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# IgM and Complement in Regulation of Antibody Responses

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**Abstract**
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Animals deficient in complement components C1q, C4, C3, and CR1/2 have severely impaired antibody responses. C1q is primarily activated by antibody-antigen complexes. Antigen-specific IgM in complex with an antigen is able to enhance the antibody response against that antigen. This is dependent on the ability of IgM to activate complement. Naïve mice have very low amounts of specific antibodies and therefore it is surprising that classical pathway activation plays a role for primary antibody responses. It was hypothesized that natural IgM, present in naïve mice, would bind an antigen with enough affinity to activate C1q. To test this, a knock-in mouse strain, Cm13, with a point mutation in m heavy chain, making its IgM unable to activate complement was constructed. Surprisingly, the antibody responses in Cm13 were normal. Puzzled by the finding that the ability of IgM to activate complement was required only for some effects, the immunization protocol was changed to mimic an infectious scenario. With this regime, Cm13 mice had an impaired antibody response compared to wildtype (WT) mice. The antibody response in WT mice to these repeated low-dose immunizations was also enhanced. These observations suggest that IgM-mediated enhancement indeed plays a physiological role in initiation of early antibody responses. IgM-mediated enhancement cannot however compensate for the dependecy of T-cell help. Although IgM from WT mice enhanced the antibody response, the T-cell response was not enhanced. The connection between classical pathway activation and CR1/2 is thought to be generation of ligands for CR1/2. In mice, CR1/2 are expressed on B cells and follicular dendritic cells (FDC). Although CR1/2 are crucial for a normal antibody response, the molecular mechanism(s) are not understood. To investigate whether CR1/2 must be expressed on B-cells or FDC to generate a normal antibody response, chimeric mice between WT and CR1/2-deficient mice were constructed. The results show that $CR1/2^+$ FDC were crucial for the generation of antibody responses. In the presence of $CR1/2^+$ FDC, both $CR1/2^+$ and $CR1/2^-$ B cells were equally good antibody producers. However, for an optimally enhanced antibody response against IgM-antigen complexes, both B cells and FDC needed to express CR1/2.

*Keywords:* IgG, GC responses, feedback regulation, T-cell responses, antigen presentation, complement receptors 1 and 2

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*Den mätta dagen den är*
*aldrig störst.*
*Den bästa dagen är en dag av*
*törst.*

The sated day is never first.
The best day is a day of thirst.

From “I rörelse” in “Härdarna”
Karin Boye

# List of Papers

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

I Rutemark, C., Alicot, E., **Bergman, A**., Ma, M., Getahun, A., Ellmerich, S., Carroll, M.C., and Heyman, B. (2011). Requirement for complement in antibody responses is not explained by the classic pathway activator IgM. *Proc. Natl. Acad. Sci. 108, E934–E942.*

II Ding, Z., **Bergman, A**., Rutemark, C., Ouchida, R., Ohno, H., Wang, J.-Y., and Heyman, B. (2013). Complement-Activating IgM Enhances the Humoral but Not the T Cell Immune Response in Mice. *PLoS ONE 8, e81299*.

III **Sörman, A.** and Heyman, B. Endogenous feedback-regulation by complement-activating IgM (*Manuscript*)

IV Rutemark, C., **Bergman, A**., Getahun, A., Hallgren, J., Henningsson, F., and Heyman, B. (2012). Complement Receptors 1 and 2 in Murine Antibody Responses to IgM-Complexed and Uncomplexed Sheep Erythrocytes. *PLoS ONE 7, e41968*.

# Contents

# Abbreviations

| | |
|---|---|
| ADCC | antibody-dependent cell-mediated cytotoxicity |
| aHUS | atypic hemolytic uremic syndrome |
| APC | antigen presenting cell |
| BCR | B-cell receptor |
| BM | bone marrow |
| CD | cluster of differentiation |
| CR1/2 | complement receptor 1 and 2 |
| CTL | cytotoxic lymphocytes |
| CVF | cobra venom factor |
| CRP | c-reactive protein |
| DC | dendritic cell |
| DZ | dark zone |
| ELISA | enzyme linked immunosorbent assay |
| ELISPOT | enzyme linked immunospot assay |
| Fc | fragmen crystallizable |
| FDCs | follicular dendritic cells |
| FO B | follicular B-cell |
| GC | germinal centre |
| IC | immune complex |
| IgM, G.. | immunoglobulin M, G… |
| IgM-IC | antigen-specific IgM in complex with its antigen |
| KLH | keyhole limpet hemocyanine |
| LZ | light zone |
| mAb | monoclonal antibody |
| MASP | mannose associated serine protease |
| MAC | membrane attack complex |
| MBL | mannose binding lectin |
| MHC | major histocompability complex |
| MZ | marginal zone |
| NKT | natural killer T-cell |
| NK | natural killer cells |
| OVA | ovalbumin |
| PBS | phosphate buffered saline |
| PFC | plaque forming cell assay |
| PNH | paroxysmal nocturnal hemoglobinurea |
| SAP | serum amyloid P component |

| | |
|---|---|
| SHM | somatic hypermutation |
| SIGN-R1 | specific intracellular adhesion molecule-grabbing non-integrin related gene 1 |
| SRBC | sheep red blood cells |
| TCR | T-cell receptor |
| $T_H$ | T-helper cell |
| TD | T-dependent antigen |
| TNP | 2, 4, 6-trinitrophenol |
| TI | T-independent antigen |
| WT | wild type |

# Introduction

In a time when immune related illnesses, such as allergies and autoimmunity, are increasing and the power of antibiotics is decreasing, it is of great importance to gain knowledge about the underlying molecular mechanisms in the generation of normal immune responses. Many therapies against e.g. cancer and autoimmunity are also directly dependent on the immune system and the immuxne response. Knowledge of the basic immunological mechanisms may provide insights into how to better tackle the issue of choice.

The immune system is roughly divided into the innate and the adaptive immune systems. The innate immune response is rapid, has a short duration, little diversity on antigen recognition and no memory whereas the adaptive has a slow onset, can respond to a large range of antigens and develops a memory that upon repeated encounter with the same antigen reacts more rapidly.

This thesis deals with the interaction between the innate and the adaptive immune system (more specifically between the complement system and antibody responses) and what regulatory properties IgM has on the antibody response. The model lymphoid organ studied is the mouse spleen.

## The mouse spleen

The spleen is the largest secondary lymphoid organ in the body and its main functions are to remove damaged/old erythrocytes and pathogens from the blood stream and to initiate an adaptive immune response against these blood-borne antigens. Individuals that for some reason have been splenectomized are highly susceptible to pneumococcal and meningococcal infections (i.e. encapsulated bacteria).

There are two main compartments in the spleen, the red and white pulp (Figure 1A). In the red pulp, the blood is filtered through sinusoids, which slows down the blood circulation and makes it possible for the macrophages to remove opsonized antigens, damaged cells and microbes. The white pulp is where the leukocytes home and is the area where the adaptive immune responses against blood borne pathogens are initiated. The white pulp is largely divided into the marginal zone (MZ) that constitutes the border between red and white pulp, the B-cell follicle and the T-cell zone (Figure 1B). The blood enters the spleen through the central splenic artery, and is further

transported into the trabecular arteries from where the central arteriole that passes through the white pulp stems. The central arteriole is further divided into several follicular arteriola which all end up in the marginal sinuses that circle around the white pulp (Figure 1C). The different compartments in the white pulp consist of different types of leukocytes with distinct functions (Figure 1C). B-cells that home in the MZ has a different phenotype from the B-cells in the follicle (FO). The MZ B-cells can circulate from the MZ into the FO and back again (1) whereas FO B-cells can recirculate between the peripheral blood and the FO (2)**.** The MZ have an outer layer of MZ macrophages (MARCO$^{+}$, SIGN-R1$^{+}$), and an inner layer of metallophilic macrophages (MOMA$^{+}$, CD169$^{+}$) (3). This inner layer forms a tight ring around the FO B-cells and can thus be used as a line, separating the MZ from the FO in histological pictures. One other important cell population in the FO is the follicular dendritic cells (FDCs). These cells are stromal cells (4) and not leukocytes and are not to be mixed up with the dendritic cells (DC). The FDCs use their dendrites to form a tight network within the B-cell follicle.

The T-cells surround the central arteriole, explaining why the T-cell zones also are named periarteriolar lymphoid sheath (PALS). The T-cell zone in turn is on one side surrounded by FO B-cells and on the other side by the MZ bridging channel.

In this thesis, the B-cell compartments are being studied. After antigen encounter, germinal centers (GCs) will be generated in the B-cell follicle. In these somatic hypermutation and affinity maturation occur.

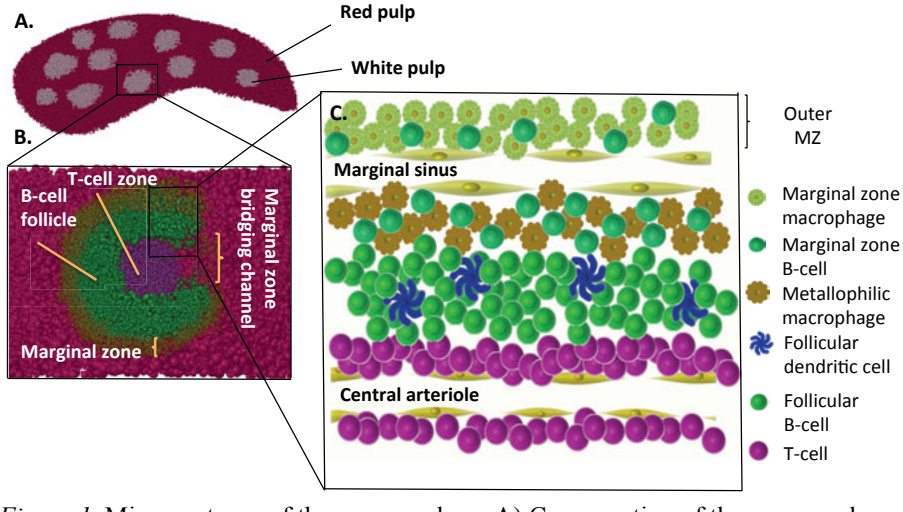


*Figure 1*. Microanatomy of the mouse spleen. A) Cross-section of the mouse spleen. B) Different compartments of the white pulp. C) Anatomical localization of different cells types of the white pulp.

## T-cells

T-cells, like B-cells, originate from the bone marrow (BM), but they mature in the thymus. In the thymus the T-cells start to express their antigen binding receptor, the T-cell receptor (TCR). The TCR recognizes protein antigens that are displayed as peptides by major histocompability complex (MHC) molecules. To be sure the TCR can recognize and bind MHC, only T-cells that bind to MHC will survive, i.e. positive selection. In the next step, to avoid auto-reactive T-cells, T-cells that bind too hard will be eliminated, i.e. negative selection. There exist two different types of MHC: class I, that is expressed on all nucleated cells in the body, and class II that is restricted to antigen presenting cells (APC). T-cells are further divided into T helper ($T_H$) cells and cytotoxic T-cells (CTL) depending on the expression of the glycoproteins CD4 ($T_H$) and CD8 (CTL).

### T helper cells

$T_H$ cells become activated when they are presented with peptide antigens by MHC class II on APCs. Once activated, they divide rapidly and secrete cytokines that regulate / assist the immune response. Depending on the signals (cytokines) the T-cell gets from the APC, they can differentiate into several different subtypes: $T_H1$, $T_H2$, $T_H17$, or $T_{FH}$ which in turn secrete different cytokines to drive the immune response in different directions depending on antigen/pathogen.

### Regulatory T-cells

Regulatory T-cells ($T_{reg}$) come in many subtypes. The most well understood subtype are those that express CD4, CD25 and Foxp3 (5). The major role of $T_{regs}$ is to shut down the T-cell mediated immunity towards the end of an immune reaction and to suppress auto-reactive T-cells that escaped the process of negative selection in the thymus.

### Cytotoxic T-cells

CTL become activated when they are presented with peptide antigens by MHC class I on a nucleated cell. Their main function is thus to recognize and destroy virus-infected cells and tumor cells.

### Natural killer T-cells

Natural killer T-cells (NKT-cells) are considered to be a link between the innate and adaptive immune system. NKT-cells express the TCR but share some other features with the innate natural killer (NK) cell. Unlike conventional T-cells, that recognize peptide antigens presented by MHC, NKT-cells recognize glycolipid antigens presented by CD1d. Once activated, NKT-cells produce cytokines and release cytolytic molecules.

## Follicular dendritic cells

FDCs are found in the lymphoid follicles of lymph nodes, spleen and mucosal lymphoid tissues. The FDCs are radiation resistant stromal cells with membranous projections/dendrites that intertwine the B-cells in the follicles. Unlike conventional dendritic cells (DCs), which are BM-derived, FDCs develop from vascular mural cells (4). FDCs produce CXCL13, the ligand to the receptor CXCR5 that are expressed on B-cells and a specific subtype of T-cells, thereby attracting these cells into the follicles (6).

The FDCs are crucial for GC maintenance, somatic hypermutation (SHM) and long-term immune memory, due to the ability to retain intact antigen/immune complexes (ICs) for extended periods (7). The cells express complement receptor 1 (CR1) (8) but also complement receptor 2 (CR2) (9, 10) and Fc receptors (11). It is through these receptors the FDCs are able to trap ICs and then recycle them in endosomal compartments, thus protecting the antigen from degradation (9). In addition FDC promote B-cell survival in GCs through production of IL-6 and BAFF (12, 13).

## B-cells

B-cells can be divided into B1- and B2 B-cells depending on their origin. Due to a specific gene rearrangement, (i.e. V(D)J recombination) each B-cell carries a unique antigen binding B-cell receptor (BCR) on its surface. The combined effect, make all B-cells together capable of recognizing more than $5 \times 10^{13}$ different antigens (reviewed in (14)). In the last step of rearranging the BCR, an IgM molecule is shaped and expressed on the B-cell surface, i.e. immature B-cells.

B1 B-cells originate from fetal precursors but are self-renewing in adults and mainly found in the peritoneum. They spontaneously produce natural antibodies, which have a low affinity but broad specificity against many different antigens. B1 cells are recognized through a combination of different surface receptors: $IgM^{high}$, $IgD^{low}$, $CD23^{low}$, $CD11b^{+}$, $B220^{low}$, and either $CD5^{+}$ (B1a) or $CD5^{-}$ (B1b) (reviewd in (15)).

B2 B-cells originate from the BM. After the final step of rearranging the BCR, the immature B-cells leave the BM to migrate to the spleen, where they continues to mature via transitional stage 1 (T1) and 2 (T2). T1 B-cells can be distinguished by the combination of $CD24^{high}$, $IgM^{high}$, $IgD^{low}$, $CD23^{low}$, $CD21^{low}$ whereas T2 B-cells are $CD24^{high}$, $IgM^{high}$, $IgD^{high}$, $CD23^{high}$ and $CD21^{high}$ (16). In the next step the B-cells have matured and now have the surface repertoire $IgM^{low}$, $IgD^{high}$, $CD21^{intermediate}$ and $CD23^{intermediate}$ (16).

### Follicular B-cells

The majority of the B2 B-cells will further develop into FO B-cells. FO B-cells are circulating B-cells defined as $IgM^{low}$, $IgD^{high}$, $CR2^{intermediate}$, $CD1d^{low}$ $CD23^{high}$ (17). The FO B-cells are located in the follicles of the lymph nodes

or in the follicles of the white pulp of the spleen. Their main function is to recognize foreign antigens, and (with T-cell help) become activated, form a specific B-cell clone (clonal selection), which is one of the cornerstones in the GC reaction. In the GC, the activated cells will further differentiate into plasma blasts (antibody producing cells) or memory B-cells. This process will be described in more detail below.

**Marginal zone B-cells**

MZ B-cells are defined as $IgM^{high}$, $IgD^{low}$, $CR1/2^{high}$, $CD1d^{high}$ $CD9^{high}$ and $CD23^{low}$ (17). They also express high levels of MHC class II, and the T-cell stimulating molecules CD80 and CD86 (18). Murine MZ B-cells are thought to be sessile cells but it has been described by Cinamon *et al.* (19) that MZ B-cells shuttle back and forth from the MZ to splenic follicles. The shuttling function of the MZ B-cells is due to the balance between their expression of CXCR5 and sphingosine 1-phosphate (S1P) receptors (1, 19).

The strategic location of the MZ B-cells in the MZ makes them an important cell in the first line of defense against blood borne antigens and pathogens (20, 21). MZ B-cells are easily activated by thymus-independent (TI) antigens and depletion of MZ B-cells resulted in an impaired IgG3 and IgG2a response against TNP-ficoll (20).

It is also suggested that MZ B-cells are important in the generation of the first wave IgM antibodies in responses against thymus dependent (TD) antigens (20, 22, 23). TD antigens are protein antigens against which the B-cells need T-cell help for complete activation and initiation of antibody production, Although B-cells are thought to be poor APCs as compared to DCs and macrophages, studies have shown that MZ B-cells can function as potent APCs and activate naïve T-cells (18, 24).

## Immune responses and germinal center formation in mouse spleen

**Antigen transportation**

Antigens that are trapped in the spleen are all delivered via the blood stream. While the blood passes through the marginal sinuses, blood circulation slows down, favoring encounter between blood-born antigens and leukocytes in the MZ. From the MZ antigens can reach the follicle via different pathways. The splenic conduit system can directly transport small blood-born antigens to the white pulp (25). Larger antigens are dependent on cell surface receptors on B-cells. As mentioned above, MZ B-cells are able to shuttle between the MZ and the B-cell follicles (19, 26). Several studies show that complement containing ICs are transported from MZ via the CR1/2 on MZ B-cells and delivered to FDCs in the follicle (19, 27, 28). In mice lacking C3 and CR1/2,

IgM-antigen complexes were trapped in the marginal zone and did not move further onto FDCs (28, 29). In mice with reduced numbers of MZ B-cells (but not with reduced numbers of FO B-cells), IgM-IC were trapped less efficiently (29). The observations that IgM-IC are transported on MZ B-cells and are deposited on FDC, and that this is dependent on IgM being able to activate complement (29), suggest that the transport of IC is dependent on CR1/2. Mice that lack the B-cell shuttling function, have higher amount of activated C4 on their MZ B-cells, indicating that offloading of complement-containing ICs does not function (19). Thus, without MZ B-cells, less antigen will be delivered into the follicle for further immune activation (19, 22, 28). However in case of IgE-IC, circulating FO B-cells are able to bind and transport IgE-IC on their low affinity receptor for IgE, CD23, (FcεRII) and transport the IC to the follicles (30, 31).

As this thesis will show, there are yet other pathways to be discovered.

**Germinal center formation**

Once the antigen is in the follicle, it is recognized by naïve specific FO B-cells. These B-cells will then migrate towards the T-cell zone, through increased expression of CCR7 and reduced expression of CXCR5 (Figure 2: 1.). Stromal cells in the T-cell zone constantly express high levels of the ligands for CCR7, CCL19 and CCL21 (32, 33). $CD4^+$ T-cells are simultaneously activated by APCs, e.g. DC that have ingested and displayed the antigen on their MHC II (Figure 2: 2.). A subgroup of T-cells will up-regulate CXCR5 and down-regulate CCR7 causing them to migrate towards the B-cell boarder (Figure 2: 2.) ((34, 35)). At the T-B-cell border, the antigen specific B-cells have internalized and processed the antigens and displayed them on their MHC II and now present them to the activated T-cells, which then will provide the B-cells with further stimulating signals (Figure 2: 2.) ((34, 35)). Some of these activated B-cells will differentiate directly to short-lived extra-follicular plasma cells, mainly producing IgM (36) (Figure 2: 3a.). The majority of the activated B-cells however, will proliferate and form the dark zone (DZ) of the GC. In the DZ B-cells will undergo high proliferation and somatic hypermutation and then migrate to the light zone (LZ) of the GC (37) (Figure 2: 4.). The organization of the GC requires CXCR4 to direct cells to the DZ, and CXCR5 to direct them to the LZ (38). In the LZ, some of the activated T-cells are further triggered by the B-cells to upregulate CXCR5 and thereby differentiate into $T_{FH}$ cells (figure 2: 3b). This upregulation of CXCR5 retains in the LZ (38–40). In the LZ, B-cells with previously mutated BCR, meet FDC that display complement-opsonized intact antigens on their dendrites (9). B-cells which, due to hypermutation of the BCR, have high affinity for the antigen, will capture the antigen, process and display it on their MHC to a limited number of $T_{FH}$ cells, which in turn provides the B-cell with the necessary survival signals (Figure 2: 5.) (41–43). This mechanism will ensure survival of B-cells with the highest affinity for

the antigens (44). The high affinity B-cells undergo class-switch recombination and some of them will return to the DZ (Figure 2: 6) whereas some of them differentiate to memory B-cells or plasma cells that secrete antibodies with high affinity (Figure 2: 7.). Although the molecular mechanisms behind affinity maturation of antibodies and access of antigen to B-cells are not fully understood, one can conclude that FDCs and $T_{FH}$ cells play very important roles.

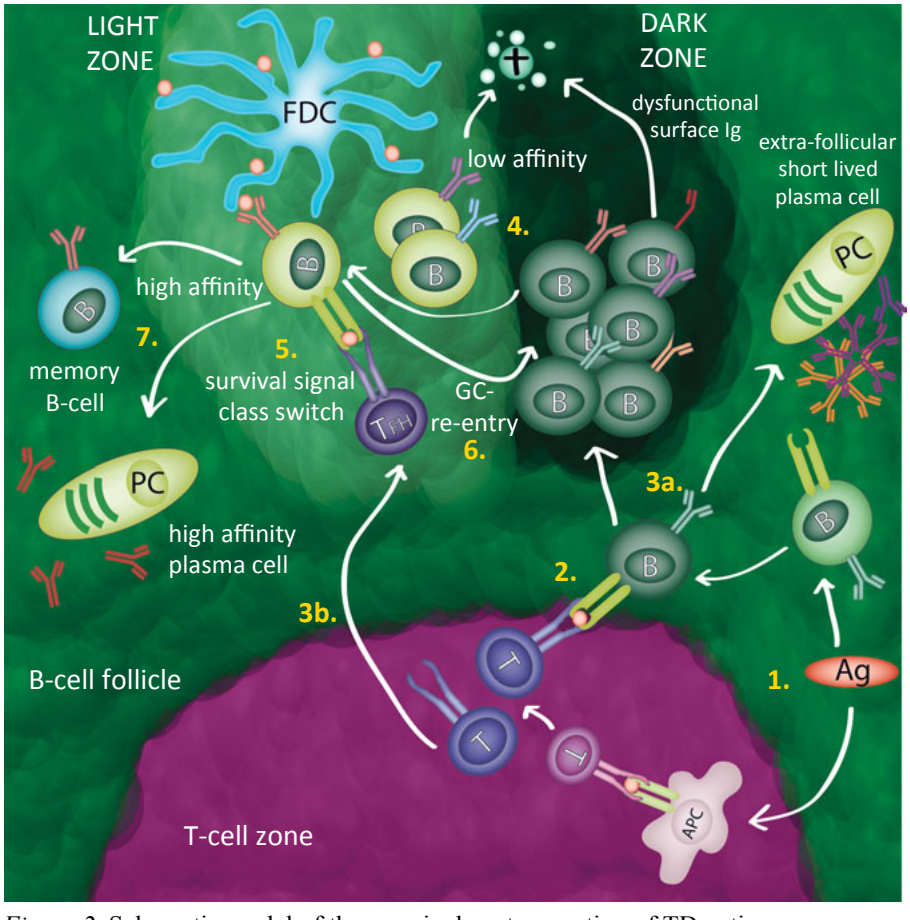


*Figure 2*. Schematic model of the germinal center reaction of TD antigens.

(1) After antigen encounter T- and B-cells are being activated.
(2) They meet at the T-B-cell border, receiving additional signals.
(3a) The B-cells differentiate into extra-follicular plasma cells or proliferate and form the DZ of the GC.
(3b) The T-cells differentiate into $T_{FH}$ and migrate into the GC.
(4) After proliferation and SHM the B-cells migrate to the LZ.
(5) In the LZ they encounter the antigen on FDCs and receive additional survival signals by $T_{FH}$. Only B-cells with high affinity BCRs survive.

(6) Some of the high affinity B-cells return to the DZ for another round of hypermutation whereas

(7) some differentiate into memory B-cells or high affinity plasma cells exiting the follicles.

# Antibodies

Antibodies, or immunoglobulins (Igs), are the secreted form of the BCR. They are built up by two heavy chains and two light chains, which in turn are divided into variable and constant regions. In the variable region the antigen-recognizing parts ($F(ab)_2$) are found, whereas part of the constant domain of the heavy chain (Fc-part) interacts with the immune system. Depending on the structure of the constant part of the heavy chain the antibodies can be divided into several different isotypes: IgA, IgD, IgE, IgG and IgM. Murine IgG can further be divided into the subclasses, IgG1, IgG2a, IgG2b (IgG2c in some mouse strains) and IgG3.

The first antibody isotype being produced during an immune response is IgM. If the B-cell receives further activating signals, it may switch the constant μ region to any other constant region (α, ε or γ) however without changing the binding specificity, determined by V(D)J recombination, towards the antigen. Once the Igs have bound to an antigen, the Fc-part may bind to Fc-receptors (FcR) and mediate an effector function, e g phagocytosis, hypersensitivity, or antibody-dependent cell-mediated cytotoxicity (ADCC). Some of the isotypes are able to activate complement and further regulate the antibody production.

This thesis focuses on IgM's regulatory functions, and therefore only IgM will be described in detail below.

**Structure of IgM**

IgM is phylogenetically the oldest antibody class and as all Igs, membrane bound IgM is built up by two μ heavy chains and two light chains (κ or λ) (Figure 3A). One μ-chain includes five domains; VH, Cμ1, Cμ2, Cμ3 and Cμ4 (figure 3A). In each of the Cμ2-Cμ4 domains there is a cysteine that can bind to other μ domains in pairs (45–48). The availability of such cysteines makes the IgM molecule unique in capacity to form covalent disulfide inter-heavy chains bonds, which makes the IgM molecule able to polymerize. Most commonly the polymerization of the IgM molecules results in a pentamer (Figure 3B). However if the J-chain is absent, the assembling of the IgM predominantly results in a hexamer (49, 50). Accordingly, factors that affect the interaction of the J-chain and the μ heavy chain, e.g. glycosylation, may influence how IgM polymerize. The tertiary structure of the IgM pentamer and hexamer have been described as a mushroom shaped structure with the Cμ2-3 as the head and Cμ4 the stem (51, 52).

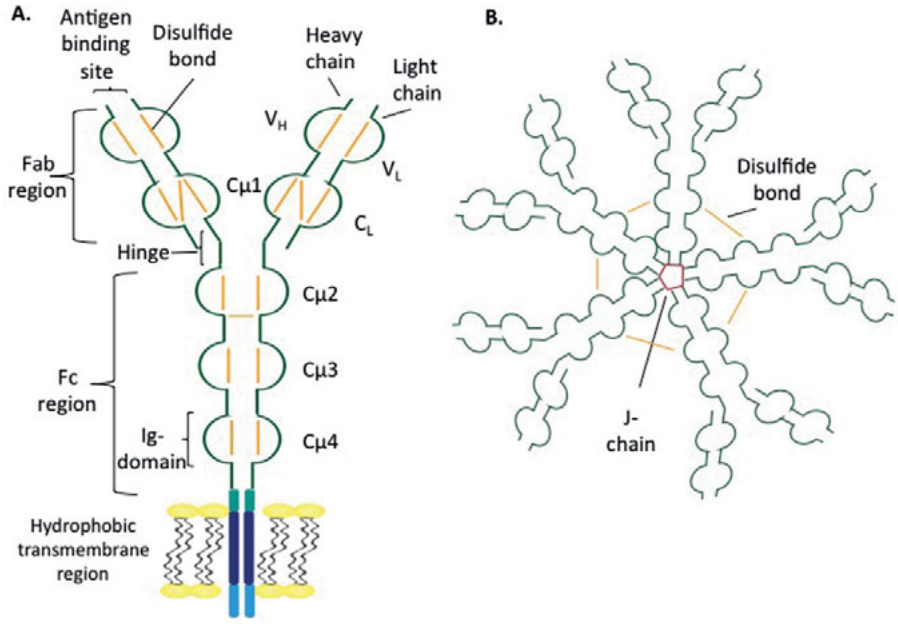


*Figure 3*. Schematic model of IgM. (A) Monomeric IgM / BCR. (B) Pentameric IgM / soluble IgM.

**Immune IgM**

After antigen encounter and stimulation of FO B-cells, IgM-antibodies specific for that specific antigen are produced. Five days after the antigen encounter the specific IgM production is at its highest. Thereafter most B-cells switch to produce another Ig class. Therefore most long-lived plasma and memory B-cells produce antibodies of switched isotypes (IgG, IgA or IgE), but long-lived IgM-producing memory B-cells also exist in both mice and humans (53, 54) with either mutated or unmutated BCRs. On the contrary, TI antigens induce IgM production but rarely lead to class switch recombination and do not result in memory B-cells. Thus, against these types of antigens IgM is the most abundant isotype (reviewed in (15)).

**Natural IgM**

Antibodies are not only produced after antigen activation of the B-cells, but are also produced without any previous antigen encounter. IgM circulating prior to antigen challenge are denoted natural IgM. Natural IgM are primarily produced by a subset of peritoneal B-cells, B1 cells (reviewed in (15)). These types of antibodies are present in germ- and antigen free newborn mice (55). Natural IgM show a low affinity against a wide range of antigens and play an important role in early defense against bacteria and viruses (56).

For instance, it has been shown that mice lacking circulating natural IgM, have a reduced viral clearance and thereby higher mortality than WT mice with normal IgM secretion (57).

In line with these observations, it has been shown that mice deficient in secretory natural IgM have impaired antibody responses (57–59) and that their responses could be restored after reconstitution with WT naïve sera (containing secretory natural IgM) (27, 57, 59).

**Fc Receptors for IgM**

FcRs are proteins that are mainly expressed on a variety of immune cells and bind the Fc part of antibodies. There exist several different types of FcRs that mediate different effector functions after ligand binding. The Fcα/μR bind both IgM and IgA, but the affinity for IgM is about 10-fold higher than for IgA (60, 61). The receptor is expressed on non-lymphoid tissue (e.g. kidney or intestine) as well as B-cells, macrophages and FDCs. It has been suggested that the Fcα/μR is important in mucosal immunity since it is expressed in the gut. It can endocytose IgM-coated particles (61), but mice that lack the receptor have normal antibody responses against TD antigens and increased GC response to some TI antigens (62).

The FcμR, formerly known as Toso/Faim3, binds IgM exclusively. The receptor has been identified both in humans and in mice, although the murine FcμR has only 54 % sequence homology with the human receptor (63). The murine FcμR is only expressed on B-cells (64) whereas the human counterpart is expressed on B-cells, T-cells and NK cells (63). Mutant mice that lack the FcμR have higher levels of IgM and IgG3, higher levels of natural autoantibodies, elevated antibody responses against T-independent type II antigens (e.g ficoll), but reduced responses to some T-dependent antigens. In addition, the $Fc\mu R^{-/-}$ mice have reduced levels of MZ B-cells and increased levels of B1-cells (64, 65).

## The complement system

The complement system is part of the innate immunity/defense against infections. It consists of several plasma proteins, mainly secreted by hepatocytes, macrophages and monocytes (66). When the complement cascade is activated, a proteolytic cascade is initiated and as the cascade proceeds, a amplification of the proteolytic products is generated. The complement system can be activated by the classical, lectin and alternative pathways (Figure 5).

*The classical pathway* is initiated upon binding and activation of component C1q. The most common C1q activators are antibodies bound to an antigen. C1q can also bind directly to antigens and other soluble proteins of the innate system (described further below). Once C1q is activated, the proteolytic cascade continues with the cleavage of C4 and C2 forming a C3 con-

vertase, cleaving C3, forming C5 convertase and finally cleaving C5 thereby initiating the lytic pathway (Figure 5).

*The lectin pathway* is activated when proteins like mannose binding lectin (MBL), the ficolins and collectin recognize their ligands, such as sugar molecules on microbes, on dying host cells or on subendothelial matrix (67, 68). Thereafter, the process continues like in the classical pathway with the cleavage of C4 and C2 forming a C3 convertase, cleaving C3, forming C5 convertase and finally cleaving C5 thereby initiating the lytic pathway.

*The alternative pathway* is continuously undergoing a low-grade activation due to hydrolysis of the internal C3 thiol-ester bond. It is further activated when there is an imbalance between activation and inhibition e.g. on foreign surfaces or structures lacking complement regulatory proteins (66). Thereafter factor B is cleaved forming a C3 convertase. Additional cleavage of C3 then results in the formation of the C5 convertase. From this point, all three pathways are united, the terminal pathway is initiated and the terminal complement complex (C5b-9) is formed. When C5b-9 is formed on a membrane, it constitutes the membrane attack complex (MAC), which penetrates the membrane and leads to lysis of cells and bacteria. However, if there is no surface to be attacked, a soluble form of the C5-9 complex (sC5b-9) (stabilized with vitronectin and clusterin) is formed. In addition to anaphylotoxins, produced from upstream cleavage products, sC5b-9 can be used as a marker for complement activation in body fluids, e.g plasma, cerebrospinal or synovial fluids (66).

All three complement pathways are regulated by soluble proteins (C1-inhibitor, factor H, factor I, C4-binding protein, carboxy-peptidase, vitronectin and clusterin) controlling the low grade of auto-activation in the soluble phase. There are also membrane bound proteins; membrane cofactor protein (CD46), CR1 (CD35), decay accelerating factor (CD55), which all control the activation of C3 and C4, and CD59 that protects from the final formation of C5b-9-complex (66). Lack of regulatory factors or dysfunctional regulation of the complement system can be associated with clinical disease, e.g. paroxysmal nocturnal hemoglobinurea (PNH) when CD55 and CD59 are missing (69)**,** or atypical haemolytic uremic syndrome (aHUS) when factor H is dysfunctional or missing (70). The regulators are thus very important to maintain complement homeostasis.

**Component C1q**

The glycoprotein C1q is built up by the polypeptides A, B and C that form a triple helix. Six of these in turn form a hexamer Two of each of the serine proteases C1r and C1s bind to the C1q molecule, forming the C1 complex (reviewed in (71)). $C1qA^{-/-}$ mice lack the A polypeptide and therefore cannot form a complete C1q molecule (72). C1q is mainly produced by macrophages and dendritic cells (73, 74) but also by FDC in GC (73). In contrast most of the other complement proteins, including C1r and C1s, are produced by

the liver, (75). When C1q recognizes and binds to a target, it undergoes a conformational change that causes C1r to auto-activate and cleave C1s (76) and thus the C1q molecule is activated. C1q are important in clearance of apoptotic cells (77), and deficiency of C1q strongly predisposes to development of the rheumatic disease systemic lupus erythomatosis (SLE) (reviewed in (78)).

**Activators of C1q**

For a long time it was believed that only IgM or IgG could initiate the classical pathway and that activation began only after one IgM pentamer or at least two IgG molecules, situated at most 30-40 nm from each other, bound to an antigen. However, further investigations of these processes have revealed a number of other activating ligands for C1q (Table 1) (reviewed in (71)). The ligands involved in this thesis will be described below.

Table 1. *C1q-activating ligands. Adapted from* (71).

| | **Activating ligand for C1q** |
|---|---|
| Immunoglobuins | Fc portion of IgM, IgG1, IgG2, IgG3 (human).<br>Fc portion of IgM, IgG1 (?) IgG2a, IgG2b and IgG3 (mouse) |
| Other proteins | Ligand bound SIGN-R1, CRP and SAP, PTX3 amyloids, prions, fibrin clots |
| Polyanions | Heparin, Chondroitin-4-sulphate, single- and double-stranded DNA, cardiolipin and other anionic phospholipids in vesicles (apoptotic cells) |
| Viruses | HIV-1, HTLV-1, polyoma virus and murine lukemia virus. |
| Gram positive bacteria | Lipoteichoic acid |
| Gram negative bacteria | Lipid A, porins |

**IgM**

Interestingly, C1q is not able to bind to monomeric IgM while pentameric IgM has a 1000-fold greater binding affinity to C1q than IgG (reviewed in (15)). For IgM to be able to activate complement, the IgM molecule needs to be in its pentameric form and bound to an antigen which induces a conformational change of the IgM pentamer exposing the binding site for C1q (51). Interestingly hexamer IgM have even higher activity then pentameric, ranging from 2-fold to >100 fold depending on species of the complement source (79).

As mentioned above, natural IgM is important in early defense against bacteria, viruses and parasites (15, 56). It has also been shown that natural IgM and C1q works together in clearance of *S. Pneumoniae* (80), to neutralize virions (81) and to help in clearance of dying cells (77, 82). Accordingly, it seems reasonable that natural IgM, would bind an antigen with high enough affinity to induce a conformational change and thereby activate C1q.

### SIGN-R1

Specific intracellular adhesion molecule-grabbing nonintegrin R1 (SIGN-R1) is the mouse homologue to the human DC-SIGN (83). It is a C-type lectin expressed on MZ macrophages in spleen and on DC and macrophages in the medullary region of the lymph nodes (83–85). The binding of carbohydrate structures (e.g. dextran (83)) to SIGN-R1 enables C1q to bind and become activated (86). SIGN-R1 captures yeast (87) and *S. Pneumoniae* from blood and the classical pathway is the dominant complement pathway for innate immunity against *S. pneumoniae* (80). Nevertheless, the antibody response against *S. pneumoniae* in mice where SIGN-R1 is conditionally blocked does not differ from that in WT animals (88).

### CRP and SAP

C-reactive protein (CRP) and serum amyloid P component (SAP) are two short pentraxins that are produced primarily in the liver during acute phase responses to tissue injury or during inflammation in mammals, mostly due to IL-6 (89–91). They recognize and bind to e.g. bacteria or host cell break down products. After binding they, like IgM, change conformation, which enables C1q to bind and become activated (89, 91, 92). SAP is the major acute phase reactant in mice whereas CRP is the major one in humans (93). Mice immunized with *S. pneumoniae* pulsed with CRP have an enhanced antibody response compared to mice immunized with antigen alone, suggesting a critical role for CRP in antibody responses (94). SAP, on the other hand, seems to be important for tolerance induction, since SAP-deficient C57BL/6 mice spontaneously develop antinuclear autoantibodies (ANA), glomerulonephritis (95) and have increased susceptibility to experimental autoimmune encephalomyelitis (96). ANA and glomerulonephritis are disease markers amongst SLE-patients. Since deficiency of C1q strongly predisposes for SLE (78) and (most probably) is important in antibody development, it could be speculated that SAP also is important in generation of the antibody response.

## Complement receptors 1 and 2

The complement cascade not only serves to attack and lyse pathogens but some of the cleavage products of C3 and C4 are the ligands for CR1/2 (Figure 4) (reviewed in (97)). In mice, CR1 and CR2 are splice products of the *Cr2* gene (98), explaining why both receptors are absent in $Cr2^{-/-}$ knockout mice (99). The receptors are expressed on B-cells, on FDCs (100) and on a small subset of activated T-cells (101, 102). They lack an intracellular signaling part, but CR2 can associate with the CD19/CD81 complex upon ligand binding on B-cells (103).

CR1 (CD35) can bind C3b, C4b, iC3b, C3dg and C3d (104) (Figure 4). After binding of the C3 split products, the amplification of the complement cascade is inhibited (in humans) by reducing the formation and stimulating the breakdown of C3 and C5 convertases (reviewed in (88, 97).

CR2 (CD21) is shorter than CR1 and binds fewer ligands, iC3b, C3dg and C3d (Figure 4). CR2 on B-cells can bind any complement-coated antigen although the BCR cannot recognize the antigen (105, 106). However, co-ligation of CR2/CD19/CD81 with the BCR via antigen complement/complexes have been shown to lower the threshold for B-cell activation (103, 107–109).

Recently work a CR1 knock-out mouse strain was constructed by deleting the exons unique for CR1 in the *Cr2* locus (8). Using those animals, it was concluded that the main complement receptor expressed by FDC was the CR1 whereas B-cells mainly expressed CR2. When the GC reaction was tested against TD antigens, the initial response was normal but not sustained over time. Surprisingly, when BM chimeras were constructed it was found that it not was lack of CR1 expression on FDC but on B-cells that mattered most for the sustained GC response (110).

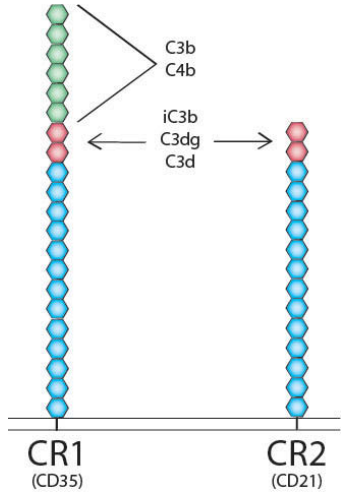


*Figure 4*. Schematic picture of the murine complement receptors 1 and 2 and their ligands and binding sites.

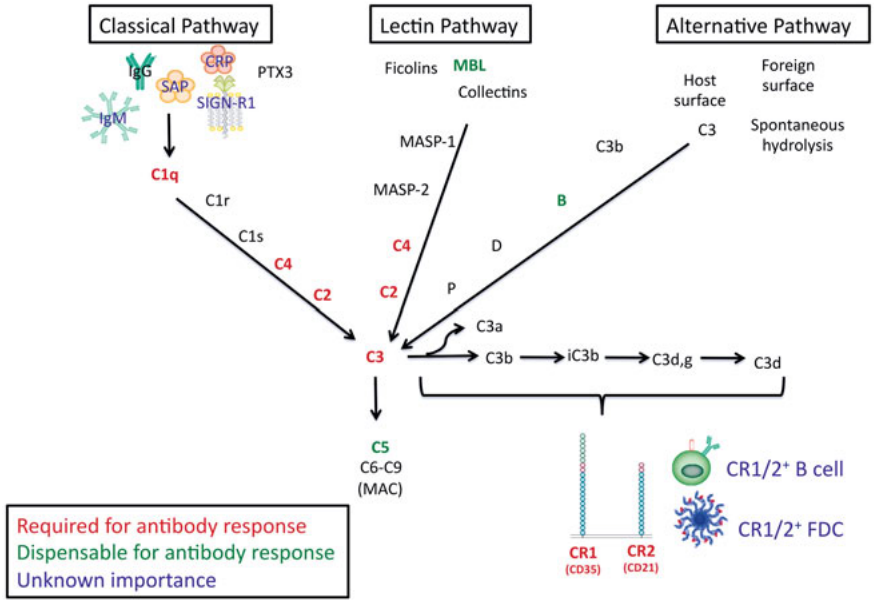


*Figure 5*. The role of complement in antibody responses. Mice lacking C1q, C4, C2, C3, CR1/2 (red, bold) have impaired antibody responses, whereas antibody responses in animals lacking MBL, factor B and C5 are fairly normal (green, bold). The involvement of IgM, SAP, CRP, SIGN-R1 and expression of CR1/2 (blue bold) will be examined in this thesis. Adapted from (111).

## Complement and antibody responses

For mammals to acquire an adequate antibody response, it is necessary to have a functional complement system (reviewed in (97, 112–115)). In general, the antibody response against lower antigen doses is more dependent on the complement system than responses against high doses (116). The antibody response against both TD and TI antigens, primary and memory responses are affected.

Mice lacking CR1/2 as well as animals deficient in complement components C1q, C2, C3, and C4 have severely impaired antibody responses (Table 2 and Figure 5). However, lack of C1q has in some situations against certain antigens (West Nile virus and malaria parasites) been associated with a normal or even higher antibody response (118, 119).

Table 2. *Complement factors important for antibody responses.*

| Complement deficiency | Species | Antibody response /memory response | Antigens tested | Reference |
|---|---|---|---|---|
| C1q | Mouse | Impaired / impaired | SRBC, and SRBC-DNP-KLH | (117) |
| C2 | Guinea pig | Impaired / impaired | Bacteriophage Φ 174 | (120) |
| C3 | Mouse, guinea pig, dog, human | Impaired / impaired | Bacteriophage Φ 174, SRBC, DNP-Ficoll, pneumococcal capsular polysaccharide | (121–126) |
| C4 | Guinea pig, mouse, human | Impaired / impaired | Bacteriophage Φ 174 | (123, 127–129) |
| CR1/2 | Mouse, human | Impaired / impaired | Bacteriophage Φ 174, SRBC, NP-KLH, KLH, pneumococcal capsular polysaccharide | (99, 130–134) |

Mice lacking the A subunit of the MBL (MBL $A^{-/-}$) have impaired IgM but normal IgG response against the *Trichuris muris* parasite and to OVA in adjuvant (135, 136). A mouse strain, lacking subunits A and C of the MBL (MBL $A/C^{-/-}$) had a lower antibody response to Hepatitis B surface antigen (HBsAg) if they were on a C57BL/6 background but normal response if they were on a SV129SvEv background (137). In conclusion MBL deficient mice display a rather complex phenotype ranging from normal to moderately higher, to moderately lower antibody responses (135–138). Yet, the occasionally moderately lower antibody responses observed in MBL-deficient mice were never as low as the response in animals lacking complement factors C2, C4, C3 or CR1/2.

Also ficolins are able to activate the MBL pathway and ficolin deficient mice or mice deficient in MASP-2 are more susceptible to *S. Pneumoniae* infections than WT mice. However in these mice antibody responses were not studied (139, 140). To my knowledge no collectin deficient mouse has been produced and the involvement of collectins in antibody responses remains unknown. Mice that lack factor B have normal antibody responses to both SRBC (141) and West Nile virus (118) excluding the alternative pathway (Figure 5) as a major player in generation of antibody responses.

C5 deficient mice have normal antibody responses (142), indicating that deficiencies of later complement factors, including the lytic pathway (Figure 5), are compatible with a normal antibody response.

## Antibody-mediated feedback regulation

Antigen specific antibodies, passively administrated or actively produced, are able to feedback regulate the antibody response to the antigen they recognize. Depending on antibody class and antigen type the result can either be a suppressed (> 99%) or an enhanced (> 1000 fold) antibody response compared to the response to antigen alone (reviewed in (114, 143)).

Table 3. *Antibody feedback regulation by specific IgM, IgG and IgE. Adapted from* (115) *pre-thesis*

| Regulating isotype | Effect on Ab/CD4+ T-cell responses | Effective against | C'-dependent | FcR dependent | Suggested mechanism(s) |
|---|---|---|---|---|---|
| IgM | Enhancement / ? | Particulate Ags, large proteins | Yes | No | FDC capture; MZB transport; B-cell signaling (?) |
| IgG3 | Enhancement / none | Proteins | Yes | No | FDC capture; MZB transport; B-cell signaling (?) |
| IgG1, IgG2a, IgG2b | Enhancement / enhancement | Small and large proteins | No | Yes | Increased Ag presentation to $CD4^+$ T-cells by DC |
| IgG1, IgG2a, IgG2b, IgG3 | Suppression / none | Particulate Ags | No | No | Epitope masking and/or increased Ag clearance |
| IgE | Enhancement / enhancement | Proteins | No | CD23 | Capture on $CD23^+$ B-cells, increased delivery of Ags to DC and presentation to $CD4^+$ T-cells |

IgG are able to suppress the response against particulate antigens, e.g. erythrocytes (Table 3) (144–147). This knowledge is used in the clinic to prevent Rhesus D negative ($RhD^-$) mothers to develop antibodies against fetal $RhD^+$ erythrocytes. The mechanism behind the suppression is not fully understood but it has been proposed (i) that antigen-specific IgG masks the epitopes of the antigen, thereby preventing recognition by B-cells, (ii) that phagocytotic cells effectively engulf IgG/antigen complexes via the Fc-gamma-receptor (FcγR) or (iii) that the IgG/antigen complexes bind the inhibitory FcγRIIB on B-cells which then would prevent B-cell activation (reviewed in (114, 143, 148)).

Antibody mediated enhancement of the antibody response can be achieved with IgE, all subclasses of IgG (in mice IgG1, IgG2a, IgG2b and IgG3) and IgM but the underlying mechanisms of the enhancement differ between the various antibody classes (114) (Table 3). Enhancement via IgE is completely dependent on the low affinity receptor for IgE, CD23. The postulated mechanism for the enhancement is transport of IgE/antigen complexes via $CD23^+$ B-cells to the spleen where the $CD11c^+$ cells present the antigen to T-cells (30).

Enhancement against soluble protein antigens via IgG1, IgG2a and IgG2b is dependent on activating FcγRs (149) and it is believed that dendritic cells and macrophages efficiently engulf IgG/antigen complexes via FcγR and present the antigens to T-cells (150). In contrast, enhancement against soluble protein antigens by IgG3 is not dependent on FcγR but on the complement system, including CR1/2 (151).

In antibody responses against TD antigens in mice, only a small fraction of the IgGs produced is IgG3. However against TI type 2 antigens IgG3 is the predominant subclass (152, 153) and IgG3 is also important in *Streptococcus pneumonia* sepsis (154). Since IgG3 antibodies are mainly produced after immunization with TI antigens, it is no surprise that IgG3 only has a minor effect on the enhancement of $CD4^+$ T-cell responses (155). IgG3 has the ability to act as a cryoglobulin and precipitate in the cold (156) and are shown self-associate and facilitate binding of other IgG3 molecules via Fc-Fc interactions (157) thus increasing the likelihood for complement activation. This may be the reason why the enhancing capacity of IgG3 is dependent on the complement system and not on binding to FcγRI (155). In conclusion, the proposed mechanism for IgG3-mediated enhancement is activation of complement and thereby generation of the ligands for CR1/2. Then MZ B-cells transport IgG3-Ag-C complexes into the follicle on their CR1/2 and deliver it to the FDC (158).

## IgM-mediated enhancement of immune responses

In 1968 it was clearly described that SRBC-specific IgM had the ability to enhance the antibody response against SRBC (146). IgM has to be administered before or simultaneously with the antigen in order to have an enhancing effect. Primary IgM, IgG and IgE as well as memory response priming are upregulated. Further investigations have shown that this enhancement primarily functions with particulate antigens (e.g. sheep red blood cells, SRBC (146, 159, 160)), malaria parasites (161) or large protein antigens (e.g. keyhole limpet hemocyanin, KLH (28, 29)) given in suboptimal doses. When high concentrations of IgM are given, the effect will be the opposite, i.e. inhibition of the immune response (162).

The enhancement is also dependent on the complement system (163), it is abolished (i) if the antigen specific IgM is unable to activate complement (163), (ii) if monomeric IgM (which cannot activate complement) is used (28, 164), (iii) in mice depleted of C3 (163) and (iv) in mice that lack CR1/2 (165).

The need for particulate/large antigens in IgM mediated enhancement is thought to be due to the conformational change of the IgM molecule needed for C1q binding, i.e. the antigen must be large enough for all of the arms of the IgM pentamer to bind (51). One of the most well known functions of the complement system is to lyse cells. It seems however unlikely that this

would explain the enhancing effect since IgM are able to enhance antibody responses in mice lacking C5 of the terminal pathway (163). These observations show that there are several similarities between the molecular mechanisms behind antibody responses to antigen alone and to IgM-complexed antigens.

## Possible mechanisms for the involvement of complement in antibody responses

Mice deficient of C4, C2 and C3 (and probably C1q) as well as mice deficient of CR1/2 have severely impaired antibody responses against a variety of antigens (reviewed in (97, 114, 115)) (Table 2) as well as lack of IgM mediated enhancement (115). Therefore it seems plausible that failure to cleave C3 results in failure to ligate CR1/2 and to carry out the processes that CR1/2 are important for. Further, mice treated with antibodies blocking both CR1/2, but not to CR1 alone, have impaired antibody responses (116). In line with this, transgenic mice expressing the human CR2 (hCR2) on a *Cr2*$^{-/-}$ background have almost a completely restored antibody response against SRBC (166). However, when the C3d binding site on the hCR2 were blocked, also the secondary antibody response was impaired (167). In addition, mice treated with a soluble CR2 molecule competing for the CR2 ligands, had impaired antibody responses (168). This suggests that CR2 plays a more prominent role in antibody response development than CR1. Yet, recently it was shown that mice selectively lacking CR1 due to deletion of the CR1 exons from the *Cr2* locus had impaired antibody responses to T dependent antigens (8), although not as severely as in CR1/2 deficient mice.

In conclusion, it appears that both CR1 and CR2 are important for normal antibody responses, although their relative importance and the mechanisms behind the involvement of the receptors are not fully understood.

Consequently, several mechanisms have been considered;

i co-ligation of CR2/CD19/CD81 with BCR via antigen complement/complexes (103, 107–109).

ii enhanced uptake of antigen-complement complexes by CR1/2$^{+}$ B-cells, followed by increased antigen presentation to T helper cells (106, 169–171).

iii transport of antigen-complement complexes on CR1/2$^{+}$ MZ B-cells into the B-cell follicle (19, 29)

iv capture (and concentration) of antigen-complement complexes on CR1/2$^{+}$ FDC in germinal centers (29, 133, 172)

(i) Co-ligation of the BCR with the CR2 co-receptor *in vitro* lowers the threshold for B-cell activation and rescues low affinity B-cells from cell death after antigen encounter (173). Thus, the facilitated activation of B-cells after ligation of CR1/2 may result in decreased number of antigen molecules

needed for activation. This might be one of the reasons why the antibody response against lower antigen doses is more dependent on the complement system (116, 174) and bypassed with high doses.

(ii) Antigen trapping and internalization via CR1/2 on B-cells, followed by presentation to T-cells, has been shown to take place *in vitro* (106, 169–171). The T-cells would then provide the B-cells with the "right" signals to get activated. However, *in vivo* the antigens tested are presented as efficiently in *Cr2*$^{-/-}$ as in WT (131, 175, 176). In addition, CR1/2 are crucial for generation of antibody responses against thymus independent antigens (177), which do not require processing and presentation to induce antibody responses.

(iii) It has been described by Cinamon *et al.* that MZ B-cells shuttle back and forth from the MZ to splenic follicles and that the amount of activated C4 is higher on MZ B-cells in mice lacking the shuttling function. Since there is no "open access" into the follicle, the shuttling of MZ B-cells provides an important entry for the antigens. IgM-IC are transported on MZ B-cells and are deposited on FDCs, and this is dependent on IgM being able to activate complement (29). Therefore it seems likely that the transport of antigen itself could be dependent on CR1/2.

(iv) B-cells undergo various stages of differentiation during the generation of an antibody response. The differentiation of the naïve B-cells to antigen producing effector cells and memory cells occurs primarily in the B-cell follicle / GC. In combination with the findings made by Cinamon *et al* (19) and Ferguson *et al* (29) (paragraph (iii)) it therefore seems logical that antigen retention on CR1/2$^{+}$ FDCs (133, 172) followed by presentation to naïve GC B-cells plays, an important role in the generation of antibody responses.

The involvement of CR1/2 in antibody responses against antigen alone, as described above, could also be applied in the case of IgM mediated enhancement, i.e. (i) co-ligation of CR2/CD19/CD81 with BCR, (ii) enhanced uptake of antigen-complement complexes by CR1/2$^{+}$ B-cells, (iii) transport of antigen-complement complexes on CR1/2$^{+}$ marginal zone B-cells in, and (iv) capture of antigen-complement complexes on CR1/2$^{+}$ FDC. Ferguson et al showed that the transport into the follicle, and thereby the formation of germinal centers, are more efficient in mice immunized with IgM immune complexes (IC) compared to antigen alone (29). This could suggest a more efficient complement deposition of IgM-IC compared to antigen alone and consequently a more efficient transport of antigen from MZ into the follicle by CR1/2$^{+}$ MZ B-cells.

# Present investigation

## Aims

The general aim was to understand the mechanisms behind the involvement of complement and IgM in antibody responses.

The specific questions were:

I Is C1q (initiator of the classical complement pathway) crucial for normal antibody responses to SRBC? Is the ability of natural IgM to enhance antibody responses dependent on its ability to activate complement?

II Does specific IgM need to activate complement for IgM mediated enhancement of humoral responses? Does specific IgM enhance $CD4^+$ T-cell responses?

III Does IgM need to activate complement for endogenous IgM mediated enhancement? Is IgM-mediated enhancement of antibody responses dependent on C1q?

IV Do B-cells and / or FDC need to express CR1/2 for normal antibody responses against SRBC? Do B-cells and / or FDC need to express CR1/2 for IgM-mediated enhancement of antibody responses against SRBC?

## Experimental setup

### Mouse strains

All studies were performed using the murine system as model. Transgenic mice were either on BALB/c or C57BL/6 genetic background. For all experiments mice were sex and age matched. Table 4 describes the different strains used.

Table 4. *The different mouse strains used.*

| Mouse strain | Annotation |
|---|---|
| BALB/c | Wild type (produces $Ig^a$ allotype antibodies) |
| $Cr2^{-/-}$ | Lacks of complement receptors 1 and 2 (BALB/c background) |
| Cμ13 | Produces IgM unable to activate complement (BALB/c background) |
| DO11.10 | Expresses OVA-specific TCR on most $CD4^+$ T-cells (BALB/c background) |
| C.BKa-*Igh$^b$*/IcrSMnJ (CB17) | BALB/c congenic (produces $Ig^b$ allotype antibodies) |
| C57BL/6 | Wild type (produces $Ig^b$ allotype antibodies) |
| $C1qA^{-/-}$ | Lacks subunit A of complement component C1q (C57BL/6 background) |
| $C3^{-/-}$ | Lacks complement component C3 (C57BL/6 background) |
| $CRP^{-/-}$ | Lacks C-reactive protein (C57BL/6 background) |
| $SAP^{-/-}$ | Lacks serum amyloid-P component (C57BL/6 background) |

The mice were immunized intravenously with antigens diluted in physiological salt solution (PBS). In studies of antibody feedback enhancement, mice were injected with purified specific antibodies diluted in PBS one hour before antigen immunization. In T-cell proliferation studies, mice were adoptively transferred with OVA-specific $CD4^+$ cells from DO11.10 mice (isolated using anti-CD4 magnetic beads) 24 h before immunization. Bone marrow chimeras between different knock out strains and wild types were constructed by first sublethally irradiating mice and then transplanting appropriate bone marrow.

To measure antibody responses, ELISA (enzyme-linked immunosorbent assay), ELISPOT (enzyme-linked immunospot assay), PFC (plaque forming cell assay), hemagglutination and hemolysis tests were used.

## Generation of the Cμ13 knock-in strain

Cμ13 was constructed in collaboration with Prof. Michael Carroll (Harvard Medical School). A codon exchange was introduced (resulting in substitution of proline for serin at position 436) in the third constant domain of the μ heavy chain (*Igh* locus) with homologous recombination in embryonic stem (ES) cells. The introduced mutation had previously been reported to abrogate the ability of IgM to activate complement (178) and to abrogate the ability to enhance antibody responses (163). The plasmid (pCμ13) used for targeting was assembled by using the information provided by Shulman et al (178). The plasmid contained a region of homology to the *Igh$^a$* allele and the neo gene cassette was flanked by LoxP sites for excision after gene targeting. Targeted ES cells were transfected with a Cre-recombinase expressing plasmid to remove the neo gene cassette. ES cells were derived from C57BL/6

(*Igh*$^{b}$) and transfected. To identify positively transfected clones, Southern blotting was used. As indication of mice carrying the transfected gene, peripheral blood was analyzed for the IgM$^{a}$ allotype. The mice were then backcrossed 12 generations to BALB/c.

## Bone marrow chimeras

To study the involvement of CR1/2 on B-cells and FDC in antibody responses, bone marrow chimeras were constructed between WT (BALB/c or CB17) and *Cr2*$^{-/-}$ mice. Since FDC are (non-haematopoetic) stromal cells they are more radiation resistant (179, 180) than the BM derived cells and therefore not replaced after BM transplantation following irradiation. Recipient mice were whole body irradiated (7.5 Gy) and then transplanted with either WT BM or *Cr2*$^{-/-}$ BM, or a mixture of both, resulting in eight different phenotypes. Since BALB/c and *Cr2*$^{-/-}$produce antibodies of Ig$^{a}$ allotype and CB17 of Ig$^{b}$ allotype, the use of CB17 as WT in some experiments enabled us to distinguish antibodies produced by recipient B-cells from those produced by donor B-cells, and to distinguish CR1/2$^{+}$ from CR1/2$^{-}$ B-cells.

## Adoptive transfer of T-cells

BALB/c mice were adoptively transferred intravenously with splenocytes or CD4$^{+}$ T-cells from DO11.10 donors. Next day the recipients were immunized and on day three post-immunization spleens were harvested to analyze OVA specific CD4$^{+}$ T-cell proliferation with flow cytometry, using a monoclonal antibody specific for the transgenic TCR.

## Immunizations

The mice were immunized intravenously with SRBC or KLH. To block SIGN-R1, mice were pretreated with a monoclonal antibody (clone 22D1) (83, 86) intravenously 24 h before immunization with SRBC. In IgM-mediated enhancement experiments, IgM anti-SRBC was administered intravenously one hour before immunization with SRBC. To mimic a biological situation, no adjuvants were used and all antigens and antibodies were given in physiological salt solutions.

## Assays

To study antibody responses, ELISA and ELISPOT were used as complement independent assays and plaque forming cell (PFC) assay as a complement dependent assay (146). To further characterize the ability of IgM to activate complement, flow cytometry of C3-deposition on SRBC incubated with IgM anti-SRBC from either BALB/c or Cμ13 and fresh mouse plasma

(as complement source) was performed. To prevent complement activation in fluid phase by the coagulation cascade, blood from the naïve mice were collected in lepirudin (trombin inhibitor) containing tubes (181). Flow cytometry was also used to analyze the B-cell compartments in bone marrow chimeric mice, in characterizing of Cμ13 mice, tracking OVA specific T-cells and GC experiments. Confocal microscopy was used to the characterize Cμ13 mouse spleens and kidneys and in GC experiments.

# Results and discussion

## Paper I

### Requirement for complement in antibody responses is not explained by the classic pathway activator IgM

*C1q is crucial for the generation of normal antibody response against SRBC*

Since deficiency of C1q in some studies resulted in impaired antibody responses (117) whereas in other studies resulted in normal or moderately higher antibody responses (118, 119), it was first tested if the protocol we used resulted in an impaired antibody response. The results showed that when mice were immunized with SRBC-doses ranging from $5\times10^5$ to $5\times10^8$ and boosted with $5\times10^6$, classical pathway activation was crucial for the generation of normal primary and secondary antibody response against SRBC.

The finding that C1q is required in primary responses might seem paradoxical since Ig are thought to be the major activator of C1q and prior to antigen encounter, very low amounts of specific antibodies are present. However, mice lacking secretory IgM have impaired antibody responses and their responses are restored after reconstitution with wild type sera (containing natural IgM) (57, 59). In addition, the ability of IgM to enhance the antibody response is dependent on its ability to activate complement (28, 163). Thus, it could be hypothesized that natural IgM in naïve animals might bind an antigen with enough affinity to induce a conformational change of the IgM molecule, exposing the binding site for C1q and thereby activate the classical pathway.

*IgM from Cμ13 mice can not activate complement, but IgG responses were not impaired*

To test the hypothesis above, a knock in mouse strain, Cμ13, was constructed (see experimental systems above). First, the mouse strain was characterized and by several different experimental approaches it could be concluded that IgM produced by Cμ13 mice failed to activate complement.

Despite the inability of the IgM to activate complement it was found in the ELISPOT assay that there were no difference in the number of antigen specific IgM producing B-cells between BALB/c and Cμ13 (Paper I Table 1) whereas the number of antigen specific B-cells in the negative control (*Cr2*$^{-/-}$) were much lower. Since the spleens were collected five days post immunization, during the IgM peak, it was first speculated that the inability of IgM to activate complement did not affect B-cell proliferation or IgM production but that the B-cell needed the extra signal from co-ligation of BCR with CR2/CD19/CD81 to induce class switch to IgG. To investigate this, BALB/c, Cμ13 and *Cr2*$^{-/-}$ were immunized with different doses of SRBC and KLH and then boosted. SRBC and KLH were chosen as antigens because both are large antigens and specific IgM are able to enhance the antibody response against them.

The overall impression of the responses seen in the Cμ13 mice was that they were as high as in BALB/c. After repeating the experiments it could be concluded that Cμ13 mice occasionally produced less antigen specific IgG and sometimes slightly higher, but the pattern was inconsistent and usually not significant. When the response in Cμ13 was lower than in BALB/c it was never as low as the response seen in *Cr2*$^{-/-}$. Data presented in paper I are from the experiments where the differences were most pronounced.

Since there are time points where the antibody response in Cμ13 was lower compared to BALB/c it cannot be excluded that complement-activating IgM in naïve mice may play a minor role in the generation of a normal antibody response against SRBC and KLH. However, it does not explain the severe phenotype seen in *Cr2*$^{-/-}$ or C1qA$^{-/-}$ mice.

*The B-cell repertoire in Cμ13 mice*

How can the results in Paper I, Figure 1, be compatible with the finding that mice lacking secretory IgM had impaired responses and that the response was restored after transfer of normal mouse sera (57, 59)?

There could be at least three independent explanations: (i) C1q could be activated by something else than IgM and (ii) the importance of natural IgM might not involve complement activation. Another explanation could be that (iii) mice deficient of secretory IgM have impaired antibody responses due to defects in their B-cell repertoire secondary to their lack of secretory IgM, i.e. lower amount of FO B-cells (182). The B-cell subpopulations in Cμ13 were analyzed and the only differences were a slightly lower total B-cell number in the spleen when gated on B220$^{+}$ cells, and an increased number of MZ B-cells. There was no difference in B-cell numbers when CD19 was used as a marker. These differences however, seemed not to have a major influence on the antibody response in Cμ13. Further, it could be argued that differences in antibody responses between the IgM secretory deficient mice (sIgM$^{-/-}$) and Cμ13 were antigen dependent. The antigens studied in the IgM secretory deficient mice were influenza virus (57) or hapten-conjugated pro-

teins (58, 59) whereas SRBC and KLH were used to study the antibody response in Cμ13. In one study (58), 1 μg NP-KLH resulted in impaired antibody response, but 2 μg KLH (Paper I, Figure 5) or 1 μg NP-KLH (Paper I, Figure S1) did not result in impaired antibody responses. Thus, the use of different antigens as an explanation for the discrepancies seems unlikely.

*SIGN-R1, SAP, or CRP are not crucial for antibody responses against SRBC*

Excluding IgM as the major C1q activator in the development of a normal antibody response, it was of interest to test other C1q activators. First IgG was considered. However IgG antibodies of all subclasses are known to feedback suppress the antibody responses against SRBC (145, 147) and presence of antigen specific IgG would most likely result in an impaired antibody response. Hence, that theory was considered to be extremely unlikely. Other possible C1q activators were SIGN-R1 (85), CRP (91) or SAP (183).

The receptor SIGN-R1 was conditionally blocked with monoclonal antibodies in BALB/c and Cμ13, the mice were immunized with SRBC and the IgG responses were analyzed (Paper I, Figure 6). In both SIGN-R1 deficient BALB/c - and Cμ13 mice, the IgG anti-SRBC response did not differ from controls. Next, $CRP^{-/-}$ and $SAP^{-/-}$ mice were immunized with SRBC and the IgG response analyzed. Again, the antibody responses were equally high as in wild type controls. Thus, neither SIGN-R1, CRP, SAP nor SIGN-R1 in combination with complement activation deficient IgM could explain the need for classical pathway activation in the generation of a normal antibody response against SRBC.

Last to consider was activation of C1q by the antigen itself. However, when SRBC were incubated with fresh mouse plasma only, there was no deposition of C3 (Paper I, Figure 3). With increasing concentration of plasma (90% plasma and 10% SRBC) some C3 were detected but the same pattern was seen with plasma from $C1qA^{-/-}$ mice (unpublished data). In addition, $C1qA^{-/-}$ mice were immunized with SRBC incubated with WT-plasma, but that did not restore the antibody response (unpublished data). Thus, the C3 detected on SRBC was probably due to MBL or alternative pathway activation. Further, the SRBC used in these experiments were fresh and non-apoptotic cells. Although the erythrocytes lack nucleus and mitochondria it has been reported that erythrocytes from humans (184) and rats (185) are able to undergo apoptosis. Hence, there is still a possibility that apoptosis of SRBC would trigger C1q activation. Other explanations could be that the other C1q activators would compensate for the ones tested.

In summary, to generate an antibody response against SRBC, C1q is indeed required, but the activator of C1q remains unknown. The initial hypothesis was based on the assumption that the importance of natural IgM to generate an antibody response (59) would depend on its ability to activate complement. However, herein complement-activation of natural IgM, as

well as SIGN-R1, CRP and SAP were tested and deficiency of any one of these molecules did not have nearly as major impact on antibody responses as deficiency of C1q, C2, C4, C3 or CR1/2. Possibly, the importance of natural IgM in other studies could be transmitted through binding to the FcμR, facilitating antigen capture by MZ B-cells and transportation into splenic follicles.

## Paper II

### Complement activating IgM enhances the humoral but not the T-cell immune response in mice

*$IgM_{C\mu 13}$ are unable to enhance antibody response, GC-formation and to induce C3 deposition in vivo but bind the FcμR*

IgM from the hybridoma cell line (178), that laid the ground for the generation of Cμ13 mice, failed to enhance antibody responses (163). In paper I it was clearly shown that IgM produced from this mutant mouse strain, Cμ13, could not activate complement and should thus be unable to feedback enhance the antibody response. However the antibody response against SRBC and KLH turned out to be fairly normal. These inconsistent results prompted the investigation of whether antigen specific IgM from Cμ13 mice could enhance antibody responses although it could not activate complement. Maybe other posttranslational properties were added to the IgM in vivo that somehow would make the IgM able to enhance antibody reponses although it could not activate complement? It was recently shown that the FcμR was important for an efficient antibody response, especially when the amount of antigen was limited (64, 65).

IgM from BALB/c and Cμ13 mice immunized 5 days before with SRBC were purified. As expected, $IgM_{BALB/c}$ enhanced antibody responses as well as the GC response whereas $IgM_{C\mu 13}$ failed to achieve this. In addition, $IgM_{BALB/c}$ but not $IgM_{C\mu 13}$, induced C3 deposition on SRBC in peripheral blood 1 min post immunization whereas IgM from both strains bound equally well to the FcμR.

*$IgM_{BALB/c}$ do not enhance $CD4^+$ T-cells*

During this work, the question was raised whether IgM could also enhance T-cell responses. This could in turn provide the FO B-cells with more help from $T_{FH}$ cells. Therefore BALB/c mice were adoptively transferred with splenocytes from DO11.10 mice. The next day they were immunized with SRBC-OVA alone or together with SRBC-specific $IgM_{BALB/c}$. On day three post immunization splenocytes were analyzed with flow cytometry regarding OVA specific $CD4^+$ T-cell proliferation and activation. Although SRBC-specific IgM enhanced the antibody responses to both SRBC and OVA, nei-

ther proliferation nor activation of antigen specific $CD4^+$ T-cells were enhanced compared to mice given SRBC-OVA alone.

*Discussion*

In conclusion, specific $IgM_{BALB/c}$ enhanced the antibody response although no enhancement of $CD4^+$ T-cells was seen. C3 was rapidly deposited onto SRBC in blood when mice were immunized with SRBC together with specific $IgM_{BALB/c}$ but no C3 deposition was seen when specific $IgM_{C_H13}$ was used. However, both $IgM_{BALB/c}$ and $IgM_{C_H13}$ bound equally well to the FcμR. These results suggest that complement-activation and not binding to FcμR is required for enhancement of antibody responses by passively administered specific IgM. This confirms previous work (28, 163) and may give clues to the involvement of CR1/2 (165) in IgM mediated enhancement. Since C3 fragments are ligands for CR1/2 and the deposition of C3 on the antigen is very rapid, the enhancement of antibody responses could be due to efficient antigen transport into the follicles via CR1/2 on MZ B-cells (28, 29) and subsequent deposition onto CR1/2 on FDCs. Presence of antigen on FDCs are important for GC development and B-cell activation (reviewed in (186)). FDCs are able to retain antigens on their surface for longer periods of time (7) and thereby increase the likelihood for antigen-specific B-cells to recognize the antigens and become activated. If increased amount of antigens are being delivered to the FDCs this sequence of events is even more likely to lead to enhanced GC reactions and antibody responses. Thus as these data suggest, when $IgM_{C_H13}$ is used, a smaller fraction of the antigens will be able to bind CR1/2 on MZ B-cells and FDC leading to reduced capacity to enhance GC reactions and antibody responses. Indeed it has been shown that specific IgM administered together with either bovine serum albumin (BSA) or virus-like particles resulted in increased antigen deposition in splenic follicles (27, 29) although antibody responses were not studied in parallel. It would thus be satisfying to show that SRBC-specific IgM, administered together with SRBC, also led to increased antigen localization in splenic follicles. However, we have tried many methods to visualize SRBC in follicles (labeling with biotin, CFSE, PKH26, Alexa Flour 647 or staining sections with hyper-immune rabbit IgG anti-SRBC) at time points ranging from 10 min to 24 h post immunization. However, all methods failed to detect any SRBC/KLH inside the follicles (data not shown). It is likely that the antigens are rapidly degraded and thereby impossible to visualize or that they are quickly internalized by FDC (9).

## Paper III

**Endogenous feedback-regulation by complement-activating IgM**

*C1q is crucial for IgM-mediated enhancement*

The phenotype (i.e. "lack of phenotype") seen in Cμ13 remained puzzling since specific IgM, also from these mice (Paper II), needed to activate complement in order to enhance antibody response. Moreover, it could not be explained by binding to the FcμR, since both WT and Cμ13 IgM bound equally well. However, the recent finding that IgM are able to activate the lectin pathway through binding of ficolin H in human sera (187) opened up for another explanation for the fairly normal antibody responses seen in Cμ13 mice. The lectin pathway could perhaps rescue complement activation by Cμ13 IgM *in vivo*. Another possibility could be that IgM-mediated enhancement can only be induced in experimental systems. First, IgM mediated enhancement was tested in *C1qA*$^{-/-}$ and C57BL/6 mice but since no enhancement was detected (with two different SRBC-doses) in *C1qA*$^{-/-}$ mice it seemed unlikly that the lectin pathway was invoved.

*Endogenous feedback-regulation*

In the previous study (Paper I), Cμ13 mice were primed with one dose, and then boosted day 21 post immunization when the amount of IgG (known to suppress the antibody response to SRBC) was high. The SRBC immunization protocol was therefore changed to mimic an infectious scenario. When Cμ13 and WT received a very low priming dose and a booster already 3 days later, there were no difference in the GC response day 6 post immunization, but the WT had a higer IgG response and GC response day 10 postimmunization as compared to Cμ13. When the protocol was applied only in WT, with a WT control group receiving the same amount of SRBC but as a single injection, the IgG response and GC response day 6 post immunization was lower in that group as compared to the group receiving a very low priming dose followed by a low booster dose.
In conclusion, IgM-mediated enhancement is dependent on classical pathway activation and in order for endogenous IgM to enhance, IgM needs to activate complement.

*MZ B-cells are found in the B-cell follicle in Cμ13 mice*

To fully understand the different kinds of antibody responses in Cμ13 mice, it was also needed to further characterize this mouse strain. In Paper I it was shown that Cμ13 hade more MZ B-cells then did BALB/c. When the spleens were studied with confocal microscopy, a pronounced difference in the distribution of CD1d$^{+}$ (MZ B-cells) could be seen, with a high proportion of the CD1d$^{+}$ cells inside the follicle in Cμ13 mice.

These observations suggest that the differention and/or migration and/or retention of MZ B-cells is perturbed in Cμ13 mice. The increased amount of

MZ B-cells may also somehow compensate for the loss of complement-activating IgM. MZ B-cells continuously shuttle between the MZ and the B-cell follicle depending on chemokine signalling (sphingosine 1-phospate (S1P) and its receptor S1PR1), and as much as 20% of the MZ B-cells relocate from the MZ to the follicle every hour (19, 26). When the MZ B-cells have the opportunity to capture an Ag in the MZ, this Ag may be delivered to FDCs in the follicle as has been directly shown for IgM-Ag and IgG3-Ag complexes (19, 28, 29, 158). The S1PR1 can be blocked by using a drug called FTY720 (Fingolimod) whereby e.g. MZ B-cells are displaced from the MZ into the follicle (1). When the treatment is only given once, the MZ B-cells return to the MZ after 24 h. When Cμ13 mice were immunized during the MZ B-cell absence their antibody response was impaired compared to in mice where no treatment was given (unpublished data), suggesting that the MZ B-cells in Cμ13 are very important to yield an antibody response.

*Deposition of immune-complexes in kidneys*

To further characterize the Cμ13 mouse strain, kidneys of old and young mice were screened for C4-containing IC. This was investigated since natural IgM together with C1q are important in the clearance of apoptotic cells (77) and deficiency of C1q strongly predisposes for development of SLE (reviewed in (78)). SLE is an autoimmune disease characterized by numerous manifestations. One clinically important sign is the presence of autoantibodies and complement in glomeruli, which may cause glomerulonephritis. In kidneys of both WT and Cμ13 one-year-old female mice there was a surprisingly high amount of $C4^+$ glomeruli. However there were no significant difference in the amount of $C4^+$ glomeruli / $mm^2$ between the two mouse strains.

## Paper IV

**Complement receptors 1 and 2 in murine antibody responses to IgM-complexed and uncomplexed sheep erythrocytes.**

*Expression of CR1/2 on FDC is important for antibody response against SRBC*

*$Cr2^{-/-}$* mice were used as a negative control throughout Paper I, since it is well documented that these mice have severely impaired antibody responses (reviewed in (97)). The connection between CR1/2 and classical pathway activation were thought to be generation of ligands for CR1/2. However the molecular mechanisms behind the involvement of CR1/2 in the generation of an antibody response remains unclear. Some studies indicate that B-cells need to express CR1/2 (130, 132) whereas other studies indicate that FDC need to express CR1/2 for a normal antibody response to occur (133, 188).

Consequently, it seems plausible that the expression on both cell types might be important to generate an antibody response**.** Chimeric mice, between BALB/c and *Cr2*$^{-/-}$ (Table 5) was therefore constructed to investigate which cell type that needed to express CR1/2 to generate a normal antibody response against SRBC.

Table 5. *CR1/2 expression in the different chimeras*

| **Chimera BM → recipient** | **B-cell CR1/2** | **FDC CR1/2** |
|---|---|---|
| BALB/c →BALB/c | + | + |
| *Cr2*$^{-/-}$ → BALB/c | - | + |
| BALB/c →*Cr2*$^{-/-}$ | + | - |
| *Cr2*$^{-/-}$→*Cr2*$^{-/-}$ | - | - |
| BALB/c → CB17 | + | + |
| *Cr2*$^{-/-}$ →CB17 | - | + |
| CB17 + *Cr2*$^{-/-}$→ CB17 | + / - | + |
| CB17 + *Cr2*$^{-/-}$ →*Cr2*$^{-/-}$ | + / - | - |

When CR1/2 were expressed on FDC, both WT and *Cr2*$^{-/-}$ B-cells were able to generate equally high antibody responses (Paper IV Figure 1). In contrast, mice lacking CR1/2 on FDC (BALB/c →*Cr2*$^{-/-}$ and *Cr2*$^{-/-}$→*Cr2*$^{-/-}$) produced equally low titers of IgG anti SRBC, unless a high antigen dose was used. Then it was favorable to have CR1/2 on the B-cells (Paper IV Figure 1C). To exclude the risk of having recipient BM surviving the radiation in WT mice receiving *Cr2*$^{-/-}$ BM, and thereby be the generator of the antibody response, chimeras were constructed with CB17 as WT recipients of BALB/c or *Cr2*$^{-/-}$ BM (Table 5). The B-cells in CB17 mice produce antibodies of Ig$^{b}$ allotype whereas BALB/c and *Cr2*$^{-/-}$ produce antibodies of Ig$^{a}$ allotype. The mice were immunized with SRBC and the IgG response showed no involvement of recipient ("original") Ig allotype (Paper IV, Figure 2). Thus, to generate a normal antibody response against SRBC, FDC needed to express CR1/2 whereas the CR1/2 expression on B-cells was less important. These results were somewhat surprising since two of the hypotheses mentioned above suggested that the CR1/2 involvement in antibody responses were due to CR1/2-expression on B-cells.

*CR1/2*$^{-}$ *B-cells are equally good producers of IgG anti-SRBC as CR1/2*$^{+}$ *B-cells*

To be certain that CR1/2-expression on B-cells was irrelevant in the generation of antibody response against SRBC as long as the receptors are expressed on FDC, chimeras were constructed with BM from both CB17 and *Cr2*$^{-/-}$ given to either CB17 or *Cr2*$^{-/-}$ (Table 5). This construction would enable B-cells with and without CR1/2 to operate in the same animal, minimiz-

ing the risk of environmental influence. Concomitantly, when competing for the same antigens, would the expression of CR1/2 on B-cells be advantageous for B-cell activation? However, it was again found that FDC needed to express CR1/2 and that CR1/2$^-$ B-cells are equally good producers of specific IgG anti-SRBC as WT B-cells (Paper IV, Figure 3). Thus, it seems that the classical pathway of the complement system is important to generate ligands for CR1/2 on the antigens so they could be trapped on CR1/2$^+$ FDC in the follicle. These results also indicate that co-ligation with CR2/CD19/CD81 would not be of importance to generate a normal antibody response against SRBC. Further, it has been suggested that CR1/2 expression on B-cells would be involved in transport of antigen-complement complex into the follicle (29). This hypothesis, however, appears not to reflect what happens with this experimental setup. The FDCs in WT mice reconstituted with *Cr2*$^{-/-}$ BM must somehow be provided with antigen-complement complexes on their CR1/2$^+$ FDC. Although, CR1/2 on B-cells does not seem to be involved in the antigen transport into the follicles, the antigens eventually end up in the follicle to enable B-cells to undergo affinity maturation and class switch recombination. It has been described by Cinamon et al. (1) that MZ B-cells shuttle back and forth from the MZ to splenic follicles and that the shuttling in itself was not dependent on CR1/2. However the amount of activated C4 was higher on MZ B-cells in mice lacking the shuttling function. Together with the finding that CR1/2 expression on B-cells was not needed (Paper IV Figures 1-3) for the antibody response, this could imply that complement-coated antigens are off-loaded on CR1/2 on FDC by MZ B-cells, but that the binding of antigen to MZ B-cells could also take place via some other receptor, e.g. the FcμR (61, 189). Another, maybe controversial but rather straightforward hypothesis, can be based on the fact that nature in general develops systems that consume the least energy and that FDCs are localized close to the MZ. Possibly the FDC's dendrites are able to surpass the follicle / MZ boarder and capture complement opsonized antigens directly on their CR1/2 in the MZ.

*CR1/2 expression on both FDCs and B-cells is important for IgM-mediated enhancement*

Surprisingly, CR1/2 expression on B-cells did not seem to be important for responses to SRBC alone. As mentioned above, the antibody response to antigen alone as well as the enhanced antibody responses detected after immunizations with IgM-IC are both dependent on the complement system (28, 163) (165). IgM-IC are transported on MZ B-cells and are deposited on FDC 16-18 h post immunization and this is dependent on IgM being able to activate complement (29). It was therefore reasoned that CR1/2 expression on B-cells would be crucial for the enhanced response to IgM-IC. The questions were through which mechanisms this took place. Would the IgM mediated enhancement of the antibody response be due to more efficient delivery of

IgM-IC complexes to FDC via CR1/2 on MZ B-cells than the CR1/2 indifferent transport of antigen alone? Or, alternatively, would the threshold for B-cell activation be lowered through crosslinking of CR2/CD19/CD81 with BCR? Or could both scenarios be involved?

To address these questions, chimeras were constructed between BALB/c and *Cr2*$^{-/-}$ (Table 5) and the mice were immunized with two different doses of SRBC alone or together with IgM anti-SRBC. As expected, enhanced antibody responses were detected with both SRBC doses in WT to WT chimeras, whereas the antibody responses were impaired in the *Cr2*$^{-/-}$ to *Cr2*$^{-/-}$ chimeras (Paper IV, Figure 4). The lower SRBC dose given to *Cr2*$^{-/-}$ to WT chimeras, resulted in a tendency towards IgM mediated enhancement but in WT to *Cr2*$^{-/-}$ chimeras, the response with and without IgM was as low as in *Cr2*$^{-/-}$ to *Cr2*$^{-/-}$ chimeras (Paper IV, Figure 4B-D). Thus, using a low dose of SRBC, FDCs were needed to express CR1/2 for an antibody response to be detected, but for IgM to enhance the response, both B-cells and FDC had to express the receptors. Further, when the mice were immunized with the higher SRBC dose, IgM could enhance antibody responses both in *Cr2*$^{-/-}$ to WT and WT to *Cr2*$^{-/-}$ chimeras. The enhancement however was not as high (day 7 and 14 post immunization) as in the WT to WT chimeras (Paper IV, Figure 4E-H). Thus, with the higher dose of SRBC in complex with IgM, CR1/2$^{+}$ B-cells were able to produce antibodies without involvement of CR1/2 on FDC.

In conclusion, CR1/2 expression on FDC was more important to generate a sustainable, enhanced response whereas CR1/2 on both cell types resulted in a more rapid enhancement of the antibody response.

# Concluding remarks and future perspectives

This work has contributed to the knowledge of factors important during initiation of antibody responses. We have concluded that (Figure 6 and 7):

- the classical pathway of the complement system is important in generation of normal antibody responses (against SRBC and KLH), as well as in IgM-mediated feedback enhancement.
- natural IgM, SIGN-R1, SAP or CRP seem not be the classical pathway activators important for normal antibody responses.
- specific $IgM_{C_{\mu}13}$ produced in vivo cannot enhance antibody responses, but bind the FcμR equally well as $IgM_{BALB/c}$
- complement activation and deposition of complement factors on IgM-IC occurs in the peripheral blood, only seconds after immunization.
- IgM mediated enhancement does not enhance the T-cell response.
- Specific IgM produced early in an immune response have endogenous enhancing effects on the GC-reaction and antibody response.
- antigen retention on CR1/2+ FDC is crucial for normal antibody responses as well as for IgM-mediated enhancement.
- early IgG-responses probably made by short lived plasma cells, seem to be dependent on CR1/2 expression on the B-cell.
- CR1/2 expression on B-cells does not seem to be crucial for normal antibody responses or IgM-mediated enhancement. However, for an optimal IgM-mediated enhancement, B-cells need to express the receptors.

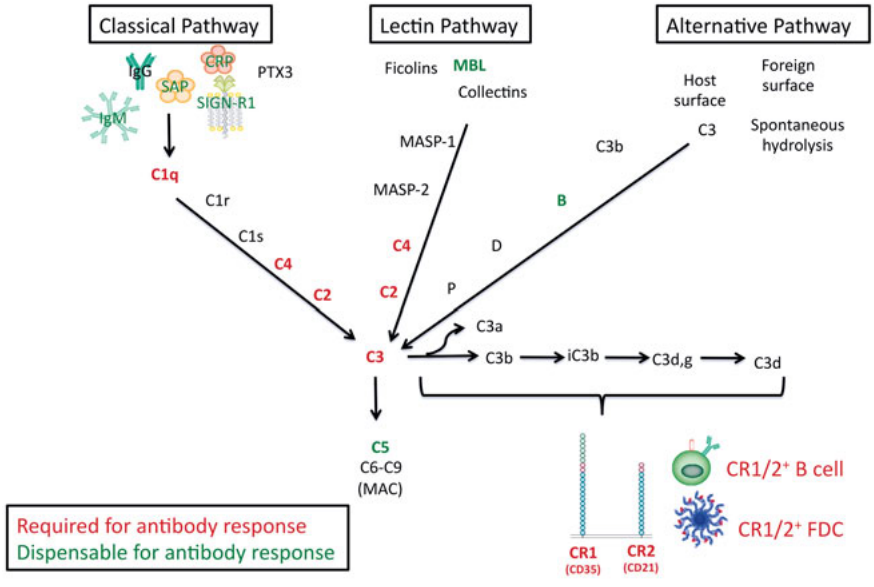


*Figure 6*. The role of complement in antibody responses (post thesis). Mice lacking C1q, C4, C2, C3, CR1/2 (red, bold) have impaired antibody responses, whereas antibody responses in animals lacking complement-activating IgM, SAP, CRP, SIGN-R1, MBL, factor B or C5 are fairly normal (green, bold). Adapted from (111).

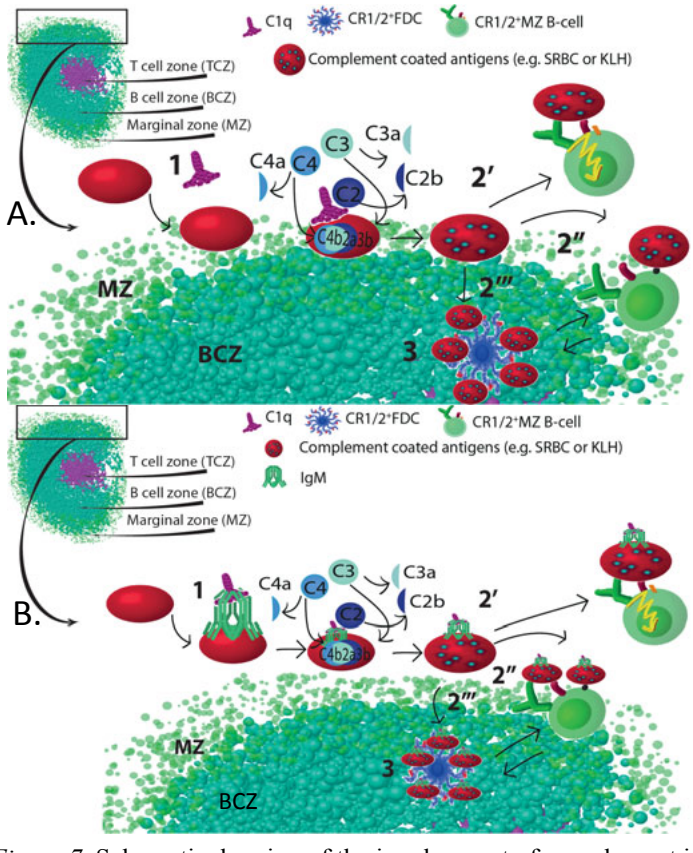


*Figure 7*. Schematic drawing of the involvement of complement in antibody responses to antigen alone (A) and IgM-ICs (B). (A) The antigen is trapped in the MZ and C1q is activated by an unknown factor, which leads to complement deposition on the antigen (1). A specific B-cell may bind the antigen-complement complex via the BCR and CR1/2 and thereby co-crosslink the BCR with the CR2/CD19/TAPA-1 coreceptor complex, lowering threshold for B-cell activation (2′). The antigen-complement complex can be transported from the MZ to the follicle via $CR1/2^+$ MZ B-cells (2′′) but also via some other unknown route (2′′′) and offloaded onto $CR1/2^+$ FDC (3). (B) The antigen is trapped in the MZ and C1q is activated by antigen specific IgM, which leads to complement deposition on the antigen (1). A specific B-cell may bind the antibody-antigen-complement complex via the BCR and CR1/2 and thereby co-crosslink the BCR with the CR2/CD19/TAPA-1 coreceptor complex, lowering threshold for B-cell activation (2′). The antigen-complement complex can be transported from the MZ to the follicle via $CR1/2^+$ MZ B-cells (2′′) but also via some other unknown route (2′′′) and offloaded onto $CR1/2^+$ FDC (3). Adapted from (115).

As often in research, when one question is answered, several others arise. The list can be made long but below is a selection

- What activates C1q when SRBC and KLH are used as antigens?
- Is it C1q and C1qR that are important in generation of normal antibody responses?
- Do the other C1q-activators add up for the one absent?
- Where does the SRBC end up?
- When CR1/2 on B-cells are absent, what cell / receptor transport the IC or SRBC alone into the follicles?
- Which role do the MZ B-cells in Cμ13 mice have?
- Why is the ability of IgM to activate complement important in MZ B-cell homeostasis?
- Would Cμ13 mice have an enhanced IgM-mediated enhancement of the antibody response, as a consequence of having more MZ B-cells?
- What would happen in Cμ13 after immunization with "real" pathogens, e.g. influenza or malaria parasites?
- Is physiological IgM-mediated enhancement important when it comes to replicating pathogens?
- Does the FcμR play a role in IgM-mediated enhancement?
- Are Cμ13-mice more susceptible for experimental autoimmune diseases (e.g. collagen induced arthritis or experimental autoimmune encephalomyelitis)?

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# References


1. Cinamon, G., M. Matloubian, M. J. Lesneski, Y. Xu, C. Low, T. Lu, R. L. Proia, and J. G. Cyster. 2004. Sphingosine 1-phosphate receptor 1 promotes B cell localization in the splenic marginal zone. *Nat. Immunol.* 5: 713–720.
2. Harwood, N. E., and F. D. Batista. 2009. The Antigen Expressway: Follicular Conduits Carry Antigen to B Cells. *Immunity* 30: 177–179.
3. Davies, L. C., S. J. Jenkins, J. E. Allen, and P. R. Taylor. 2013. Tissue-resident macrophages. *Nat. Immunol.* 14: 986–995.
4. Krautler, N. J., V. Kana, J. Kranich, Y. Tian, D. Perera, D. Lemm, P. Schwarz, A. Armulik, J. L. Browning, M. Tallquist, T. Buch, J. B. Oliveira-Martins, C. Zhu, M. Hermann, U. Wagner, R. Brink, M. Heikenwalder, and A. Aguzzi. 2012. Follicular Dendritic Cells Emerge from Ubiquitous Perivascular Precursors. *Cell* 150: 194–206.
5. Wing, J. B., and S. Sakaguchi. 2014. Foxp3+ Treg cells in humoral immunity. *Int. Immunol.* 26: 61–69.
6. Ansel, K. M., V. N. Ngo, P. L. Hyman, S. A. Luther, R. Förster, J. D. Sedgwick, J. L. Browning, M. Lipp, and J. G. Cyster. 2000. A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature* 406: 309–314.
7. Mandel, T. E., R. P. Phipps, A. P. Abbot, and J. G. Tew. 1981. Long-term antigen retention by dendritic cells in the popliteal lymph node of immunized mice. *Immunology* 43: 353–362.
8. Donius, L. R., J. M. Handy, J. J. Weis, and J. H. Weis. 2013. Optimal Germinal Center B Cell Activation and T-Dependent Antibody Responses Require Expression of the Mouse Complement Receptor Cr1. *J. Immunol.* 191: 434–447.
9. Heesters, B. A., P. Chatterjee, Y.-A. Kim, S. F. Gonzalez, M. P. Kuligowski, T. Kirchhausen, and M. C. Carroll. 2013. Endocytosis and Recycling of Immune Complexes by Follicular Dendritic Cells Enhances B Cell Antigen Binding and Activation. *Immunity* 38: 1164–1175.
10. Heesters, B. A., R. C. Myers, and M. C. Carroll. 2014. Follicular dendritic cells: dynamic antigen libraries. *Nat. Rev. Immunol.* 14: 495–504.
11. Allen, C. D. C., and J. G. Cyster. 2008. Follicular dendritic cell networks of primary follicles and germinal centers: Phenotype and function. *Semin. Immunol.* 20: 14–25.
12. Garin, A., M. Meyer-Hermann, M. Contie, M. T. Figge, V. Buatois, M. Gunzer, K.-M. Toellner, G. Elson, and M. H. Kosco-Vilbois. 2010. Toll-like Receptor 4 Signaling by Follicular Dendritic Cells Is Pivotal for Germinal Center Onset and Affinity Maturation. *Immunity* 33: 84–95.
13. Wu, Y., M. E. M. E. Shikh, R. M. E. Sayed, A. M. Best, A. K. Szakal, and J. G. Tew. 2009. IL-6 produced by immune complex-activated follicular dendritic cells promotes germinal center reactions, IgG responses and somatic hypermutation. *Int. Immunol.* 21: 745–756.
14. Pieper, K., B. Grimbacher, and H. Eibel. 2013. B-cell biology and development. *J. Allergy Clin. Immunol.* 131: 959–971.

15. Ehrenstein, M. R., and C. A. Notley. 2010. The importance of natural IgM: scavenger, protector and regulator. *Nat. Rev. Immunol.* 10: 778–786.
16. Loder, B. F., B. Mutschler, R. J. Ray, C. J. Paige, P. Sideras, R. Torres, M. C. Lamers, and R. Carsetti. 1999. B Cell Development in the Spleen Takes Place in Discrete Steps and Is Determined by the Quality of B Cell Receptor–Derived Signals. *J. Exp. Med.* 190: 75–90.
17. Oliver, A. M., F. Martin, G. L. Gartland, R. H. Carter, and J. F. Kearney. 1997. Marginal zone B cells exhibit unique activation, proliferative and immunoglobulin secretory responses. *Eur. J. Immunol.* 27: 2366–2374.
18. Oliver, A. M., F. Martin, and J. F. Kearney. 1999. IgMhighCD21high Lymphocytes Enriched in the Splenic Marginal Zone Generate Effector Cells More Rapidly Than the Bulk of Follicular B Cells. *J. Immunol.* 162: 7198–7207.
19. Cinamon, G., M. A. Zachariah, O. M. Lam, F. W. Foss, and J. G. Cyster. 2008. Follicular shuttling of marginal zone B cells facilitates antigen transport. *Nat. Immunol.* 9: 54–62.
20. Guinamard, R., M. Okigaki, J. Schlessinger, and J. V. Ravetch. 2000. Absence of marginal zone B cells in Pyk-2–deficient mice defines their role in the humoral response. *Nat. Immunol.* 1: 31–36.
21. Martin, F., A. M. Oliver, and J. F. Kearney. 2001. Marginal Zone and B1 B Cells Unite in the Early Response against T-Independent Blood-Borne Particulate Antigens. *Immunity* 14: 617–629.
22. Rubtsov, A., P. Strauch, A. DiGiacomo, J. Hu, R. Pelanda, and R. M. Torres. 2005. Lsc Regulates Marginal-Zone B Cell Migration and Adhesion and Is Required for the IgM T-Dependent Antibody Response. *Immunity* 23: 527–538.
23. Song, H., and J. Cerny. 2003. Functional Heterogeneity of Marginal Zone B Cells Revealed by Their Ability to Generate Both Early Antibody-forming Cells and Germinal Centers with Hypermutation and Memory in Response to a T-dependent Antigen. *J. Exp. Med.* 198: 1923–1935.
24. Attanavanich, K., and J. F. Kearney. 2004. Marginal Zone, but Not Follicular B Cells, Are Potent Activators of Naive CD4 T Cells. *J. Immunol.* 172: 803–811.
25. Nolte, M. A., J. A. M. Beliën, I. Schadee-Eestermans, W. Jansen, W. W. J. Unger, N. van Rooijen, G. Kraal, and R. E. Mebius. 2003. A Conduit System Distributes Chemokines and Small Blood-borne Molecules through the Splenic White Pulp. *J. Exp. Med.* 198: 505–512.
26. Arnon, T. I., R. M. Horton, I. L. Grigorova, and J. G. Cyster. 2013. Visualization of splenic marginal zone B-cell shuttling and follicular B-cell egress. *Nature* 493: 684–688.
27. Link, A., F. Zabel, Y. Schnetzler, A. Titz, F. Brombacher, and M. F. Bachmann. 2012. Innate Immunity Mediates Follicular Transport of Particulate but Not Soluble Protein Antigen. *J. Immunol.* 188: 3724–3733.
28. Youd, M. E., A. R. Ferguson, and R. B. Corley. 2002. Synergistic roles of IgM and complement in antigen trapping and follicular localization. *Eur. J. Immunol.* 32: 2328–2337.
29. Ferguson, A. R., M. E. Youd, and R. B. Corley. 2004. Marginal zone B cells transport and deposit IgM-containing immune complexes onto follicular dendritic cells. *Int. Immunol.* 16: 1411–1422.
30. Henningsson, F., Z. Ding, J. S. Dahlin, M. Linkevicius, F. Carlsson, K.-O. Grönvik, J. Hallgren, and B. Heyman. 2011. IgE-Mediated Enhancement of CD4+ T Cell Responses in Mice Requires Antigen Presentation by CD11c+ Cells and Not by B Cells. *PLoS ONE* 6: e21760.

31. Hjelm, F., M. C. I. Karlsson, and B. Heyman. 2008. A Novel B Cell-Mediated Transport of IgE-Immune Complexes to the Follicle of the Spleen. *J. Immunol.* 180: 6604–6610.
32. Cyster, J. G. 2005. Chemokines, Sphingosine-1-Phosphate, and Cell Migration in Secondary Lymphoid Organs. *Annu. Rev. Immunol.* 23: 127–159.
33. Mueller, S. N., and R. N. Germain. 2009. Stromal cell contributions to the homeostasis and functionality of the immune system. *Nat. Rev. Immunol.* 9: 618–629.
34. Victora, G. D. 2014. SnapShot: The Germinal Center Reaction. *Cell* 159: 700–700.e1.
35. Vinuesa, C. G., M. A. Linterman, C. C. Goodnow, and K. L. Randall. 2010. T cells and follicular dendritic cells in germinal center B-cell formation and selection. *Immunol. Rev.* 237: 72–89.
36. MacLennan, I. C. M., K.-M. Toellner, A. F. Cunningham, K. Serre, D. M.-Y. Sze, E. Zúñiga, M. C. Cook, and C. G. Vinuesa. 2003. Extrafollicular antibody responses. *Immunol. Rev.* 194: 8–18.
37. Victora, G. D., T. A. Schwickert, D. R. Fooksman, A. O. Kamphorst, M. Meyer-Hermann, M. L. Dustin, and M. C. Nussenzweig. 2010. Germinal Center Dynamics Revealed by Multiphoton Microscopy with a Photoactivatable Fluorescent Reporter. *Cell* 143: 592–605.
38. Allen, C. D. C., K. M. Ansel, C. Low, R. Lesley, H. Tamamura, N. Fujii, and J. G. Cyster. 2004. Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. *Nat. Immunol.* 5: 943–952.
39. Hardtke, S., L. Ohl, and R. Förster. 2005. Balanced expression of CXCR5 and CCR7 on follicular T helper cells determines their transient positioning to lymph node follicles and is essential for efficient B-cell help. *Blood* 106: 1924–1931.
40. Haynes, N. M., C. D. C. Allen, R. Lesley, K. M. Ansel, N. Killeen, and J. G. Cyster. 2007. Role of CXCR5 and CCR7 in Follicular Th Cell Positioning and Appearance of a Programmed Cell Death Gene-1High Germinal Center-Associated Subpopulation. *J. Immunol.* 179: 5099–5108.
41. Gitlin, A. D., Z. Shulman, and M. C. Nussenzweig. 2014. Clonal selection in the germinal centre by regulated proliferation and hypermutation. *Nature* 509: 637–640.
42. Schwickert, T. A., G. D. Victora, D. R. Fooksman, A. O. Kamphorst, M. R. Mugnier, A. D. Gitlin, M. L. Dustin, and M. C. Nussenzweig. 2011. A dynamic T cell–limited checkpoint regulates affinity-dependent B cell entry into the germinal center. *J. Exp. Med.* 208: 1243–1252.
43. Shulman, Z., A. D. Gitlin, S. Targ, M. Jankovic, G. Pasqual, M. C. Nussenzweig, and G. D. Victora. 2013. T Follicular Helper Cell Dynamics in Germinal Centers. *Science* 341: 673–677.
44. Chan, T. D., and R. Brink. 2012. Affinity-based selection and the germinal center response. *Immunol. Rev.* 247: 11–23.
45. Beale, D., and A. Feinstein. 1969. Studies on the reduction of a human 19s immunoglobulin M. *Biochem. J.* 112: 187–194.
46. Davis, A. C., K. H. Roux, J. Pursey, and M. J. Shulman. 1989. Intermolecular disulfide bonding in IgM: effects of replacing cysteine residues in the mu heavy chain. *EMBO J.* 8: 2519–2526.
47. Milstein, C. P., N. E. Richardson, E. V. Dieverson, and A. Feinstein. 1975. Interchain disulphide bridges of mouse immunoglobulin M. *Biochem. J.* 151: 615–624.

48. Wiersma, E. J., and M. J. Shulman. 1995. Assembly of IgM. Role of disulfide bonding and noncovalent interactions. *J. Immunol.* 154: 5265–5272.
49. Kownatzki, E. 1973. Reassociation of IgM subunits in the presence and absence of J chain. *Immunol. Commun.* 2: 105–113.
50. Kownatzki, E., and M. Drescher. 1973. Antigen binding and complement fixing activity of IgM molecules reassociated in the presence and absence of J chains. *Clin. Exp. Immunol.* 15: 557–563.
51. Czajkowsky, D. M., and Z. Shao. 2009. The human IgM pentamer is a mushroom-shaped molecule with a flexural bias. *Proc. Natl. Acad. Sci. U. S. A.* 106: 14960–14965.
52. Müller, R., M. A. Gräwert, T. Kern, T. Madl, J. Peschek, M. Sattler, M. Groll, and J. Buchner. 2013. High-resolution structures of the IgM Fc domains reveal principles of its hexamer formation. *Proc. Natl. Acad. Sci.* 110: 10183–10188.
53. Capolunghi, F., M. M. Rosado, M. Sinibaldi, A. Aranburu, and R. Carsetti. 2013. Why do we need IgM memory B cells? *Immunol. Lett.* 152: 114–120.
54. Reynaud, C.-A., M. Descatoire, I. Dogan, F. Huetz, S. Weller, and J.-C. Weill. 2012. IgM memory B cells: a mouse/human paradox. *Cell. Mol. Life Sci.* 69: 1625–1634.
55. Haury, M., A. Sundblad, A. Grandien, C. Barreau, A. Coutinho, and A. Nobrega. 1997. The repertoire of serum IgM in normal mice is largely independent of external antigenic contact. *Eur. J. Immunol.* 27: 1557–1563.
56. Ochsenbein, A. F., T. Fehr, C. Lutz, M. Suter, F. Brombacher, H. Hengartner, and R. M. Zinkernagel. 1999. Control of Early Viral and Bacterial Distribution and Disease by Natural Antibodies. *Science* 286: 2156–2159.
57. Baumgarth, N., O. C. Herman, G. C. Jager, L. E. Brown, L. A. Herzenberg, and J. Chen. 2000. B-1 and B-2 Cell–Derived Immunoglobulin M Antibodies Are Nonredundant Components of the Protective Response to Influenza Virus Infection. *J. Exp. Med.* 192: 271–280.
58. Boes, M., C. Esau, M. B. Fischer, T. Schmidt, M. Carroll, and J. Chen. 1998. Enhanced B-1 Cell Development, But Impaired IgG Antibody Responses in Mice Deficient in Secreted IgM. *J. Immunol.* 160: 4776–4787.
59. Ehrenstein, M. R., T. L. O'Keefe, S. L. Davies, and M. S. Neuberger. 1998. Targeted gene disruption reveals a role for natural secretory IgM in the maturation of the primary immune response. *Proc. Natl. Acad. Sci.* 95: 10089–10093.
60. Ohno, T., H. Kubagawa, S. K. Sanders, and M. D. Cooper. 1990. Biochemical nature of an Fc mu receptor on human B-lineage cells. *J. Exp. Med.* 172: 1165–1175.
61. Shibuya, A., N. Sakamoto, Y. Shimizu, K. Shibuya, M. Osawa, T. Hiroyama, H. J. Eyre, G. R. Sutherland, Y. Endo, T. Fujita, T. Miyabayashi, S. Sakano, T. Tsuji, E. Nakayama, J. H. Phillips, L. L. Lanier, and H. Nakauchi. 2000. Fc alpha/mu receptor mediates endocytosis of IgM-coated microbes. *Nat. Immunol.* 1: 441–446.
62. Honda, S., N. Kurita, A. Miyamoto, Y. Cho, K. Usui, K. Takeshita, S. Takahashi, T. Yasui, H. Kikutani, T. Kinoshita, T. Fujita, S. Tahara-Hanaoka, K. Shibuya, and A. Shibuya. 2009. Enhanced humoral immune responses against T-independent antigens in Fcα/μR-deficient mice. *Proc. Natl. Acad. Sci.* 106: 11230–11235.
63. Kubagawa, H., S. Oka, Y. Kubagawa, I. Torii, E. Takayama, D.-W. Kang, G. L. Gartland, L. F. Bertoli, H. Mori, H. Takatsu, T. Kitamura, H. Ohno, and J.-Y. Wang. 2009. Identity of the elusive IgM Fc receptor (FcμR) in humans. *J. Exp. Med.* 206: 2779–2793.

64. Honjo, K., Y. Kubagawa, D. M. Jones, B. Dizon, Z. Zhu, H. Ohno, S. Izui, J. F. Kearney, and H. Kubagawa. 2012. Altered Ig levels and antibody responses in mice deficient for the Fc receptor for IgM (FcμR). *Proc. Natl. Acad. Sci.* 109: 15882–15887.
65. Ouchida, R., H. Mori, K. Hase, H. Takatsu, T. Kurosaki, T. Tokuhisa, H. Ohno, and J.-Y. Wang. 2012. Critical role of the IgM Fc receptor in IgM homeostasis, B-cell survival, and humoral immune responses. *Proc. Natl. Acad. Sci.* 109: E2699–E2706.
66. Hovland, A., L. Jonasson, P. Garred, A. Yndestad, P. Aukrust, K. T. Lappegård, T. Espevik, and T. E. Mollnes. 2015. The complement system and toll-like receptors as integrated players in the pathophysiology of atherosclerosis. *Atherosclerosis* 241: 480–494.
67. Garred, P., C. Honoré, Y. J. Ma, L. Munthe-Fog, and T. Hummelshøj. 2009. MBL2, FCN1, FCN2 and FCN3—The genes behind the initiation of the lectin pathway of complement. *Mol. Immunol.* 46: 2737–2744.
68. Ma, Y. J., M.-O. Skjoedt, and P. Garred. 2013. Collectin-11/MASP Complex Formation Triggers Activation of the Lectin Complement Pathway - The Fifth Lectin Pathway Initiation Complex. *J. Innate Immun.* 5: 242–250.
69. Luzzatto, L., and G. Gianfaldoni. 2006. Recent Advances in Biological and Clinical Aspects of Paroxysmal Nocturnal Hemoglobinuria. *Int. J. Hematol.* 84: 104–112.
70. Atkinson, J. P., and T. H. J. Goodship. 2007. Complement factor H and the hemolytic uremic syndrome. *J. Exp. Med.* 204: 1245–1248.
71. Kang, Y.-H., L. A. Tan, M. V. Carroll, M. E. Gentle, and R. B. Sim. 2009. Target pattern recognition by complement proteins of the classical and alternative pathways. *Adv. Exp. Med. Biol.* 653: 117–128.
72. Botto, M., C. Dell' Agnola, A. E. Bygrave, E. M. Thompson, H. T. Cook, F. Petry, M. Loos, P. P. Pandolfi, and M. J. Walport. 1998. Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies. *Nat. Genet.* 19: 56–59.
73. Schwaeble, W., M. K. Schäfer, F. Petry, T. Fink, D. Knebel, E. Weihe, and M. Loos. 1995. Follicular dendritic cells, interdigitating cells, and cells of the monocyte-macrophage lineage are the C1q-producing sources in the spleen. Identification of specific cell types by in situ hybridization and immunohistochemical analysis. *J. Immunol.* 155: 4971–4978.
74. Whaley, K. 1980. Biosynthesis of the complement components and the regulatory proteins of the alternative complement pathway by human peripheral blood monocytes. *J. Exp. Med.* 151: 501–516.
75. Gulati, P., C. Lemercier, D. Guc, D. Lappin, and K. Whaley. 1993. Regulation of the synthesis of C1 subcomponents and C1-inhibitor. *Behring Inst. Mitt.* 196–203.
76. Dodds, A. W., R. B. Sim, R. R. Porter, and M. A. Kerr. 1978. Activation of the first component of human complement (C1) by antibody-antigen aggregates. *Biochem. J.* 175: 383–390.
77. Quartier, P., P. K. Potter, M. R. Ehrenstein, M. J. Walport, and M. Botto. 2005. Predominant role of IgM-dependent activation of the classical pathway in the clearance of dying cells by murine bone marrow-derived macrophages in vitro. *Eur. J. Immunol.* 35: 252–260.
78. Leffler, J., A. A. Bengtsson, and A. M. Blom. 2014. The complement system in systemic lupus erythematosus: an update. *Ann. Rheum. Dis.* 73: 1601–1606.

79. Collins, C., F. W. L. Tsui, and M. J. Shulman. 2002. Differential activation of human and guinea pig complement by pentameric and hexameric IgM. *Eur. J. Immunol.* 32: 1802–1810.
80. Brown, J. S., T. Hussell, S. M. Gilliland, D. W. Holden, J. C. Paton, M. R. Ehrenstein, M. J. Walport, and M. Botto. 2002. The classical pathway is the dominant complement pathway required for innate immunity to Streptococcus pneumoniae infection in mice. *Proc. Natl. Acad. Sci.* 99: 16969–16974.
81. Jayasekera, J. P., E. A. Moseman, and M. C. Carroll. 2007. Natural Antibody and Complement Mediate Neutralization of Influenza Virus in the Absence of Prior Immunity. *J. Virol.* 81: 3487–3494.
82. Notley, C. A., M. A. Brown, G. P. Wright, and M. R. Ehrenstein. 2011. Natural IgM Is Required for Suppression of Inflammatory Arthritis by Apoptotic Cells. *J. Immunol.* 186: 4967–4972.
83. Kang, Y.-S., S. Yamazaki, T. Iyoda, M. Pack, S. A. Bruening, J. Y. Kim, K. Takahara, K. Inaba, R. M. Steinman, and C. G. Park. 2003. SIGN-R1, a novel C-type lectin expressed by marginal zone macrophages in spleen, mediates uptake of the polysaccharide dextran. *Int. Immunol.* 15: 177–186.
84. Gonzalez, S. F., V. Lukacs-Kornek, M. P. Kuligowski, L. A. Pitcher, S. E. Degn, Y.-A. Kim, M. J. Cloninger, L. Martinez-Pomares, S. Gordon, S. J. Turley, and M. C. Carroll. 2010. Capture of influenza by medullary dendritic cells via SIGN-R1 is essential for humoral immunity in draining lymph nodes. *Nat. Immunol.* 11: 427–434.
85. Kang, Y.-S., J. Y. Kim, S. A. Bruening, M. Pack, A. Charalambous, A. Pritsker, T. M. Moran, J. M. Loeffler, R. M. Steinman, and C. G. Park. 2004. The C-type lectin SIGN-R1 mediates uptake of the capsular polysaccharide of Streptococcus pneumoniae in the marginal zone of mouse spleen. *Proc. Natl. Acad. Sci.* 101: 215–220.
86. Kang, Y.-S., Y. Do, H.-K. Lee, S. H. Park, C. Cheong, R. M. Lynch, J. M. Loeffler, R. M. Steinman, and C. G. Park. 2006. A Dominant Complement Fixation Pathway for Pneumococcal Polysaccharides Initiated by SIGN-R1 Interacting with C1q. *Cell* 125: 47–58.
87. Taylor, P. R., G. D. Brown, J. Herre, D. L. Williams, J. A. Willment, and S. Gordon. 2004. The Role of SIGNR1 and the β-Glucan Receptor (Dectin-1) in the Nonopsonic Recognition of Yeast by Specific Macrophages. *J. Immunol.* 172: 1157–1162.
88. Moens, L., A. Jeurissen, G. Wuyts, P. G. Fallon, B. Louis, J. L. Ceuppens, and X. Bossuyt. 2007. Specific Intracellular Adhesion Molecule-Grabbing Nonintegrin R1 Is Not Involved in the Murine Antibody Response to Pneumococcal Polysaccharides. *Infect. Immun.* 75: 5748–5752.
89. Agrawal, A., P. P. Singh, B. Bottazzi, C. Garlanda, and A. Mantovani. 2009. Pattern Recognition by Pentraxins. *Adv. Exp. Med. Biol.* 653: 98–116.
90. Kopf, M., H. Baumann, G. Freer, M. Freudenberg, M. Lamers, T. Kishimoto, R. Zinkernagel, H. Bluethmann, and G. Köhler. 1994. Impaired immune and acute-phase responses in interleukin-6-deficient mice. *Nature* 368: 339–342.
91. Suresh, M. V., S. K. Singh, D. A. Ferguson, and A. Agrawal. 2006. Role of the Property of C-Reactive Protein to Activate the Classical Pathway of Complement in Protecting Mice from Pneumococcal Infection. *J. Immunol.* 176: 4369–4374.
92. Sørensen, I. J., E. H. Nielsen, O. Andersen, B. Danielsen, and S. E. Svehag. 1996. Binding of complement proteins C1q and C4bp to serum amyloid P component (SAP) in solid contra liquid phase. *Scand. J. Immunol.* 44: 401–407.

93. Whitehead, A. S., K. Zahedi, M. Rits, R. F. Mortensen, and J. M. Lelias. 1990. Mouse C-reactive protein. Generation of cDNA clones, structural analysis, and induction of mRNA during inflammation. *Biochem. J.* 266: 283–290.
94. Thomas-Rudolph, D., T. W. D. Clos, C. M. Snapper, and C. Mold. 2007. C-Reactive Protein Enhances Immunity to Streptococcus pneumoniae by Targeting Uptake to FcγR on Dendritic Cells. *J. Immunol.* 178: 7283–7291.
95. Gillmore, J. D., W. L. Hutchinson, J. Herbert, A. Bybee, D. A. Mitchell, R. P. Hasserjian, K.-I. Yamamura, M. Suzuki, C. A. Sabin, and M. B. Pepys. 2004. Autoimmunity and glomerulonephritis in mice with targeted deletion of the serum amyloid P component gene: SAP deficiency or strain combination? *Immunology* 112: 255–264.
96. Ji, Z., Z.-J. Ke, and J.-G. Geng. 2012. SAP suppresses the development of experimental autoimmune encephalomyelitis in C57BL/6 mice. *Immunol. Cell Biol.* 90: 388–395.
97. Carroll, M. C. 2008. Complement and Humoral Immunity. *Vaccine* 26: I28–I33.
98. Kurtz, C. B., E. O'Toole, S. M. Christensen, and J. H. Weis. 1990. The murine complement receptor gene family. IV. Alternative splicing of Cr2 gene transcripts predicts two distinct gene products that share homologous domains with both human CR2 and CR1. *J. Immunol.* 144: 3581–3591.
99. Molina, H., V. M. Holers, B. Li, Y. Fung, S. Mariathasan, J. Goellner, J. Strauss-Schoenberger, R. W. Karr, and D. D. Chaplin. 1996. Markedly impaired humoral immune response in mice deficient in complement receptors 1 and 2. *Proc. Natl. Acad. Sci. U. S. A.* 93: 3357–3361.
100. Kinoshita, T., J. Takeda, K. Hong, H. Kozono, H. Sakai, and K. Inoue. 1988. Monoclonal antibodies to mouse complement receptor type 1 (CR1). Their use in a distribution study showing that mouse erythrocytes and platelets are CR1-negative. *J. Immunol.* 140: 3066–3072.
101. Kaya, Z., M. Afanasyeva, Y. Wang, K. M. Dohmen, J. Schlichting, T. Tretter, D. Fairweather, V. M. Holers, and N. R. Rose. 2001. Contribution of the innate immune system to autoimmune myocarditis: a role for complement. *Nat. Immunol.* 2: 739–745.
102. Molnár, E., J. Prechl, and A. Erdei. 2008. Novel roles for murine complement receptors type 1 and 2: II. Expression and function of CR1/2 on murine mesenteric lymph node T cells. *Immunol. Lett.* 116: 163–167.
103. Matsumoto, A. K., D. R. Martin, R. H. Carter, L. B. Klickstein, J. M. Ahearn, and D. T. Fearon. 1993. Functional dissection of the CD21/CD19/TAPA-1/Leu-13 complex of B lymphocytes. *J. Exp. Med.* 178: 1407–1417.
104. Molina, H., T. Kinoshita, C. B. Webster, and V. M. Holers. 1994. Analysis of C3b/C3d binding sites and factor I cofactor regions within mouse complement receptors 1 and 2. *J. Immunol.* 153: 789–795.
105. Arvieux, J., H. Yssel, and M. G. Colomb. 1988. Antigen-bound C3b and C4b enhance antigen-presenting cell function in activation of human T-cell clones. *Immunology* 65: 229–235.
106. THORNTON, B. P., V. VĚTVIČKA, and G. D. ROSS. 1996. Function of C3 in a humoral response: iC3b/C3dg bound to an immune complex generated with natural antibody and a primary antigen promotes antigen uptake and the expression of co-stimulatory molecules by all B cells, but only stimulates immunoglobulin synthesis by antigen-specific B cells. *Clin. Exp. Immunol.* 104: 531–537.
107. Carter, R. H., M. O. Spycher, Y. C. Ng, R. Hoffman, and D. T. Fearon. 1988. Synergistic interaction between complement receptor type 2 and membrane IgM on B lymphocytes. *J. Immunol.* 141: 457–463.

108. Dempsey, P. W., M. E. Allison, S. Akkaraju, C. C. Goodnow, and D. T. Fearon. 1996. C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. *Science* 271: 348–350.
109. Fearon, D. T., and R. H. Carter. 1995. The CD19/CR2/TAPA-1 Complex of B Lymphocytes: Linking Natural to Acquired Immunity. *Annu. Rev. Immunol.* 13: 127–149.
110. Donius, L. R., J. J. Weis, and J. H. Weis. 2014. Murine complement receptor 1 is required for germinal center B cell maintenance but not initiation. *Immunobiology* 219: 440–449.
111. Rutemark, C., E. Alicot, A. Bergman, M. Ma, A. Getahun, S. Ellmerich, M. C. Carroll, and B. Heyman. 2011. Requirement for complement in antibody responses is not explained by the classic pathway activator IgM. *Proc. Natl. Acad. Sci.* 108: E934–E942.
112. Carroll, M. C. 2004. The complement system in regulation of adaptive immunity. *Nat. Immunol.* 5: 981–986.
113. Carroll, M. C., and D. E. Isenman. 2012. Regulation of Humoral Immunity by Complement. *Immunity* 37: 199–207.
114. Heyman, B. 2000. Regulation of Antibody Responses via Antibodies, Complement, and Fc Receptors. *Annu. Rev. Immunol.* 18: 709–737.
115. Sörman, A., L. Zhang, Z. Ding, and B. Heyman. 2014. How antibodies use complement to regulate antibody responses. *Mol. Immunol.* 61: 79–88.
116. Heyman, B., E. J. Wiersma, and T. Kinoshita. 1990. In vivo inhibition of the antibody response by a complement receptor-specific monoclonal antibody. *J. Exp. Med.* 172: 665–668.
117. Cutler, A. J., M. Botto, D. van Essen, R. Rivi, K. A. Davies, D. Gray, and M. J. Walport. 1998. T Cell–dependent Immune Response in C1q-deficient Mice: Defective Interferon γ Production by Antigen-specific T Cells. *J. Exp. Med.* 187: 1789–1797.
118. Mehlhop, E., and M. S. Diamond. 2006. Protective immune responses against West Nile virus are primed by distinct complement activation pathways. *J. Exp. Med.* 203: 1371–1381.
119. Taylor, P. R., E. Seixas, M. J. Walport, J. Langhorne, and M. Botto. 2001. Complement Contributes to Protective Immunity against Reinfection by Plasmodium chabaudi chabaudiParasites. *Infect. Immun.* 69: 3853–3859.
120. Böttger, E. C., T. Hoffmann, U. Hadding, and D. Bitter-Suermann. 1985. Influence of genetically inherited complement deficiencies on humoral immune response in guinea pigs. *J. Immunol.* 135: 4100–4107.
121. Böttger, E. C., S. Metzger, D. Bitter-Suermann, G. Stevenson, S. Kleindienst, and R. Burger. 1986. Impaired humoral immune response in complement C3-deficient guinea pigs: absence of secondary antibody response. *Eur. J. Immunol.* 16: 1231–1235.
122. Burger, R., J. Gordon, G. Stevenson, G. Ramadori, B. Zanker, U. Hadding, and D. Bitter-Suermann. 1986. An inherited deficiency of the third component of complement, C3, in guinea pigs. *Eur. J. Immunol.* 16: 7–11.
123. Fischer, M. B., M. Ma, S. Goerg, X. Zhou, J. Xia, O. Finco, S. Han, G. Kelsoe, R. G. Howard, T. L. Rothstein, E. Kremmer, F. S. Rosen, and M. C. Carroll. 1996. Regulation of the B cell response to T-dependent antigens by classical pathway complement. *J. Immunol.* 157: 549–556.
124. Hazlewood, M. A., D. S. Kumararatne, A. D. B. Webster, M. Goodall, P. Bird, and M. Daha. 1992. An association between homozygous C3 deficiency and low levels of anti-pneumococcal capsular polysaccharide antibodies. *Clin. Exp. Immunol.* 87: 404–409.

125. O'Neil, K. M., H. D. Ochs, S. R. Heller, L. C. Cork, J. M. Morris, and J. A. Winkelstein. 1988. Role of C3 in humoral immunity. Defective antibody production in C3-deficient dogs. *J. Immunol.* 140: 1939–1945.
126. Pepys, M. B. 1974. Role of Complement in Induction of Antibody Production in Vivo Effect of Cobra Factor and Other C3-Reactive Agents on Thymus-Dependent and Thymus-Independent Antibody Responses. *J. Exp. Med.* 140: 126–145.
127. Finco, O., S. Li, M. Cuccia, F. S. Rosen, and M. C. Carroll. 1992. Structural differences between the two human complement C4 isotypes affect the humoral immune response. *J. Exp. Med.* 175: 537–543.
128. Jackson, C. G., H. D. Ochs, and R. J. Wedgwood. 1979. Immune Response of a Patient with Deficiency of the Fourth Component of Complement and Systemic Lupus Erythematosus. *N. Engl. J. Med.* 300: 1124–1129.
129. Ochs, H. D., R. J. Wedgwood, M. M. Frank, S. R. Heller, and S. W. Hosea. 1983. The role of complement in the induction of antibody responses. *Clin. Exp. Immunol.* 53: 208–216.
130. Ahearn, J. M., and D. T. Fearon. 1989. Structure and function of the complement receptors, CR1 (CD35) and CR2 (CD21). *Adv. Immunol.* 46: 183–219.
131. Carlsson, F., A. Getahun, C. Rutemark, and B. Heyman. 2009. Impaired Antibody Responses but Normal Proliferation of Specific CD4+ T Cells in Mice Lacking Complement Receptors 1 and 2. *Scand. J. Immunol.* 70: 77–84.
132. Croix, D. A., J. M. Ahearn, A. M. Rosengard, S. Han, G. Kelsoe, M. Ma, and M. C. Carroll. 1996. Antibody response to a T-dependent antigen requires B cell expression of complement receptors. *J. Exp. Med.* 183: 1857–1864.
133. Fang, Y., C. Xu, Y.-X. Fu, V. M. Holers, and H. Molina. 1998. Expression of Complement Receptors 1 and 2 on Follicular Dendritic Cells Is Necessary for the Generation of a Strong Antigen-Specific IgG Response. *J. Immunol.* 160: 5273–5279.
134. Thiel, J., L. Kimmig, U. Salzer, M. Grudzien, D. Lebrecht, T. Hagena, R. Draeger, N. Völxen, A. Bergbreiter, S. Jennings, S. Gutenberger, A. Aichem, H. Illges, J. P. Hannan, A.-K. Kienzler, M. Rizzi, H. Eibel, H.-H. Peter, K. Warnatz, B. Grimbacher, J.-A. Rump, and M. Schlesier. 2012. Genetic CD21 deficiency is associated with hypogammaglobulinemia. *J. Allergy Clin. Immunol.* 129: 801–810.e6.
135. Carter, T., M. Sumiya, K. Reilly, R. Ahmed, P. Sobieszczuk, J. A. Summerfield, and R. A. Lawrence. 2007. Mannose-Binding Lectin A-Deficient Mice Have Abrogated Antigen-Specific IgM Responses and Increased Susceptibility to a Nematode Infection. *J. Immunol.* 178: 5116–5123.
136. Lawrence, R. A., T. Carter, L. V. Bell, K. J. Else, J. Summerfield, and Q. Bickle. 2009. Altered antibody responses in mannose-binding lectin-A deficient mice do not affect Trichuris muris or Schistosoma mansoni infections. *Parasite Immunol.* 31: 104–109.
137. Ruseva, M., M. Kolev, F. Dagnaes-Hansen, S. B. Hansen, K. Takahashi, A. Ezekowitz, S. Thiel, J. C. Jensenius, and M. Gadjeva. 2009. Mannan-binding lectin deficiency modulates the humoral immune response dependent on the genetic environment. *Immunology* 127: 279–288.
138. Guttormsen, H.-K., L. M. Stuart, L. Shi, M. C. Carroll, J. Chen, D. L. Kasper, R. A. B. Ezekowitz, and K. Takahashi. 2009. Deficiency of mannose-binding lectin greatly increases antibody response in a mouse model of vaccination. *Clin. Immunol.* 130: 264–271.
139. Ali, Y. M., N. J. Lynch, K. S. Haleem, T. Fujita, Y. Endo, S. Hansen, U. Holmskov, K. Takahashi, G. L. Stahl, T. Dudler, U. V. Girija, R. Wallis, A.

Kadioglu, C. M. Stover, P. W. Andrew, and W. J. Schwaeble. 2012. The Lectin Pathway of Complement Activation Is a Critical Component of the Innate Immune Response to Pneumococcal Infection. *PLoS Pathog* 8: e1002793.
140. Endo, Y., M. Takahashi, D. Iwaki, Y. Ishida, N. Nakazawa, T. Kodama, T. Matsuzaka, K. Kanno, Y. Liu, K. Tsuchiya, I. Kawamura, M. Ikawa, S. Waguri, I. Wada, M. Matsushita, W. J. Schwaeble, and T. Fujita. 2012. Mice Deficient in Ficolin, a Lectin Complement Pathway Recognition Molecule, Are Susceptible to Streptococcus pneumoniae Infection. *J. Immunol.* 189: 5860–5866.
141. Matsumoto, M., W. Fukuda, A. Circolo, J. Goellner, J. Strauss-Schoenberger, X. Wang, S. Fujita, T. Hidvegi, D. D. Chaplin, and H. R. Colten. 1997. Abrogation of the alternative complement pathway by targeted deletion of murine factor B. *Proc. Natl. Acad. Sci.* 94: 8720–8725.
142. Martinelli, G. P., T. Matsuda, H. S. Waks, and A. G. Osler. 1978. Studies on Immunosuppression by Cobra Venom Factor III. On Early Responses to Sheep Erythrocytes in C5-Deficient Mice. *J. Immunol.* 121: 2052–2055.
143. Heyman, B. 2014. Antibodies as natural adjuvants. *Curr. Top. Microbiol. Immunol.* 382: 201–219.
144. Clarke, C. A., R. Finn, D. Lehane, R. B. McConnell, P. M. Sheppard, and J. C. Woodrow. 1966. Dose of anti-D gamma-globulin in prevention of Rh-haemolytic disease of the newborn. *Br. Med. J.* 1: 213–214.
145. Getahun, A., and B. Heyman. 2009. Studies on the Mechanism by Which Antigen-Specific IgG Suppresses Primary Antibody Responses: Evidence for Epitope Masking and Decreased Localization of Antigen in the Spleen. *Scand. J. Immunol.* 70: 277–287.
146. Henry, C., and N. K. Jerne. 1968. Competition of 19s and 7s Antigen Receptors in the Regulation of the Primary Immune Response. *J. Exp. Med.* 128: 133–152.
147. Karlsson, M. C. I., S. Wernersson, T. D. de Ståhl, S. Gustavsson, and B. Heyman. 1999. Efficient IgG-mediated suppression of primary antibody responses in Fcγ receptor-deficient mice. *Proc. Natl. Acad. Sci.* 96: 2244–2249.
148. Hjelm, F., F. Carlsson, A. Getahun, and B. Heyman. 2006. Antibody-Mediated Regulation of the Immune Response. *Scand. J. Immunol.* 64: 177–184.
149. Wernersson, S., M. C. I. Karlsson, J. Dahlström, R. Mattsson, J. S. Verbeek, and B. Heyman. 1999. IgG-Mediated Enhancement of Antibody Responses Is Low in Fc Receptor γ Chain-Deficient Mice and Increased in FcγRII-Deficient Mice. *J. Immunol.* 163: 618–622.
150. Getahun, A., J. Dahlström, S. Wernersson, and B. Heyman. 2004. IgG2a-Mediated Enhancement of Antibody and T Cell Responses and Its Relation to Inhibitory and Activating Fcγ Receptors. *J. Immunol.* 172: 5269–5276.
151. Ståhl, T. D. de, J. Dahlström, M. C. Carroll, and B. Heyman. 2003. A Role for Complement in Feedback Enhancement of Antibody Responses by IgG3. *J. Exp. Med.* 197: 1183–1190.
152. Perlmutter, R. M., D. Hansburg, D. E. Briles, R. A. Nicolotti, and J. M. David. 1978. Subclass Restriction of Murine Anti-Carbohydrate Antibodies. *J. Immunol.* 121: 566–572.
153. Rubinstein, L. J., and K. E. Stein. 1988. Murine immune response to the Neisseria meningitidis group C capsular polysaccharide. I. Ontogeny. *J. Immunol.* 141: 4352–4356.
154. McLay, J., E. Leonard, S. Petersen, D. Shapiro, N. S. Greenspan, and J. R. Schreiber. 2002. γ3 Gene-Disrupted Mice Selectively Deficient in the Dominant IgG Subclass Made to Bacterial Polysaccharides. II. Increased Susceptibility to Fatal Pneumococcal Sepsis Due to Absence of Anti-Polysaccharide IgG3 Is

Corrected by Induction of Anti-Polysaccharide IgG1. *J. Immunol.* 168: 3437–3443.
155. Hjelm, F., F. Carlsson, S. Verbeek, and B. Heyman. 2005. IgG3-Mediated Enhancement of the Antibody Response is Normal in FcγRI-Deficient Mice. *Scand. J. Immunol.* 62: 453–461.
156. Abdelmoula, M., F. Spertini, T. Shibata, Y. Gyotoku, S. Luzuy, P. H. Lambert, and S. Izui. 1989. IgG3 is the major source of cryoglobulins in mice. *J. Immunol.* 143: 526–532.
157. Cooper, L. J., J. C. Schimenti, D. D. Glass, and N. S. Greenspan. 1991. H chain C domains influence the strength of binding of IgG for streptococcal group A carbohydrate. *J. Immunol.* 146: 2659–2663.
158. Zhang, L., Z. Ding, H. Xu, and B. Heyman. 2014. Marginal Zone B Cells Transport IgG3-Immune Complexes to Splenic Follicles. *J. Immunol.* 193: 1681–1689.
159. Heyman, B., G. Andrighetto, and H. Wigzell. 1982. Antigen-dependent IgM-mediated enhancement of the sheep erythrocyte response in mice. Evidence for induction of B cells with specificities other than that of the injected antibodies. *J. Exp. Med.* 155: 994–1009.
160. Wason, W. M. 1973. Regulation of the Immune Response with Antigen Specific IgM Antibody: A Dual Role. *J. Immunol.* 110: 1245–1252.
161. Harte, P. G., A. Cooke, and J. H. Playfair. 1983. Specific monoclonal IgM is a potent adjuvant in murine malaria vaccination. *Nature* 302: 256–258.
162. Dennert, G., and J. Salk. 1973. Effects of IgM on the in vivo and in vitro immune response. *Proc. Soc. Exp. Biol. Med. Soc. Exp. Biol. Med. N. Y. N* 143: 889–893.
163. Heyman, B., L. Pilström, and M. J. Shulman. 1988. Complement activation is required for IgM-mediated enhancement of the antibody response. *J. Exp. Med.* 167: 1999–2004.
164. Chen, F. H., S. K. Arya, A. Rinfret, D. E. Isenman, M. J. Shulman, and R. H. Painter. 1997. Domain-switched mouse IgM/IgG2b hybrids indicate individual roles for C mu 2, C mu 3, and C mu 4 domains in the regulation of the interaction of IgM with complement C1q. *J. Immunol.* 159: 3354–3363.
165. Applequist, S. E., J. Dahlström, N. Jiang, H. Molina, and B. Heyman. 2000. Antibody Production in Mice Deficient for Complement Receptors 1 and 2 Can Be Induced by IgG/Ag and IgE/Ag, But Not IgM/Ag Complexes. *J. Immunol.* 165: 2398–2403.
166. Marchbank, K. J., C. C. Watson, D. F. Ritsema, and V. M. Holers. 2000. Expression of Human Complement Receptor 2 (CR2, CD21) in Cr2−/− Mice Restores Humoral Immune Function. *J. Immunol.* 165: 2354–2361.
167. Kulik, L., K. Chen, B. T. Huber, and V. M. Holers. 2011. Human complement receptor type 2 (CR2/CD21) transgenic mice provide an in vivo model to study immunoregulatory effects of receptor antagonists. *Mol. Immunol.* 48: 883–894.
168. Hebell, T., J. M. Ahearn, and D. T. Fearon. 1991. Suppression of the immune response by a soluble complement receptor of B lymphocytes. *Science* 254: 102–105.
169. Boackle, S. A., V. M. Holers, and D. R. Karp. 1997. CD21 augments antigen presentation in immune individuals. *Eur. J. Immunol.* 27: 122–129.
170. Cherukuri, A., P. C. Cheng, and S. K. Pierce. 2001. The Role of the CD19/CD21 Complex in B Cell Processing and Presentation of Complement-Tagged Antigens. *J. Immunol.* 167: 163–172.

171. Prechl, J., D. C. Baiu, A. Horváth, and A. Erdei. 2002. Modeling the presentation of C3d-coated antigen by B lymphocytes: enhancement by CR1/2–BCR coligation is selective for the co-ligating antigen. *Int. Immunol.* 14: 241–247.
172. Papamichail, M., C. Gutierrez, P. Embling, P. Johnson, E. J. Holborow, and M. B. Pepys. 1975. Complement dependence of localisation of aggregated IgG in germinal centres. *Scand. J. Immunol.* 4: 343–347.
173. Barrington, R. A., M. Zhang, X. Zhong, H. Jonsson, N. Holodick, A. Cherukuri, S. K. Pierce, T. L. Rothstein, and M. C. Carroll. 2005. CD21/CD19 Coreceptor Signaling Promotes B Cell Survival during Primary Immune Responses. *J. Immunol.* 175: 2859–2867.
174. Thyphronitis, G., T. Kinoshita, K. Inoue, J. E. Schweinle, G. C. Tsokos, E. S. Metcalf, F. D. Finkelman, and J. E. Balow. 1991. Modulation of mouse complement receptors 1 and 2 suppresses antibody responses in vivo. *J. Immunol.* 147: 224–230.
175. Costa, X. J. D., M. A. Brockman, E. Alicot, M. Ma, M. B. Fischer, X. Zhou, D. M. Knipe, and M. C. Carroll. 1999. Humoral response to herpes simplex virus is complement-dependent. *Proc. Natl. Acad. Sci.* 96: 12708–12712.
176. Gustavsson, S., T. Kinoshita, and B. Heyman. 1995. Antibodies to murine complement receptor 1 and 2 can inhibit the antibody response in vivo without inhibiting T helper cell induction. *J. Immunol.* 154: 6524–6528.
177. Haas, K. M., M. Hasegawa, D. A. Steeber, J. C. Poe, M. D. Zabel, C. B. Bock, D. R. Karp, D. E. Briles, J. H. Weis, and T. F. Tedder. 2002. Complement Receptors CD21/35 Link Innate and Protective Immunity during Streptococcus pneumoniae Infection by Regulating IgG3 Antibody Responses. *Immunity* 17: 713–723.
178. Shulman, M. J., C. Collins, N. Pennell, and N. Hozumi. 1987. Complement activation by IgM: evidence for the importance of the third constant domain of the mu heavy chain. *Eur. J. Immunol.* 17: 549–554.
179. Humphrey, J. H., D. Grennan, and V. Sundaram. 1984. The origin of follicular dendritic cells in the mouse and the mechanism of trapping of immune complexes on them. *Eur. J. Immunol.* 14: 859–864.
180. Jaroslow, B. N., and G. J. Nossal. 1966. Effect of x-irradiation on antigen localization in lymphoid follicles. *Aust. J. Exp. Biol. Med. Sci.* 44: 609–627.
181. Hamad, O. A., K. N. Ekdahl, P. H. Nilsson, J. Andersson, P. Magotti, J. D. Lambris, and B. Nilsson. 2008. Complement activation triggered by chondroitin sulfate released by thrombin receptor-activated platelets. *J. Thromb. Haemost.* 6: 1413–1421.
182. Baker, N., and M. R. Ehrenstein. 2002. Cutting Edge: Selection of B Lymphocyte Subsets Is Regulated by Natural IgM. *J. Immunol.* 169: 6686–6690.
183. Hicks, P. S., L. Saunero-Nava, T. W. D. Clos, and C. Mold. 1992. Serum amyloid P component binds to histones and activates the classical complement pathway. *J. Immunol.* 149: 3689–3694.
184. D Bratosin. 2001. Programmed cell death in mature erythrocytes: a model for investigating death effector pathways operating in the absence of mitochondria. *Publ. Online 06 Dec. 2001 Doi101038sjcdd4400946* 8.
185. Agalakova, N. I., and G. P. Gusev. 2011. Fluoride-induced death of rat erythrocytes in vitro. *Toxicol. In Vitro* 25: 1609–1618.
186. Tew, J. G., R. P. Phipps, and T. E. Mandel. 1980. The maintenance and regulation of the humoral immune response: persisting antigen and the role of follicular antigen-binding dendritic cells as accessory cells. *Immunol. Rev.* 53: 175–201.

187. Lei, X., C. Liu, K. Azadzoi, C. Li, F. Lu, A. Xiang, J. Sun, Y. Guo, Q. Zhao, Z. Yan, and J. Yang. 2015. A novel IgM–H-Ficolin complement pathway to attack allogenic cancer cells in vitro. *Sci. Rep.* 5.
188. Brockman, M. A., A. Verschoor, J. Zhu, M. C. Carroll, and D. M. Knipe. 2006. Optimal Long-Term Humoral Responses to Replication-Defective Herpes Simplex Virus Require CD21/CD35 Complement Receptor Expression on Stromal Cells. *J. Virol.* 80: 7111–7117.
189. Shima, H., H. Takatsu, S. Fukuda, M. Ohmae, K. Hase, H. Kubagawa, J.-Y. Wang, and H. Ohno. 2010. Identification of TOSO/FAIM3 as an Fc receptor for IgM. *Int. Immunol.* 22: 149–156.

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