after immunization when the IgM titer peaks, and tested for IgM anti-SRBC antibodies in direct plaque-forming cell assay (PFC, complement dependent) and ELISPOT (complement independent). At the same time point mice were bled and sera were tested in hemagglutination and hemolysis. The results are shown in Table 1.

Dose†		PFC‡	ELISPOT§	Hemagglut*	Hemolysis¶
10^6					
	Wildtype	2.98 ± 0.49 (1580) (n=5)**	ND	1:307 (n=5)	< 1:4
	Cmu13	1.89 ± 0.26 (90) (n=5) p<0.005	ND	1:218 (n=5)	< 1:4
	Cmu13Z	1.699 ± 0 (50) (n=5) p<0.001	ND	1:384 (n=5)	< 1:4
10^8					
	Wildtype	4.73 ± 0.04 (53320) (n=5)	4.92 ± 0.006 (82396) (n=2)	1:4096 (n=5)	1:2150 (n=5)
	Cmu13	2.24 ± 0.54 (300) (n=5) p<0.001	4.85 ± 0.01 (70382) (n=2)	1:3277 (n=5)	1:86 (n=5)
	Cmu13Z	1.99 ± 0.30 (120) (n=5) p<0.001	4.88 ± 0.12 (76424) (n=2)	1:4096 (n=5)	1:96 (n=5)

TABLE 1. Comparison of antibody responses in wildtype and mutant mice 5 days after immunization with SRBC.
† Dose is given in SRB cells per mouse.
‡ ^{10}log of direct PFC per spleen ± SD. Actual number of plaques as geometric mean in parenthesis.
§ ^{10}log of ELISPOT data. Actual number of cells producing anti-SRBC IgMs in parenthesis.
* Mean value of agglutination titer. Titer was determined as the highest dilution that still could cause agglutination.
¶ Mean value of hemolysis titer. Titer was determined as the highest dilution that still could cause hemolysis.
** n is number of mice

The PFC data shows a significant difference between control and mutant mice in complement activation by anti-SRBC IgM: there is about a 20-fold lower number of hemolytic plaque-forming cells after immunization with 10^6 SRBC per mouse and a 200-fold difference after immunization with 10^8 SRBC. No obvious difference in PFC data between Cmu13 and the Cmu13Z strains was observed. These PFC results suggest that the endogenous IgM antibodies in the mutant mice are unable to activate complement. The lack of PFC found could however be a consequence of that the mutant mice have low numbers of IgM-producing B-cells. Therefore, as a supplement to this test, ELISPOT was done on chosen samples. I found that the number of B-cells producing IgM anti-SRBC were the same in both wildtype and mutant mice. The ELISPOT was repeated with doses $5x10^5$ and $5x10^6$ SRBC per mouse using Cmu13Z and wildtype mice indicating the same results (data not shown).

The presence of SRBC-specific IgM antibodies in sera was also tested with a hemagglutination test. The mean titer value of hemagglutination was equally high in all mouse groups and was elevated with the higher immunization dose. This shows that there is a normal production of IgM in the mutant mice and that the antibodies produced do not lack hemagglutination properties. To assess whether these serum antibodies also could activate complement, a hemolysis test was done. In the group immunized with a low dose of SRBC, no hemolysis could be detected. However, in the group with mice immunized with 10^8 SRBC per mouse, mutant mice exhibit a 20-fold lower hemolytic titer.

4.2 Antibody response

Three similar but independent experiments were done in order to test the IgG antibody response in mutant mice upon immunization with SRBC (see Figure 8). In the first two experiments doses $5x10^5$ and $5x10^6$ SRBC per mouse were used and in the third experiment, only dose $5x10^5$ was used.

$5x10^5$ SRBC per mouse **$5x10^6$ SRBC per mouse**

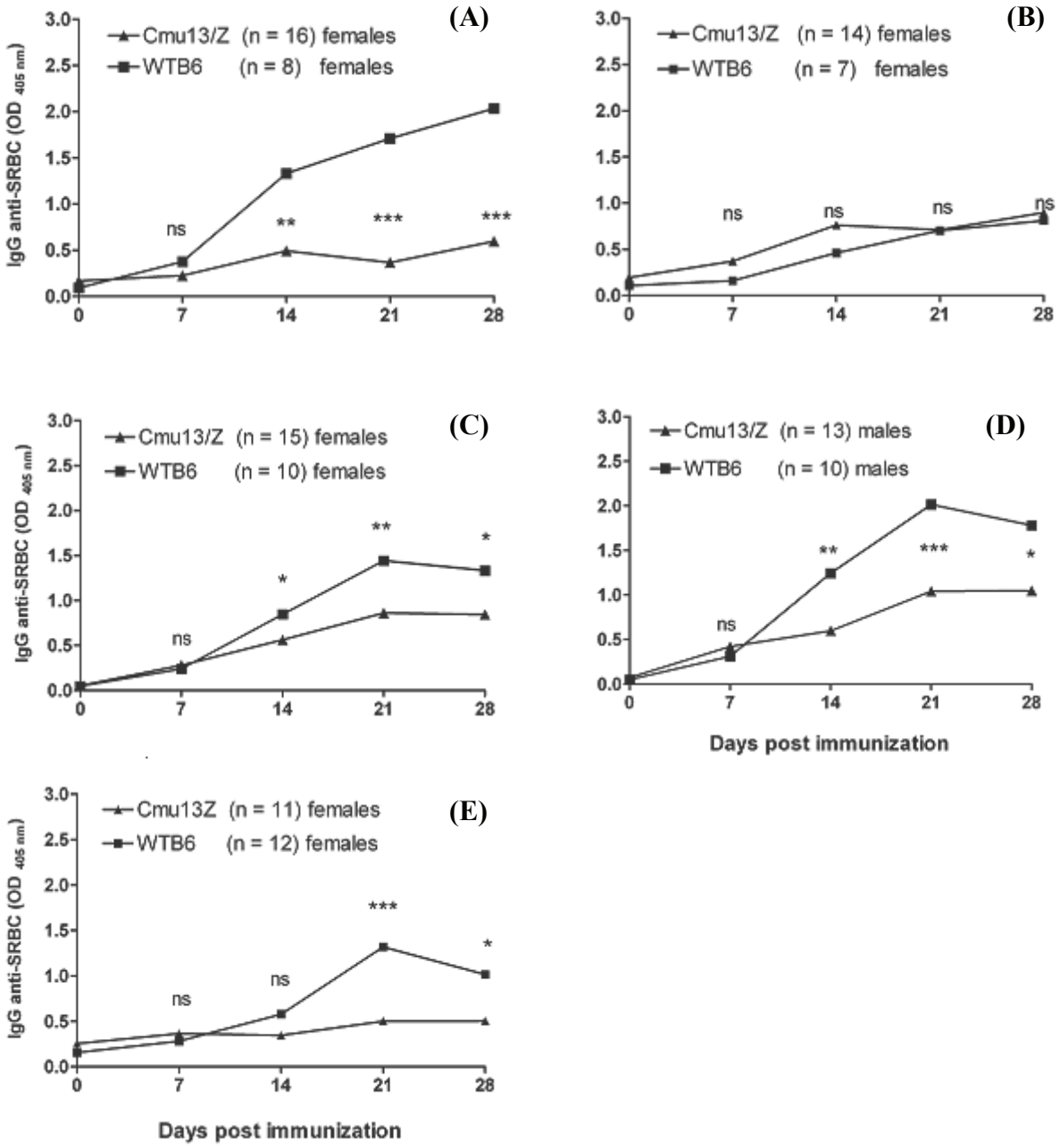


FIGURE 8. IgG response after immunization in wild type and mutant mice. Mean OD-values are given for samples diluted 1:25, doses are $5x10^5$ (graphs in left column) and $5x10^6$ (graphs in right column). P values are presented as: not significant (ns); $P < 0.05$ (*); $P < 0.01$ (**); $P < 0.001$ (***).

Pre-immune sera were obtained from individual mice approximately one week before they were immunized intravenously in tail veins with 0.2 ml (0.1 ml in experiments 2 and 3) of $5x10^5$ or $5x10^6$ SRBC per mouse. Mice were then bled once a week for four weeks and then sacrificed on day 28 post immunization. Sera from all mice were tested for IgG anti-SRBC using ELISA (see Figure 8A through 8E). Data generated by Cmu13 and Cmu13Z were pooled since no significant difference between them was seen (this was done after having seen data from several independent experiments). The left graphs show the IgG anti-SRBC response in mice immunized with $5x10^5$. Here, a significant difference in antibody response is seen in all three experiments. Wildtype mice have an expected increase in IgG responses with time, while the response in mutant mice is low. Mice immunized with $5x10^6$ SRBC per mouse (right column) have a more ambiguous response. In the first experiment (Figure 8B) there is a low response in both wildtype and mutant mice. Spleens of all 45 animals were checked for possible necrosis that might cause erroneous values, e.g. a wildtype having abnormally low response, but no correlation was found. In the second experiment (Figure 8D), the response in wildtype mice was significantly higher than the response in mutants.

4.3 IgM purification

Attempts were made to purify IgM from immune serum using an affinity column with goat IgG anti-mouse-IgM. All fractions were checked for OD-values using a spectrophotometer. However, according to spectrophotometric data no protein was eluted in the expected fractions. The serum was further incubated for longer periods of time and some refinements of buffers were done but without changes in result.

The euglobulin method for purification was then chosen. The precipitate formed after dialysis of serum in distilled water was positive for hemagglutination (i.e. the occurrence of IgM). However, an ELISA control test for IgG showed high amounts of contaminating IgG antibodies in the solution.

5. DISCUSSION

This work shows that endogenous IgM from the mutant mouse strain Cmu13 has a severely impaired ability to activate complement. This has been shown in the two complement-dependent tests PFC and hemolysis. In mice immunized with 10^6 SRBC per mouse, few conclusions can be drawn because of the low immune response generated in all mice. However, with 10^8 SRBC per mouse there is an obvious difference in test results between wildtype and mutant mice in both PFC and hemolysis. Sera from mutant mice surprisingly exhibit some hemolytic activity. The reason for this is not clear but a possibility is that IgG3, known to yield an early response to antigens containing carbohydrates [38], causes the lysis. The chance that several IgG3s bind to the same antigen sufficiently close enough to be able to activate complement factor C1, is relatively small. Interestingly, IgG3 possesses the property of cooperative binding, which means that once one IgG3 molecule has bound to a surface, it will facilitate the binding of other IgG3 molecules through Fc-Fc interactions [39].

An alternative explanation for the residual complement activation in sera from mutant mice is the presence of mannose-binding lectin (MBL). It has been known for a while that murine [40] and more recently human [41] IgM associates with rabbit MBL *in vitro*. This fact has greatly been used to purify IgM [40]. In 2006 M. Zhang and M.C. Carroll showed evidence that murine IgM bound to auto-antigen also bound and activated MBL, thereby engaging the complement system [42].

My results indicate that the mutation in IgM does not have major effect on the production of IgM in the knock-in mice. The hemagglutination titers for both immunization groups (10^6 and 10^8 SRBC per mouse) are similar in mutant and wildtype mice. The ELISPOT results are also good indicators, but not conclusive because only two mice per group were tested. Additional experiments are required to address this question, e.g. by using large mouse groups immunized with doses between 10^6 and 10^8 and test for IgM anti-SRBC production in ELISPOT or ELISA.

The most interesting result from this study was the reproducible finding that mutant mice produce less IgG anti-SRBC than wildtype. The three experiments were done following the exact same protocol in order to minimize errors. Reasons for the conflicting results seen in Figure 8B, that wildtype and mutant mice have the same IgG response, may be a

miscalculation or a mix up in doses used. In these experiments we do not expect a total lack of antibody response in mutant mice. If that would be the case, the mice would probably not survive a normal environment.

The presented data are compatible with the main theory of how natural IgM can activate complement, co-crosslink the B-cell receptor (BCR) and complement receptor (CR 2), and thereby enhance an antibody response (see Figure 4). There are two alternative, not mutually exclusive, theories, for how the co-crosslinking of BCR and CR 2 can be initiated. First, in the cell surface IC (immune complex) model [43], investigators have found that soluble monovalent antigens initially bind to the B-cell receptor, expressed on antigen-specific B-cells. This results in a multimeric array of antigens on the B-cell surface. Soluble IgM can then bind to the antigen matrix, undergo its conformational change when binding and thereby recruit complement factor C1q. Main advantages to this model, according to the authors, are that the model does not expect the formation of soluble IC, as with our model, which are quickly cleared from the circulation. Moreover, the multimeric array formed by antigens binding to BCRs helps natural IgM to bind, in spite of its low affinity for soluble antigens. Finally, membrane bound monomeric IgM (mIgM) can be forced to aggregate to other monomeric IgM by the cross-linkage caused by a polyvalent ligand and activate complement on the B-cell surface [44].

The purification of IgM will be continued, finding optimal conditions for the methods presented here or trying new methods. The main focus will be to deplete contaminating IgG from the euglobulin precipitates. With this accomplished the pure wildtype IgM solution will be used in experiments in attempts to “cure” the mutant mice and restore their antibody response. Purifications with mutant IgM will be tested for hemolysis, where the expected result would be a very low or no titer of hemolysis at all. Finally, the antibody response in mutant mice will be examined using other types of antigens such as KLH (thymus-dependent) and the thymus independent substance dextran.

7. ACKNOWLEDGEMENTS

I would especially like to thank my supervisors at Experimental Immunology, Department of Genetics and Pathology, Uppsala University:

Professor Birgitta Heyman for the opportunity to carry out my degree project, for interesting experimental ideas and endless enthusiasm. Also, huge thanks for feedback during the time for report compilation.

Post Doc Andrew Getahun for being the best supervisor a newcomer can have; always having the time to answer questions and having outstanding educational skills.

Lars Hellman at Department of Cell and Molecular Biology for the scientific review.

I would also like to thank all the guys in the writing room for well needed serious and less serious discussions.

And finally Michael Carroll at Harvard University, for giving us the Cmu13 mice.

8. APPENDIX

8.1 Buffers and media

1 L 10xPBS pH ~ 7.4

80.0 g	NaCl
2.0 g	KCl
14.40 g	Na_2HPO_4 x $2H_2O$
2.0 g	KH_2PO_4
1 L	MQ ultra pure water

Substrate buffer for ELISA pH ~ 9.8

800 ml	dH_2O
97 ml	diethanolamine
100 mg	$MgCl_2$ x 6 H_2O

Hank's balanced salt solution (BSS)

Stock solution A (x10)

10 g	glucose
0.6 g	KH_2PO_4
2.5 g	Na_2HPO_4 x $2H_2O$

Dilute to 1 liter with distilled H_2O, sterile filter and store at 4°C

Stock solution B (x10)

1.86 g	$CaCl_2$ x $2H_2O$
4.0 g	KCl
80 g	NaCl
2.0 g	$MgCl_2$ x 6 H_2O
2.0 g	$MgSO_4$ x 7 H_2O

Dilute to 1 liter with distilled H_2O, sterile filter and store at 4°C

Preparation of ready-to-use solution
8 parts distilled water and add 1 part of solution A and 1 part of solution B. Set pH = 6.9.

DMEM 500 ml for ELISPOT

2.5 ml	Hepes 2M
5 ml	PG Penicillin/Streptomyosin/L-Glutamin (Sigma-Aldrich)
0.25 ml	2-Mercaptoethanol 0.1M
5 ml	Na-pyruvat 0.1M
25 ml	FCS

8.2 IgM purification

Equilibration buffer PB 1L
29.22 g NaCl
1 L MQ ultra pure water, gives 0.5M NaCl
Add
1.38 g NaH_2PO_4
Adjust pH to 7.2 with 1 M NaOH.

Elution buffer 1L
75.07 g glycine
58.44 g NaCl
1 L MQ ultra pure water, gives 0.1M glycine and 0.5M NaCl
Adjust pH to 2.4 with 1 M HCl.

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