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IgG3 Complements IgM in the Complement-Mediated Regulation of Immune Responses

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Abstract
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An intact complement system is essential for the initiation of a normal antibody response. Antibodies can regulate their own production against the antigens that they are specific for. Both IgG3 and IgM are able to enhance the antibody response via complement. Here, we have compared the fate of OVA-TNP (ovalbumin-2,4,6-trinitrophenyl) administered intravenously to mice either alone or in complex with monoclonal IgG3 anti-TNP. IgG3-antigen complexes bind to marginal zone (MZ) B cells via complement receptors 1 and 2 (CR1/2) and are transported into splenic follicles. The majority (50% - 90%) of the antigens is deposited on follicular dendritic cells (FDC) and the antigen distribution pattern is strikingly similar to peripheral dendrites/ processes of FDC already 2 h after immunization. The development of germinal centers (GC) induced by IgG3-antigen complexes is impaired in mice lacking CR1/2. Experiments on bone marrow chimeric mice show that CR1/2 expression on both MZ B cells and FDC is required for optimal IgG3-mediated enhancement of antibody responses. Complement factors C3 and C1q are essential for OVA-TNP delivery and deposition on splenic FDC. The production of IgG anti-OVA is abrogated in mice lacking CR1/2, C1q, and C3. Further, IgG3-antigen complexes dramatically upregulate the memory response against OVA-TNP by inducing OVA-specific memory cells. Besides small protein OVA, IgG3 can also upregulate humoral responses against large soluble keyhole limpet hemocyanin.

To further study the role of MZ B-cells and CR1/2 in enhancement of antibody responses, a knock-in mouse strain, Cμ13, was used. IgM in this mouse strain is unable to activate complement due to a point mutation in the constant μ-heavy chain. Cμ13 mice have a higher proportion of MZ B cells, with higher CR1/2 expression, than wild-type mice. More IgG3-immune complexes are captured by MZ B cells and deposited on FDC in Cμ13 than in WT mice. In spite of this, IgG3 did not enhance the primary antibody response more efficiently in Cμ13 mice. The existence of endogenous IgM-mediated feedback regulation was suggested by the observation that GC development and antibody responses, after priming and boosting with suboptimal doses of SRBC, was lower in Cμ13 than in WT mice.

Keywords: IgG3, IgM, marginal zone B cells, follicular dendritic cells, complement receptors 1 and 2, C1q, C3, antigen transport

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山重水复疑无路，柳暗花明又一村。
--宋朝诗人陆游

Through toils of wilderness, past endless ridges and rapids,
behind shady willows, lie bright flowers and a lovely secret hamlet.
--You Lu, poet of the Song dynasty
(translated by Jin Zhao)

To my family and love

List of Papers

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

I **Zhang, L.**, Ding, Z., Xu, H. and Heyman, B. Marginal Zone B Cells Transport IgG3-Immune Complexes to Splenic Follicles. *J. Immunol.* **193,** 1681–1689 (2014).

II **Zhang, L.**, Ding, Z., and Heyman, B. IgG3-Antigen Complexes Are Deposited on Follicular Dendritic Cells in the Presence of C1q and C3. *(Manuscript)*

III **Zhang, L.***, Sörman, A.*, Westin, A., and Heyman, B. Mice Producing IgM Unable to Activate Complement Have Impaired Endogenous Feedback Regulation but Increased Antigen Trapping in Follicles. *(Manuscript)* *equal contribution

Review article (not included in the thesis)

Sörman, A., **Zhang, L.**, Ding, Z., and Heyman, B. (2014). How Antibodies Use Complement to Regulate Antibody Responses. *Molecular Immunology* 61, 79-88.

Contents

Abbreviations

APC	antigen presenting cells
BAFF	B-cell activating factor
BCR	B-cell receptors
BSA	bovine serum albumin
C	complement
CCL	chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
CD40L	CD40 ligand
CR	complement receptor
CR1/2	complement receptors 1 and 2
CVF	cobra venom factor
CXCL	chemokine (C-X-C motif) ligand
CXCR	C-X-C chemokine receptor
DC	dendritic cells
DZ	dark zone
Fc	fragment crystallizable
FcαR	Fc alpha receptor
Fcα/μR	Fc alpha/mu receptor
FcεR	Fc epsilon receptor
FcγR	Fc gamma receptor
FcμR	Fc mu receptor
FcRs	Fc receptors
FcRγ	Fc receptor gamma chain
FDC	follicular dendritic cells
FO	follicular
FTY720	2-amino-2-[2-(4-octylphenyl) ethyl]-1,3-propanediol hydrochloride
GC	germinal center
i.v.	intravenously
KLH	keyhole limpet hemocyanin
KO	knock-out
LZ	light zone
MHC	major histocompability complex
MZ	marginal zone
OVA	ovalbumin
RhD	Rhesus D antigen

SHM	somatic hypermutation
SRBC	sheep red blood cells
S1P	sphingosine 1-phosphate
S1PR1	S1P receptor 1
TCR	T-cell receptor
TD	thymus-dependent
Tfh	T follicular helper cells
TI	thymus-independent
TNP	2,4,6-trinitrophenyl
WT	wild-type

Introduction

It is a truth universally acknowledged, that all living organisms must possess defense mechanisms against infection. In more recently evolved organisms such as mammals, a specialized and sophisticated defense system called the immune system is evolved to eliminate threats from either the outside or the inside environment. The immune system can be classified into the following two subsystems: innate immunity and adaptive immunity. Innate immunity addresses a broad spectrum of pathogens without a high specificity and can be stimulated in a rapid but short-term manner. In contrast, adaptive immunity slowly adapts to infections, resulting in an exquisite specificity, and memorizes the infections for subsequent encounters with the same pathogens.

Lymphoid organs

The lymphoid organs can be classified as primary lymphoid organs, comprising the bone marrow and the thymus, and secondary lymphoid organs, such as the lymph nodes, spleen, and Peyer's patches. In the primary lymphoid organs, lymphocytes are generated and matured. In the secondary lymphoid organs, the mature naïve lymphocytes are maintained, and the immune responses are initiated. This thesis focuses on studies regarding immune responses that occur in the mouse spleen.

Spleen

As the largest blood filter in the body, the spleen is where the massive production of antibodies against blood-borne antigens occurs. The murine spleen consists of the following two major parts: the red pulp in which the blood is filtered and the irons are recycled, and the lymphatic white pulp in which the immune responses initiate (reviewed in reference[1]).

The white pulp is composed of the following relatively separated compartments: the B-cell follicle(s) ('follicles' in short is used below), the T-cell zone, and the marginal zone (MZ), which encloses both the follicle(s) and T-cell zone and separates them from the red pulp (reviewed in reference[1]). White pulp comprises many different types of cells as follows: follicular (FO) B cells, MZ B cells, T cells, dendritic cells (DC), macrophages, and stromal cells (including follicular dendritic cells (FDC)).

The MZ area is a unique microenvironment in which blood is released from arterioles, and the adaptive immune responses against blood-borne antigens are initiated. Various resident immune cells are involved, such as MZ B cells, DC, MZ macrophages, and metallophilic macrophages. The MZ macrophages form an outer boundary that separates the MZ from the red pulp, while the metallophilic macrophages form an inner boundary on the border between the MZ area and the follicle (reviewed in reference[2]).

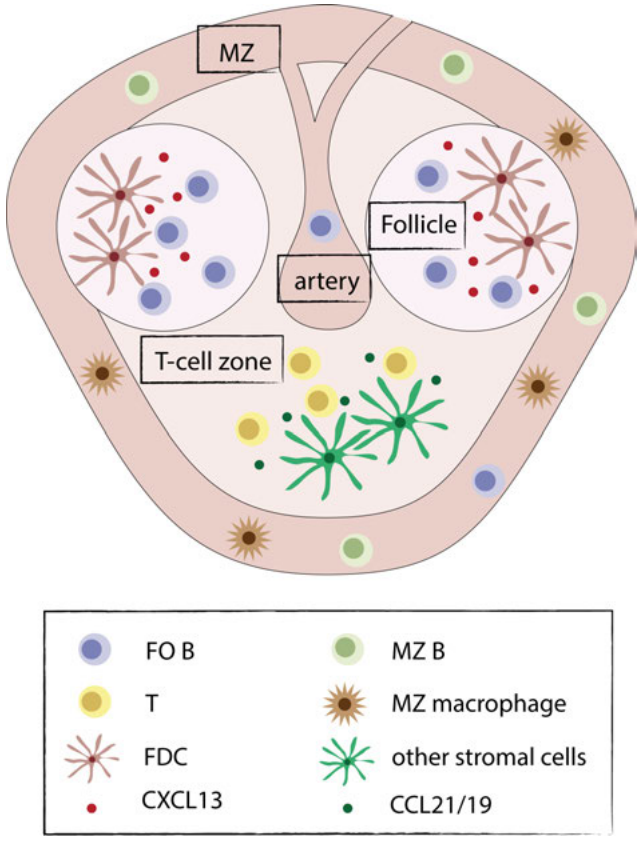

Figure 1. Scheme of splenic white pulp. The splenic white pulp contains different types of cells as follows: MZ B cells, which mainly reside in the MZ area but also shuttle across the border between the MZ area and the follicle in a semi-motile fashion; MZ macrophages, which are found in the MZ area; circulating FO B cells, which are found in the blood and follicles; CXCL13-releasing FDC, which are in the follicles; T cells, which are found in the T zone; and CCL21- and CCL19-releasing stromal cells (e.g., fibroblastic reticular cells), which are found in the T zone.

The follicle contains a high gradient of chemokine (C-X-C motif) ligand 13 (CXCL13), also known as B-lymphocyte chemoattractant, which attracts C-X-C chemokine receptor 5 (CXCR5)-expressing cells, such as B cells. The CXCL13-expressing cells are colocalized with cells that are positive for the FDC markers. Therefore, it is likely that the FDC are the cells that produce

CXCL13. However, this hypothesis does not exclude the possibility that cells that are tightly associated with the FDC can also produce CXCL13 (reviewed in reference[2]).

The T-cell zone contains a high gradient of chemokine (C-C motif) ligand 21 (CCL21) and CCL19, which are released by the stromal cells, e.g., the fibroblastic reticular cells. CCL21 and CCL19 attract and retain C-C chemokine receptor 7 (CCR7) -expressing cells, such as T cells and DC (Fig. 1) (reviewed in reference[2]).

Immune cells

Many different types of cells defend the host when it encounters pathogens as follows: innate immune cells, including natural killer cells, mast cells, eosinophils, basophils, macrophages, neutrophils, and DC, and adaptive immune cells, including B and T lymphocytes. In addition, there are also connective tissues, including stromal cells, that support the immune system. Here, we focus on the following three major cell types in the spleen, which are closely related to the research focus of this thesis: B cells, FDC, and T cells.

B cells

The following two major types of B cells can be found in the splenic white pulp: FO B cells and MZ B cells. FO B cells are defined as cells with a marker profile of $B220^{+}$ IgM^{hi} IgD^{hi} $CD23^{hi}$ $CD21^{lo}$, while MZ B cells are defined as $B220^{+}$ IgM^{hi} IgD^{lo} $CD23^{lo}$ $CD21^{hi}$ $CD1d^{hi}$ cells (reviewed in reference[3]).

FO B cells are circulating cells that are present in the blood and follicles. The following two states of follicles can be found in the splenic white pulp: primary and secondary follicles. B cells that have never encountered any antigens, which are known as naïve B cells, are the major components of the primary follicles in which they are extensively searching for cognate antigens. B cells that have previously encountered antigens, which are also called activated B cells, mainly exist in the secondary follicles in a central area called the germinal center (GC) (reviewed in reference[4]).

Unlike their FO counterparts, MZ B cells are generally assumed to comprise a ‘residing’ cell population in the MZ. These cells are retained in the MZ area by integrin-mediated adhesion[5]. Recently, it has been discovered that MZ B cells shuttle between the MZ area and the follicle with a cellular exchange rate of at least 20% per hour[6]. The following two opposing chemoattraction scenarios appear to influence the shuttling activity of MZ B cells: (i) CXCL13 attracts CXCR5-expressing cells to the follicle and (ii) sphingosine 1-phosphate (S1P) attracts S1P receptor 1 (S1PR1)-expressing cells to

the MZ area. All mature B cells (including MZ B cells) express CXCR5 and, thus, are constantly attracted to the follicles due to the high gradient of CXCL13 that is released by stromal cells, e.g., FDC. In addition, the MZ B cells express high levels of S1PR1. In the white pulp, the MZ area is rapidly and continuously filled with blood that contains a high concentration of S1P, while the follicle contains quite low levels of S1P due to the presence of the S1P-degrading enzyme S1P lyase. Normally, the attraction between S1P and S1PR1 overcomes the CXCR5-CXCL13 interaction, which attracts MZ B cells to the MZ area. However, S1P bound to S1PR1 will lead to S1PR1 internalization into the MZ B cells when they reside in the MZ area. Consequently, the MZ B cells will lose their attraction to the MZ area and migrate to the follicle in response to the high gradient of CXCL13. With low S1P concentrations in the follicles, the MZ B cells will gradually regain S1PR1 expression and move back to the MZ area (Fig. 2) (reviewed in reference[7]). The shuttling activity of MZ B cells facilitates the transport of the IgM-antigen-complement complexes to which they bind[8].

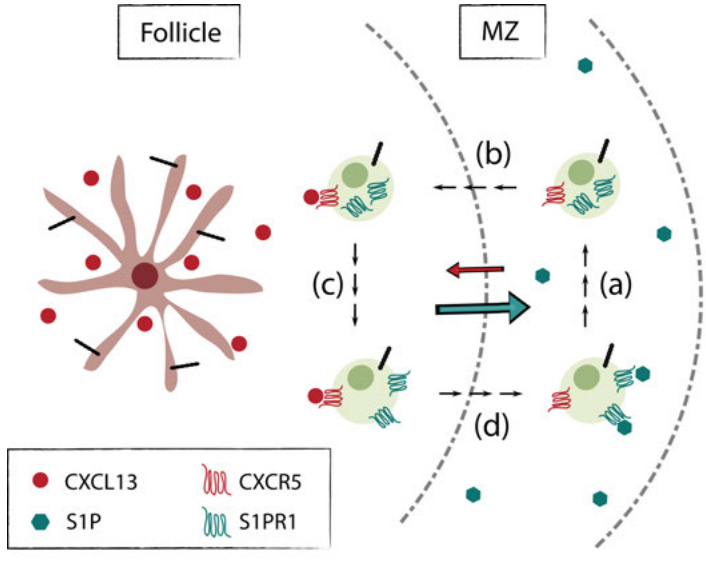

Figure 2. MZ B shuttling activity. MZ B cells express CXCR5 and high levels of S1PR1, which are the receptors for S1P and CXCL13, respectively. The concentration of S1P is high in the blood flow but low in tissues due to the presence of S1P lyase. CXCL13, which is generated by stromal cells, including FDC, is present in high concentrations in the follicle. The chemoattraction between S1PR1 and S1P (green arrow) overcomes that between CXCR5 and CXCL13 (red arrow), and therefore, the MZ B cells are prone to move toward the MZ area. (a) S1PR1 is internalized due to S1P binding. (b) MZ B cells with internalized S1PR1 migrate to the follicle in response to CXCL13. (c) MZ B cells regain S1PR1 expression due to the low gradient of S1P within the follicle. (d) MZ B cells with regained S1PR1 migrate back to the MZ area.

As an antagonist of S1PR1, 2-amino-2-[2-(4-octylphenyl) ethyl]-1,3-propanediol hydrochloride (FTY720) can transiently down-regulate the S1PR1 expression on MZ B cells. Treatment with FTY720 abolishes the attraction between S1P and S1PR1, which leads to a rapid and reversible dislocation of the MZ B cells from the MZ area to the follicle[9]. Additionally, exposure to cognate antigens and lipopolysaccharides can also induce S1PR1 internalization in MZ B cells[9].

Follicular dendritic cells

The existence of FDC, that retain immune complexes on their surfaces, in the follicles has been known since the 1960s[10–13]. In these early literatures, FDC were often referred to as 'follicular reticular cells,' 'dendritic reticular cells' or 'antigen-retaining reticular cells'. In 1978, these cells were termed 'follicular dendritic cells' for the first time[14], which was recommended as the nomenclature in 1982[15].

FDC are distinct in their morphology and their capacity to retain immune complexes. Morphologically, these cells are large and have irregularly shaped nuclei that contain very little heterochromatin. The cytoplasm has a stellate shape, beginning near the nuclei with long, thin processes, which can fold and coil, extending in several directions. The FDC cytoplasm contains few organelles, such as the rough endoplasmic reticulum and lysosomes, and therefore, FDC are distinct from other cells that are also extended in shape. Their processes retain particulates and are coated with electron-dense materials[14].

The cellular origin of FDC remained puzzling for quite a long time. Chimeric experiments show that FDC are radio-resistant[16] and are derived from host precursors instead of donor bone-marrow cells[17]. It was recently discovered that FDC arise from perivascular mural cells that express platelet-derived growth factor receptor β not only in lymphoid organs, but also in non-lymphoid organs such as the kidney and the white adipose tissue of the perigonadal fat pad[18]. FDC maturation requires help from lymphocytes that express tumor necrosis factor and lymphotoxin[19,20].

FDC are non-migratory cells that locate centrally in the follicles and support the GC reaction. Their cell profile varies during the GC development. In the primary follicles, FDC express high levels of complement receptor (CR) 1/CD35[21,22], FDC-M1/milk fat globule-EGF factor 8 protein[23], FDC-M2/C4[24], intercellular adhesion molecule 1, and vascular cell adhesion protein 1 (reviewed in reference[4]). In the secondary GC follicle, FDC up-regulate the expression level of fragment crystallizable (Fc) gamma receptor (γR) IIB[25]. The kinetics suggest that FcγRIIB is not involved in the early stages of antigen trapping in the primary follicles, but is instead believed to play a role in the activation of FDC by engaging with the immune complexes and further regulating the B cell recall response[26,27]. In addition to FcγRIIB,

the expression level of CD23 in FDC is also increased in the secondary follicles in certain tissues, such as the lymph nodes, under certain immunization conditions[28].

FDC actively shape humoral immunity by acquiring immune complexes from non-cognate B cells and displaying intact antigens for both naïve B cells and GC B cells during prolonged periods. Antigen trapping by FDC is mainly mediated by the binding of opsonized antigens to complement receptors 1 and 2 (CR1/2) in both primary and secondary follicles[29–32]. The trapped immune complexes will be rapidly internalized in an actin-dependent manner and retained in non-degradative cycling compartments by FDC[32]. The internalized immune complexes can return to the FDC surface and are displayed for cognate B-cells in a periodically cycling manner[32]. In the absence of complement opsonization, antigen-IgG complexes can also bind to FcγRIIB in the secondary follicles[21,27]. The role of FDC in GC B-cell development will be discussed in detail in section 'Germinal center reaction'.

In addition to trapping and displaying antigens, FDC also (i) recruit $CXCR5^+$ lymphocytes (B and T cells) by releasing CXCL13; (ii) provide survival signals, such as B-cell activating factor (BAFF), for B cells; (iii) remove apoptotic cells; (iv) modulate GC responses through innate toll-like receptor signaling; and (v) contribute to autoimmune diseases by displaying auto-antigens (reviewed in reference[33]).

T cells

In the splenic white pulp, $CD4^+$ T cells are mainly retained in the CCL21- and CCL19-abundant T-cell zone due to their high level of CCR7 expression (reviewed in reference[2]). $CD4^+$ T cells can be activated by antigen presenting cells, such as DC. The major function of the activated $CD4^+$ T cells in the white pulp is to activate naïve B cells and initiate a GC reaction, as discussed below in sections 'Antigen recognition and lymphocytes activation' and 'Germinal center reaction'.

Induction of an immune response

The substances that stimulate the induction of an immune response are called antigens. Antigens can be divided into the following two categories: (i) thymus-independent (TI) antigens, which directly activate B cells without help from T cells due to their intrinsic B-cell activating features or highly repetitive surface structures, such as lipopolysaccharides and *Streptococcus pneumonia*, and (ii) thymus-dependent (TD) antigens, which require help from T cells, such as proteins. Here, we focus on the induction of humoral responses by TD antigens.

Antigen transport to the follicles

The best-described entry route of foreign antigens into the lymph nodes is the delivery of antigens to the T-cell zone by migratory DCs (reviewed in reference[34]). However, how the antigens reach the follicles remained an unsolved question for quite a long time. Recently, the processes of antigen delivery to FDCs in the lymph nodes have begun to be understood (reviewed in reference[35]). Antigen delivery occurs through multiple pathways as follows: (i) small antigens enter the follicles and reach FDCs directly through conduits[36]; (ii) particulate antigens are captured and transported to the lymph nodes by $CD169^+$ macrophages in the subcapsular sinus[37–39]; and (iii) particulate antigens are sampled by resident DC in the medulla via the lectin receptor specific intracellular adhesion molecule-grabbing nonintegrin R1[40]. These particulate antigens, when opsonized with complement factor(s), will be delivered to B cells and, subsequently, FDC[38]. For un-opsonized antigens, the delivery to FDCs is poorly understood but likely occurs via migratory DC.

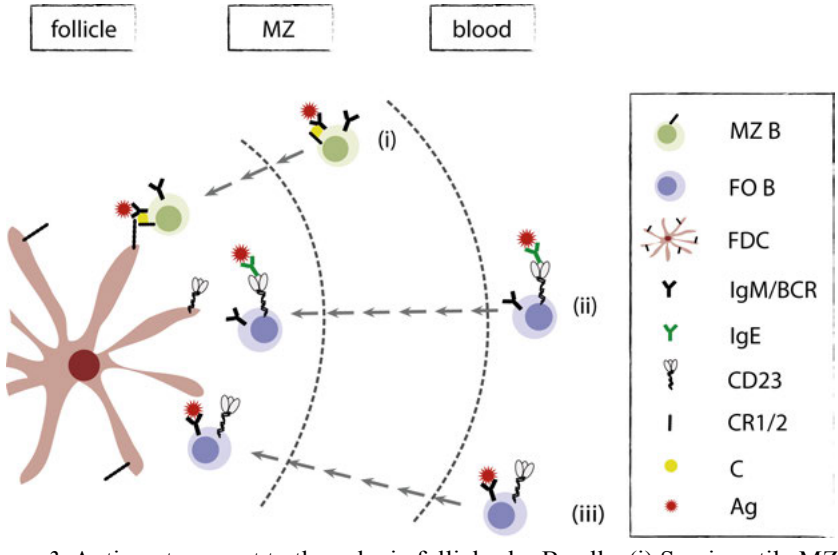

Figure 3. Antigen transport to the splenic follicles by B cells. (i) Semi-motile MZ B cells capture the IgM-antigen complex through CR1/2 and deliver it to the $CR1/2^+$ FDC through their shuttling activity. (ii) Circulating FO B cells capture the IgE-antigen complex through CD23 and transport it into the follicles. (iii) Circulating FO B cells capture the virus-like particles through BCR and transport them into the follicles.

Compared to the well-characterized antigen delivery to the lymph nodes, much less is known regarding the transport of antigens to the splenic follicles. There are a few publications that describe the following three different methods of cell-mediated antigen delivery to the splenic follicles (Fig. 3,

reviewed in reference[41]): (i) particulate antigens that are complexed with IgM, thus presumably activating complement, can be transported and deposited onto FDCs by MZ B cells in a complement and CR1/2-dependent manner[8]; (ii) soluble antigen-IgE complexes can be captured by re-circulating B cells via CD23, which is the low-affinity receptor for IgE, and subsequently transported into the splenic follicles[42]; and (iii) intranasally administered virus-like particles can be captured by re-circulating B cells in the lung through their B-cell receptors (BCR) and then delivered to the splenic follicles[43]. Regarding (ii) and (iii), it has not been formally proven that the antigens that are inside the follicles are delivered to FDC.

Antigen recognition and lymphocytes activation

Upon reaching the spleen, the antigens will be captured by antigen presenting cells (APC), including DC, macrophages and B cells, and will then be processed and presented on the major histocompatibility complex (MHC) class II molecules to activate naïve T cells. In the follicles, naïve B cells will be activated through the antigen-BCR interaction, which leads to antigen internalization and presentation on B cell MHC-II molecules. Activated B cells will up-regulate CCR7 and down-regulate CXCR5, which allows the B cells to migrate toward the T-cell zone. In the T-cell zone, naïve T cells will be activated by APC. In contrast to B cells, activated T cells reduce their CCR7 expression, increase their expression of CXCR5 and CD40 ligand (CD40L), and migrate toward the follicles. The activated T and B cells meet at the border between the follicle and the T-cell zone. The antigen peptides on the B cell MHC-II molecules will be engaged with the T-cell receptor (TCR) on cognate T cells. In addition, the up-regulated CD40L on the T cells will ligate with the constitutively expressed CD40 on the B cells. The engagement of TCR with the antigen peptide on MHC-II, together with the CD40-CD40L ligation, promotes B cell activation and differentiation (reviewed in references[2,44]).

Germinal center reaction

Once the engagement between the cognate B and T cells occurs at the border between the follicles and T-cell zone, a few T cells will be activated and differentiated into CXCR5-expressing T follicular helper (Tfh) cells, which migrate to the follicles, while others remain in the T-cell zone. Additionally, some of the activated B cells will become (mostly IgM^+) GC-independent memory B cells[45] or exit the follicles to become short-lived antibody-secreting plasma cells[46], while some other activated B cells that are committed to become GC B cells will migrate back to the follicles. These B cells will migrate to an anatomical structure called the GC in the center of the follicle, which consists of a dark zone (DZ) and a light zone (LZ), named

after the histological staining features. The DZ is situated in the GC pole near the T zone and is tightly packed with B cells that undergo a rapid clonal expansion and somatic hypermutation (SHM). The LZ, which is on the opposite side of the GC near the MZ area, contains less densely packed cells, including FDC, B cells, and a limited number of Tfh cells, and is the spot in which affinity-based B-cell selection occurs.

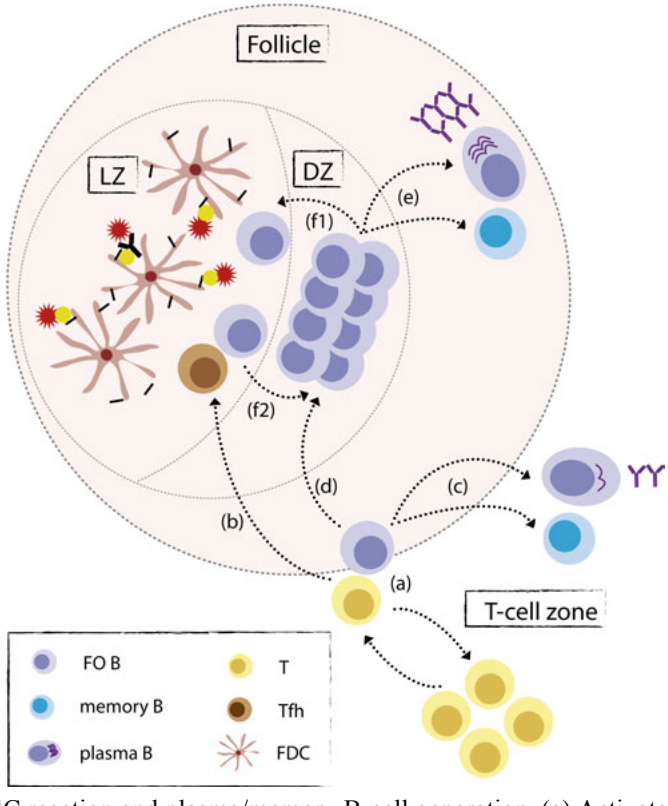

Figure 4. GC reaction and plasma/memory B-cell generation. (a) Activated T and B cells engage at the border between the follicles and T-cell zone. (b) Some of the activated T cells differentiate into Tfh and migrate to the follicle. (c) Some of the activated B cells exit the follicle and become extrafollicular plasma cells or memory B cells. (d) Some of the activated B cells move back to the FO DZ and undergo a clonal expansion and SHM. (e) Some of the B cells exit the GC and become antibody-secreting plasma cells, which will later locate in the bone marrow, while some of them become memory B cells, which will remain in the tissue or enter the circulation. (f1) Some of the B cells enter the LZ and survey for antigens retained on FDC. Those with the highest-affinity BCR receive the limited survival signal from Tfh, while others undergo apoptosis. (f2) The survived B cells re-enter the DZ and undergo further round(s) of clonal expansion and SHM. Some of these B cells will continue the cycling (f1 and f2) procedure to obtain increasingly higher affinities. During each cycle, some of them will exit the loop and differentiate into plasma or memory B cells (d and e).

Previously, it was believed that FDC, serving as antigen libraries, was the limiting factor for BCR-based selection: B cells bearing high affinity antigen-specific BCR were thought to be selected when surveying the FDC network, while B cells with lower affinity were believed to be unsuccessful in the competition for antigens and to undergo apoptosis. Recent studies favor Tfh as the limiting factor for providing crucial survival signals for B cells: B cells with both high- and low-affinity BCRs acquire antigens accordingly and present antigen peptides on their MHC-II molecules, but only those B cells with higher amount of antigen peptides on MHC-II molecules will win the competition for T cell help while the rest will undergo apoptosis. These two hypotheses of B cell selection based on 'competition for antigen binding' and 'competition for T cell help' are not mutually exclusive but rather synergistic, because BCR signaling is not dispensable for GC development and loss of BCR signaling will induce B cell death in GC area.

Once selected by Tfh cells, the high-affinity B cells will re-enter the DZ to undergo further round(s) of clonal expansion and SHM. These B cells will either repeat the cycling between the DZ and the LZ, thus obtaining even higher affinities, or exit the GC to become plasma cells or memory cells (Fig. 4, reviewed in references[47,48]). In addition to providing survival signals, $CD4^+$ Tfh cells also shape the antibody repertoire by secreting cytokines, e.g., interleukin 4[49].

Although it was not discussed here, the GC reaction can also occur in a T-cell independent manner (reviewed in reference[50]).

Generation of plasma and memory B cells

As mentioned above, plasma and memory cells with a low affinity and a short life span can be generated in the pre-GC phase. Here, we focus on the GC-dependent generation of these cell types.

Some of the selected B cells exit the GC and differentiate into antibody-secreting plasma cells. These cells acquire the ability to home to the bone marrow in which they will be maintained by the BAFF family cytokines and survive for long periods. The plasma cells do not circulate. Instead, they stay in the bone marrow and continuously secrete antibodies into the circulation for months or even years until the antigens are cleared. In parallel, some of the selected B cells differentiate into GC-dependent memory B cells. These cells express high levels of the anti-apoptotic protein Bcl-2, which affords them a long life span even without additional surviving signals. The memory B cells can remain in the lymphoid organs in which they are generated or circulate in the blood. These cells are capable of initiating rapid antibody responses when they re-encounter their specific antigens (Fig. 4, reviewed in reference[44]).

Antibodies

Antibodies are large, Y-shaped proteins that are produced by plasma cells. The monomeric antibody has a symmetric core structure consisting of the following four peptide chains: two identical heavy chains and two identical light chains. The antibodies recognize pathogens through the Fab region and function through the Fc region. Murine and human antibodies can be divided into the following five classes or isotypes: IgM, IgD, IgA, IgG, and IgE. IgM is the first isotype that is produced by B cells when encountering antigens. If additional signals are received, B cells will undergo isotype switching through V(D)J recombination by switching the constant μ region to another constant region (α, γ or ε) without changing their variable regions.

IgD is primarily co-expressed with IgM on the surface of B cells and functions as a transmembrane antigen receptor. The serum level of IgD is very low. T cells express receptors for IgD[51]. IgD does not bind to complement factors (reviewed in reference[52]).

IgA is usually found in a secreted dimeric form and plays a pivotal role in the local (mucosal) immunity. There are several receptors for IgA, such as the Fc alpha receptor (FcαR) I (CD89), the polymeric Ig receptor (transporting IgM and IgA through the epithelial barriers), the Fc alpha/mu receptor (Fcα/μR), and at least two other alternative receptors (reviewed in reference[53]). IgA is a poor complement activator (reviewed in reference[54]).

IgG

IgG exists as a monomer and has a molecular weight of approximately 150 kDa. IgG is the most abundant isotype in the circulating blood and extracellular fluid. There are four types of murine IgG subclasses as follows: IgG1, IgG2a, IgG2b, and IgG3.

All IgG subclasses bind to the neonatal Fc receptor, which is involved in the recycling of IgG and transporting of the IgG-antigen complex. All subclasses, except for IgG3, bind to the inhibitory FcγRIIB. In addition to its well-known inhibitory effect, FcγRIIB, such as that on DC, is also involved in the endocytosis of the IgG-antigen complex, which preserve the intact antigen for subsequent interaction with BCR on B cells[55]. Furthermore, IgG1, IgG2a, and IgG2b also bind to several activating FcγRs that promote antigen degradation, processing and presentation as follows: (i) IgG1 preferentially binds to FcγRIII; (ii) IgG2a has a high affinity for FcγRI and FcγRIV and a medium level affinity for FcγRIII; and (iii) IgG2b favors FcγRIV but also binds to FcγRI and FcγRIII (reviewed in reference[56]).

IgG1 is generally a poor complement activator[57]. IgG2a, IgG2b and IgG3 are all efficient at activating the complement system[57–60].

IgG3

Murine IgG3, which was first described in 1971[61], is a cryoglobulin that precipitates in cold temperatures[62]. It has the unique feature of self-aggregation due to Fc-Fc cooperative binding (Fig. 5 (i))[63–66]. Similarly to IgM, IgG3 appears early in an immune response and sometimes does not require help from T cells. It is the predominant IgG subclass in the responses against TI type II antigens, such as *Streptococcus pneumonia*[63,67], and it only plays a minor role in the responses to TD antigens[68,69].

The Fc receptor for IgG3 remained elusive for many years. Earlier studies observed that monoclonal IgG3-coated sheep red blood cells (SRBC) could be phagocytosed by macrophages through a Fc receptor[70]. Later, FcγRI, a receptor that also binds IgG2a and IgG2b, was shown to bind IgG3[71].

IgG3 is efficient at activating the complement system, which is likely due to its aggregation capacity. It has been long believed that IgG3 activates the complement system only via the alternative pathway. However, it is currently known that a high affinity monoclonal anti-erythrocyte IgG3 antibody can activate the complement system through the classical pathway[60].

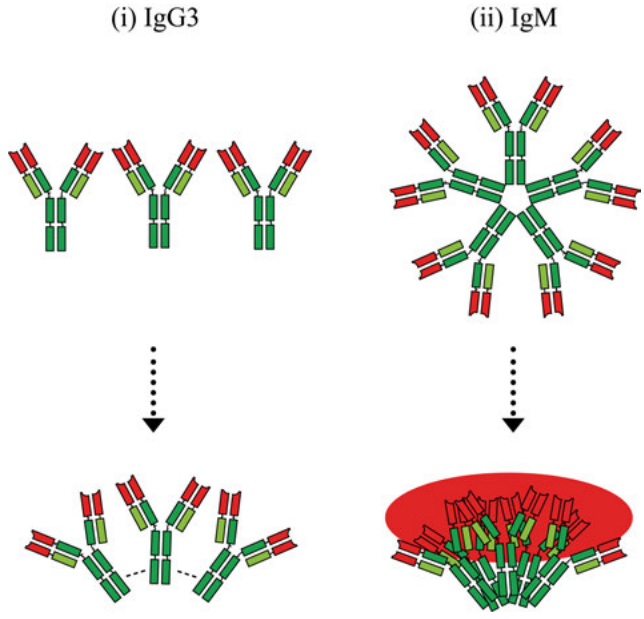

Figure 5. The IgG3 and IgM molecules. (i) single monomeric IgG3 molecule attracts other IgG3 molecules through Fc-Fc interaction. (ii) pentameric IgM changes its conformation from a planar form to a staple form when binding to a multivalent large antigen.

IgE

IgE is a monomer either expressed on the surface of B cells or secreted in the circulation. Secreted IgE binds tightly to the Fc receptors on the surface of mast cells and basophils, even without binding to its antigen first. There are two types of receptors for IgE as follows: the high-affinity receptor Fc epsilon receptor (Fcε) RI, which is expressed on mast cells and basophils, stimulates cellular degranulation and causes allergic symptoms, and the low-affinity receptor FcεRII (also known as CD23) facilitates antigen trapping and presentation[72]. CD23 is expressed mainly on B cells[73] and FDC[28] in mice, but in humans, it is also expressed on monocytes/macrophages[74], eosinophils[75], and epithelial cells[76]. IgE does not activate the complement system.

IgM

IgM exists either as a membrane-bound monomer or as a secreted polymer. The secreted IgM is mostly found in a pentameric form with a molecular weight of 970 kDa. Therefore, it usually possesses a high avidity even when the Fab part has a low affinity.

Although the polymeric Ig receptor and Fcα/μR serve as receptors for IgM (and IgA), the bona fide receptor for IgM remained unknown until the Fc mu receptor (FcμR), also known as Toso/Faim3, was recently discovered[77]. Regarding the surface expression of FcμR, some groups claim that this receptor is restrictedly expressed on murine B cells and human B, T, and NK cells[77–79], some other groups claim that it is more widely expressed on various leukocyte populations[80–82]. FcμR in mice is critical for IgM homeostasis and B-cell survival. It is also involved in the promotion of the humoral immune response and the suppression of autoantibody production[83].

Pentameric IgM is a very potent complement activator. A single pentameric IgM molecule, when forms an immune complex with antigens, is able to recruit and activate C1q in the C1-complex. This initiates the classical complement activation pathway as discussed in greater detail in the next section. Antigens must be large enough for the pentameric IgM molecule to bind to them with several of its arms. This binding changes the conformation of IgM from a planar form to a staple form (Fig. 5 (ii)), which is believed to be required for C1q binding[84]. Monomeric IgM cannot activate the complement system.

Complement-opsonized IgM-antigen complexes can be recognized by CR1/2 as discussed in section ‘Complement receptors’.

Complement system

Complement activation pathways

The complement system can be activated through the classical, lectin, and alternative pathways (reviewed in references[85,86]). The sequential events in the classical pathway activation are as follows: (i) when in complex with antigens, one single pentameric IgM or several (ideally six) IgG molecules recruit and activate the C1q molecule in the C1-complex ($C1qr^2s^2$). This activation is initiated by a conformational change in the C1q molecule through binding to the Fc portion of IgM or IgG; (ii) The conformational change of C1q activates $C1r^2s^2$, which, in turn, splits C4 and C2 into C4a/4b and C2a/2b; and (iii) C4b and C2a together form the classical pathway C3 convertase C4bC2a. Analogously, the lectin pathway is triggered through a C1q-like component (mannose-binding lectin or ficolin) binding to the surface of the pathogens and, in turn, splitting C4 and C2 to form the C3 convertase C4bC2a. In contrast, the alternative pathway is initiated in the absence of specific antibodies through a direct binding of C3 to the microbial surface. This subsequently leads to the formation of a distinct C3 convertase C3bBbP. All pathways lead to the generation of C3-convertases, which cleave the central component C3 into C3a and C3b. C3a is released and acts as a chemoattractant. C3b, together with the other components, forms C5 convertase and initiates the formation of a membrane attack complex. The C3 split product C3b can further be broken down into iC3b, C3dg, and C3d, which constitute the ligands for CR1/2. CR1/2 are important immune-regulatory CRs as discussed in the following section.

Complement receptors

The complement facilitates the antigen uptake and clearance through binding to CR-expressing phagocytes. The best-characterized CRs in the murine system are the following receptors for the C3 and C4 fragments: CR1, 2, 3 and 4.

Murine CR1/2 are different splice forms encoded by a single gene, *Cr2*. Their expression are limited to B cells, FDC and a few activated T cells[87,88]. A recent study has shown that murine FDC almost exclusively express CR1, while B cells predominantly express CR2[89]. Murine CR1/2 are receptors for C3b and its splits factors (iC3b, C3dg, and C3d) and C4b (Fig. 6). The larger product, CR1, preferentially binds to C3b but also binds to C4b, iC3b, C3dg and C3d with a lower affinity. Murine CR1 mainly functions as a facilitator of phagocytosis of immune complexes and is also involved in the cleavage of C3b and C4b (reviewed in references[85,86]). The smaller product, CR2, preferentially binds to C3dg and C3d but also binds to iC3b, although less efficiently[90–92]. CR2 facilitates antigen trapping and functions as a co-

receptor for B cell activation (reviewed in references[85,86]). Human CR1/2 are encoded by two distinct genes. Human CR1 is expressed on FDCs, erythrocytes, mononuclear phagocytes, neutrophils, B cells, T cells, and eosinophils, and human CR2 is mainly expressed on B cells and FDC (reviewed in references[85,86]).

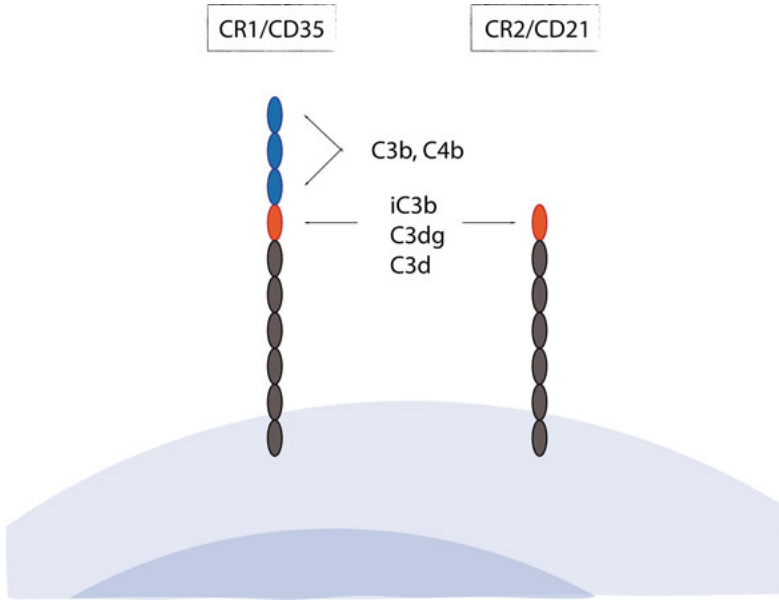

Figure 6. Murine CR1 (CD35) and CR2 (CD21). Murine CR1/2 are different splice forms encoded by the same gene, *Cr2*. CR1 is larger than CR2. CR2 mainly binds to iC3b, C3dg, and C3d. CR1 also recognizes these ligands, but it exhibits a higher affinity for C3b and C4b.

CR3 (CD11bCD18) and CR4 (CD11cCD18) are expressed on the surface of mononuclear phagocytes, neutrophils, and NK cells and facilitate the recognition of iC3b-tagged pathogens by phagocytes (reviewed in reference[93]).

There are also receptors for C3a and C5a. The C3a- and C5a-receptors are mainly expressed on endothelial cells, mast cells and phagocytes. These receptors function in the stimulation of inflammation, regulation of the complement activation and activation of G-proteins[85,86].

Complement and antibody response

The connection between the complement system and the antibody response was established when it was observed that mice depleted of C3 due to a treatment with cobra venom factor (CVF) exhibited severely impaired antibody responses[94]. Subsequent studies have confirmed that animals (including humans, mice, guinea pigs and dogs) with a deficiency of C1q, C2, C4 and C3 all develop poor antibody responses (reviewed in reference[95]). Almost no

alteration in the antibody response have been observed in mice lacking factor B of the alternative pathway or mannose-binding lectin of the lectin pathway[96–98]. These findings suggest that the classical pathway activation is crucial for the maintenance of normal antibody production, while the alternative and lectin pathways are dispensable in this respect.

Antibody-mediated feedback regulation

Small amounts of antibodies can regulate the humoral immune response against their specific antigens. Emil von Behring, who received the first Nobel Prize in physiology or medicine in 1901, first noticed this phenomenon, which is currently known as antibody feedback regulation. Antibodies, regardless of passively administered or endogenously produced, when in complex with their specific antigens, can induce an almost total suppression or a 10-1000-fold higher antibody responses than antigens administered alone[95,99]. The feedback regulation that is initiated by an epitope-specific antibody often affects the responses to all epitopes on the antigen, which is called non-epitope specific regulation (reviewed in reference[95]). However, the IgG-mediated feedback suppression can also be epitope specific[100].

All antibody isotypes, except for IgD, are able to feedback regulate antibody responses. However, regarding IgA-mediated feedback regulation, there is only one study showed that IgA enhances immunological memory but not the primary responses against soluble antigens. This thesis focuses on the ability of IgG3, IgE, and IgM to enhance immune responses.

Antibody-mediated feedback suppression

The most well-known antibody-mediated suppression is the clinical use of IgG anti-erythrocytes, to prevent hemolysis of Rhesus D antigen (RhD)-positive erythrocytes in fetuses and newborns, who are delivered by RhD-negative mothers[101]. This application is based on the findings that the response to erythrocytes is suppressed by erythrocyte-specific IgGs of all subclasses[57,102–104]. In contrast to the almost entirely suppressed humoral response, the antigen-specific T cell proliferation is only marginally reduced[104,105]. The mechanism underlying the IgG-mediated suppression is still under investigation. Neither FcγRs nor the complement are required for the suppressive effect[104,106–108]. This finding suggests that the FcγR- and complement-mediated antigen clearance is not involved in the suppression. The most likely explanation thus far is epitope masking, i.e., specific IgG antibodies block the epitopes and prevent cognate B-cells from responding to the antigen[100].

In addition to an intact IgG, IgG $F(ab')_2$ fragments and IgE are also able to suppress the primary response against SRBC[104,109]. Furthermore, specific

IgM can also induce a significant suppression of the primary response against SRBC, but only when IgM is administered one or two days after SRBC administration[110].

IgG-mediated enhancement of antibody responses

IgG possesses dual functions in feedback regulation. In addition to its suppressive effect, murine IgG1, IgG2a, and IgG2b can also enhance the primary immune responses against protein antigens, such as ovalbumin (OVA), bovine serum albumin (BSA), and the large protein keyhole limpet hemocyanin (KLH)[57,111–115]. This enhancement is impaired in Fc receptor gamma chain (FcRγ)-deficient mice (lacking the activating receptors FcγRI, FcγRIII, and FcγRIV) and is augmented in FcγRIIB knock-out (KO) mice (lacking the inhibitory FcγRIIB)[114]. Murine IgG1, which activates the complement system very poorly[57], can still lead to an enhancement, thus showing that its enhancing effect is not dependent on the complement activation[116]. Both IgG2a and IgG2b have the capacity to activate the complement[57]. However, it is unlikely that the complement activation is required for the IgG2a- and IgG2b-mediated enhancement due to the facts that (i) mutant IgG2a that cannot activate the complement leads to an enhancement that is equal to what observed when using wild-type (WT) IgG2a[57]; (ii) IgG2a can enhance the antibody responses in Cr2 KO mice[117]; and (iii) IgG2b can induce the enhancement in mice depleted of C3 due to a CVF treatment[116].

Polyclonal IgG and monoclonal IgG2a can enhance $CD4^+$ T helper cell proliferation through activating the IgG Fc receptors in the presence of $CD11c^+$ cells[115,118–120].

IgG3-mediated enhancement of antibody responses

IgG3 is the final IgG subclass to be found to induce an immune enhancement against small soluble antigens, such as OVA and BSA[121,122]. Unlike the other IgG subclasses, IgG3 does not function through the Fc receptors (FcRs), but rather, functions through activating the complement system, which is very similar to IgM. As previously mentioned, the complement system can be activated by a single pentameric IgM molecule. However, for IgG to activate the complement, two or more IgG molecules must act together. The distinctive self-aggregation feature of IgG3 makes it very prominent regarding complement activation. When binding to the surface of antigens, e.g., group A streptococci, one monomeric IgG3 molecule can attract more IgG3 molecules via Fc-Fc interactions[63,123]. *In vivo* studies have demonstrated that the IgG3-induced immune enhancement is impaired in mice depleted of C3 or lacking CR1/2[121]. However, FcγRs, including the IgG3-binding FcγRI, do not appear to be involved in the IgG3-mediated enhancement. Normal antibody responses were observed in FcγRI-deficient mice and FcRγ-deficient

mice[121,122]. The mechanism underlying the IgG3-mediated enhancement is not currently understood.

Unlike the other IgG subclasses, IgG3 is a poor enhancer of $CD4^+$ T cell proliferation both *in vitro* and *in vivo*[122].

IgE-mediated enhancement of antibody responses

The role of IgE in antibody feedback regulation was first established by the observation that IgE enhances antibody responses against BSA via the low affinity receptor for IgE, FcεRII (CD23)[124]. Further studies have shown that IgE can enhance the response against small soluble antigens, such as BSA, OVA, and tetanus toxoid, but not against large antigens, such as SRBC and KLH[42,125,126]. IgE enhances the responses of all antibody isotypes with no obvious skewing of the Th1- or Th2-prone responses[125,127]. IgE also augments the proliferation of $CD4^+$ T cells[42,128–130]. IgE binds to several FcRs as follows: FcεRI, FcεRII/CD23, FcγRIIB, FcγRIII, and FcγRIV; however, interestingly, only CD23 is required for the IgE-mediated enhancement (reviewed in references[95,131]). Furthermore, the IgE-mediated enhancement is unperturbed in complement-deficient mice[117,121].

As mentioned above, the mechanism underlying the IgE-mediated enhancement is most likely that $CD23^+$ FO B cells capture the IgE-antigen complexes and transport them to the splenic follicles in which $CD8\alpha^-$ cDCs take over the antigen and activate $CD4^+$ T cells, subsequently leading to B cell activation[42,130,132].

IgM-mediated enhancement of antibody responses

Specific IgM that is passively administered a few hours before the antigen administration can enhance both the primary and memory antibody responses against the antigen[110,117,133–137]. The enhancing effect of IgM is prominent when suboptimal doses of large particulate antigens, such as SRBC and malaria parasites, or the large soluble protein KLH are used[111,133–135,137,138]. However, IgM does not enhance the responses against small soluble antigens, such as OVA and BSA. The reason why IgM selectively enhances responses against large antigens is likely due to the requirement of complement activation. As mentioned above, the conformational change in the pentameric IgM enables it to recruit and activate C1q. Several observations lead to the assumption that the IgM-mediated enhancement is complement-dependent as follows: (i) monomeric IgM cannot activate the complement and fails to enhance antibody responses[137]; (ii) mutant IgM with a point mutation in the heavy constant chain (Cμ13 IgM) results in an inability to activate the complement and a loss of its enhancing effect[139–141]; and (iii) the IgM-mediated enhancement is impaired in mice depleted of C3 or lacking C3, C1q, or CR1/2[117,139,142,143]. The Fc receptor for IgM (FcμR) does not

appear to be involved in the enhancing effect as follows: Cμ13 IgM, which cannot activate the complement and has lost its ability to enhance antibody responses, nevertheless binds equally well to FcμR as WT IgM[141].

Regarding the mechanism underlying the IgM-mediated enhancement, some *in vitro* studies have suggested that the co-crosslinking of BCR and CR2/CD19/CD81 on the B cell surface by the complement-opsonized antigens lowers the threshold for B cell activation *in vitro*, which may explain the enhancing effect by IgM *in vivo*[144–146]. However, it was shown *in vivo* that WT B cells and B cells lacking CR1/2 expression produce equal amounts of antibodies against SRBC in the presence of $CR1/2^+$ FDC[142], rendering the crosslinking theory unlikely. Based on the *in vivo* studies, others propose that the enhancing effect might correlate to the increased antigen concentration in the follicles, due to the transport of the IgM-antigen-complement complexes by $CR1/2^+$ MZ B cells, and the increased antigen deposition on FDC[8,147].

In naive mice, natural IgM is present. It has been observed that mice lacking natural/secretory IgM have impaired antibody responses and that these responses can be rescued by transferring normal mouse serum[148,149]. However, it appears that the ability of natural IgM to activate the complement system is not required for maintaining a normal level of humoral immunity because mice lacking the complement-activating IgM exhibit normal antibody responses[140].

IgM does not enhance T cell proliferation[141]. However, T cell help is required for the IgM-mediated enhancement of antibody responses against TD antigens because no enhancing effect of IgM is observed in T cell-deficient mice[150].

Present Investigation

Aims and questions

The general aim of this thesis is to elucidate the mechanism underlying the IgG3- and IgM- mediated immune enhancement and the involvement of the complement system.

The following specific questions have been addressed:

I Which cells capture and transport the IgG3-antigen complexes into the splenic follicles? How are CR1/2 involved in this process and in GC development and antibody production?

II Where are the IgG3-antigen complexes located in the splenic follicles? Do they locate on FDC? How are C3 and C1q involved in this process? Is IgG3 able to induce immunological memory against small and large soluble antigens (OVA and KLH)?

III Can endogenous IgM enhance the antibody responses? If yes, is complement activation involved? Do Cμ13 mice have larger numbers of MZ B cells? Do these cells express more CR1/2 than MZ B cells from WT mice? Do these cells bind the IgG3-antigen complexes better? Do IgG3-immune complexes deposit on FDC more efficiently in Cμ13 mice?

Experimental setup

Mouse strains, antigens and antibodies

All animal experiments were performed according to permits C146/10 and C25/13 and were approved by the Uppsala Animal Research Ethics Committee. The mice used in this thesis are listed below (see Table 1). Age- and gender-matched mice were used in all experiments, except for in Figure 1 in Paper III. The different antigens and antibodies that were used in this thesis are listed below (see Table 2).

Table 1. *Different mouse strains used in this thesis*

Name (Background)	Annotation	Appeared in paper
BALB/c	WT (from Bommice)	I, II, III
Cr2 KO (BALB/c)	Lack CR1/2[151]	I, III
CD23 KO (BALB/c)	Lack CD23[126]	
C57BL/6	WT (from Taconic Bioscience, Inc)	II, III
C1qA KO (C57BL/6)	Lack C1qA and, thus, the entire C1q molecule[152]	II, III
C3 KO (C57BL/6)	Lack C3 (from Jackson Laboratories)	II, III
Cμ13 KI (BALB/c)	Lack the complement-activation capacity by IgM due to an amino acid substitution in the constant part of the μ-heavy chain[140]	III

Table 2. *Different antigens and antibodies used in this thesis*

Antigen	Antibody	Appeared in Paper
OVA-2,4,6-trinitrophenyl (TNP)	IgG3 anti-TNP (clone: IMF10 and IMH11)	I, II, III
Biotin-OVA-TNP	IgG3 anti-TNP (clone: IMF10 and IMH11)	I, II, III
KLH-TNP	IgG3 anti-TNP (clone: IMF10)	II
SRBC	IgM anti-SRBC	III

Immunization and blood sampling

Mice were immunized intravenously (*i.v.*) through the tail vein with the indicated amounts of antigens, antibodies or immune complexes in a total amount of 0.2 ml. Blood samples were collected through tail bleeding. Sera were prepared and stored at -20°C.

Treatment for the dislocation of MZ B cells

To dislocate the MZ B cells, the indicated amount of FTY720 was administered *i.p.* to WT mice 4 h before the immunization.

Flow cytometry

The blood and spleen samples were prepared as previously described[141]. Briefly, the cell suspensions were depleted of erythrocytes and treated with an Fc block before the staining with the indicated mixtures of antibodies. The stained cells were immediately analyzed or pre-fixed with 4% paraformaldehyde before analyzed by flow cytometry.

Confocal microscopy

The spleens were prepared as previously described[141]. Briefly, the spleens were snap frozen in liquid nitrogen and cut into 8 μm sections. The slides were pre-fixed, rehydrated, blocked, and then stained as indicated in each figure. All slides were mounted in Fluoromount G and analyzed by confocal microscopy.

Bone marrow transplantation

Bone marrow chimeric mice were generated as previously described[142]. Briefly, female naïve WT and Cr2 KO mice were first whole-body irradiated with a sub-lethal dose of 7.5 Gy and then received bone marrow cells from non-irradiated naïve WT or Cr2 KO mice 24 hours after the irradiation. This produced four groups of mice that express CR1/2 on both the MZ B cells and FDC, on either cell type, or on none of the cell types.

Splenocytes transplantation

Splenocyte chimeric mice were generated as previously described[142]. Briefly, female naïve BALB/c mice were whole-body irradiated with a sub-lethal dose of 7.5 Gy and rested for 24 hours. Fifteen million splenocytes from primed mice were freshly prepared and *i.v.* administered to the irradiated mice.

Enzyme-linked immunosorbent assay

High-binding 96-well plates were coated with OVA, KLH, or SRBC, blocked with dry milk, and sampled with properly diluted sera. After washing, the plates were incubated with alkaline phosphatase-conjugated sheep anti-mouse IgG. After washing, the substrate solution was added to the plates before analysis.

Statistical analysis

The statistical differences between the indicated groups were determined by unpaired parametric student's *t*-tests (two tailed) or unpaired non-parametric Mann-Whitney tests (two tailed) as follows: ns (not significant), $p>0.05$; *, $p<0.05$; **, $p<0.01$; ***, $p< 0.001$. The colocalization of antigens on FDC is shown as Manders' coefficients + SEM, which indicates the proportion of the FDC area that is also positively stained for the antigen.

Results and discussion

Paper I

Marginal zone B cells transport IgG3-immune complexes to splenic follicles

Passively administered IgG3 enhances the immune responses against the small soluble antigens, and this enhancement is dependent on the complement but not on the FcγRs[121,122]. In the present study, we sought to investigate whether the IgG3-antigen immune complexes are captured and transported to the splenic follicles and whether they induce GC development. The involvement of CR1/2 in these processes has also been investigated.

To initiate an efficient immune response, antigens must obtain access to the splenic follicles. Unlike the IgE-OVA complexes, which have been shown to be captured by circulating FO B cells[42], a massive amount of IgG3-OVA complexes was found on splenic MZ B cells, and a very small amount was found on FO B cells in WT mice. No binding of the IgG3-OVA complexes to any B cell types was found in the Cr2 KO mice. These observations indicate that the MZ B cells capture the IgG3-OVA complexes in the presence of CR1/2. Furthermore, the confocal analysis showed clusters of antigens inside the follicles of WT mice that were immunized with the IgG3-antigen complexes. However, antigens were trapped in the MZ area and did not enter the follicles in the WT mice that were immunized with antigen alone. Very few antigens were detected in the follicles of the Cr2 KO mice regardless of whether they were immunized with the antigen alone or IgG3-antigen complexes. Therefore, the following task was performed to determine which cell type(s) transported the IgG3-OVA complexes to the splenic follicles.

The MZ B cells were thought to be a residential cell type in the MZ area. However, a recent study has shown that a treatment with FTY720 (the antagonist of S1P) induced the re-localization of the MZ B cells to the follicles[9]. Subsequent studies have shown that the MZ B cells shuttle between the MZ area and the follicles[6,153]. Inspired by these findings and findings that the MZ B cells transport the IgM-antigen complexes to the splenic follicles and deposit them on FDC[8], we speculated that the MZ B cells might also play a role in transporting the IgG3-OVA complexes into the follicles. To investigate this hypothesis, the antigen capture and localization were investigated in FTY720-treated WT mice in which the MZ B cells were temporarily relocated to the follicles. As expected, impaired binding of the IgG3-OVA complexes to the MZ B cells and impaired antigen localization in the splenic follicles were observed in FTY720-treated mice. These results suggest that MZ B cells act as transporter cells for the IgG3-antigen complexes.

FDC play an important role in the GC formation by rapidly internalizing the acquired immune complexes, retaining them intact in an intracellular recycling compartment, and displaying them periodically on the cell surface for antigen recognition[32]. In the WT mice that were immunized with the IgG3-OVA complexes, antigens were detected in the periphery of the FDC network by two hours after the immunization. Eight hours after the immunization, antigens had moved further into the follicles, resulting in a dimmer staining of the antigens in the central FDC network. However, very few antigens co-localized with FDC in the WT mice that were immunized with antigens alone. Furthermore, in the WT mice, the IgG3-OVA complexes increased the GC formation and stimulated antibody production compared to mice treated with the antigen alone. Both the GC reaction and antibody responses were markedly reduced in the IgG3-OVA-immunized Cr2 KO mice compared to those in the similarly immunized WT mice.

CR1/2 are primarily expressed on two types of murine cells – MZ B cells and FDC[154,155]. According to a recent study, MZ B cells mainly express CR2, while FDC primarily express CR1[156]. CR1/2 are required for the IgG3-induced enhancement of antibody responses[121]. However, the mechanism by which CR1/2 contribute to such reactions remains unrevealed. To investigate which CR1/2-expressing cell type(s) is crucial for the IgG3-mediated immune enhancement, four groups of bone marrow chimeric mice were generated based on the knowledge that FDC are radio-resistant cell types (listed in Table 3). The antibody responses showed that mice expressing CR1/2 on both cell types produced an optimal antibody response after the immunization with the IgG3-antigen complexes. Mice that did not express CR1/2 on either cell type exhibited a severely impaired enhancement with only residual responses remaining. In mice lacking CR1/2 expression on either MZ B cells or FDC, intermediate antibody responses were observed.

Table 3. *CR1/2 expression in different bone-marrow chimeric groups*

Donor → recipient	**B cells**	**FDC**
WT → WT	+	+
Cr2 KO → WT	-	+
WT → Cr2 KO	+	-
Cr2 KO → Cr2 KO	-	-

In summary, our data suggest that passively administered IgG3-antigen complexes are flushed into the splenic MZ area in which they are captured by CR1/2$^+$ MZ B cells. Due to the constant shuttling of MZ B cells between the MZ area and the follicles, antigens are transported to the splenic follicles. In the follicles, CR1/2$^+$ FDC capture the IgG3-antigen complexes and present the antigens to GC B cells, resulting in an enhanced GC development and antibody responses. CR1/2 appear to be involved in the antigen transport by CR1/2$^+$ MZ B cells and the antigen presentation by FDC. CR1/2 expression on both MZ B cells and FDC is required for optimal antibody responses to the IgG3-antigen complexes. This finding is remarkably similar to findings demonstrating the IgM-mediated enhancement of the antibody responses. IgM, when forming complexes with its specific antigen, activates the complement, increases the antigen capturing by the MZ B cells (via CR1/2) and increases the antigen deposition on FDC[8]. Although the absence of CR1/2 on B cells impairs the complement-opsonized antigen trapping, the B cell activation does not appear to be affected *in vivo*, because CR1/2$^-$ B cells produce equal amounts of antibodies compared to WT B cells [142]. However, the lack of CR1/2 on FDC will impair the antigen retention, which might, in turn, affect the GC B cell maturation and differentiation into antibody-secreting plasma or memory B cells[22]. Furthermore, neither IgG3 nor IgM enhance CD4$^+$ T cell proliferation[122,141]. IgG2a and IgE, in contrast, also enhance the antibody responses against the same TD antigens as IgG3. In

contrast, these isotypes increase the antigen presentation by DC and enhance the $CD4^+$ T cell proliferation via FcγRs[42,115,128].

Paper II

IgG3-antigen complexes are deposited on follicular dendritic cells in the presence of C1q and C3

We have demonstrated that CR1/2 are critically important for the IgG3-mediated antigen transport by MZ B cells in paper I. CR1/2 are receptors for the C3 split products (generated with the help of C1q, C2 and C4) and C4b. C3 is the central component of all three complement-activation pathways. The C1-complex activation (initiated by the C1q recruitment) by the immune complex is the first step of the classical pathway activation. Moreover, the antigen deposition on FDC is essential for antibodies undergoing affinity maturation[157]. Here, we sought to investigate the role of the classical and total complement activation in the IgG3-mediated antigen localization to FDC in the splenic follicles. We also investigated whether IgG3 could enhance the antibody response to the large protein KLH and induce immunological memory.

As previously reported, $CR1/2^+$ MZ B cells are the major cell type that captures biotin-OVA-TNP that is administered together with the TNP-specific IgG3[158]. Strikingly, 50% - 90% of the antigen in the splenic follicle was exclusively co-localized with the peripheral dendrites of FDC and covered one-third of the FDC network in WT mice 2 h after the immunization. As reported in Paper I, the antigens will reach deeper into the central FDC network over time as demonstrated by the staining 8 h after the immunization[158]. This antigen distribution pattern is distinct from that observed in mice that were immunized with the IgE-OVA/OVA-TNP complexes. The IgE-antigen complexes were transported from the circulation to the spleen by $CD23^+$ FO B cells and diffusely distributed over the entire follicle[42]. $CD8\alpha^-$ conventional DC were recently found to take up the IgE-OVA complexes and present them to $CD4^+$ T cells in the spleen[132]. Unlike the immune responses to the IgG3-antigen complexes, which required the expression of CR1/2 on both FDC and B cells, CD23 expression on B cells, but not on FDC, is required and sufficient for the IgE-induced enhancement of the antibody responses[159].

No increased antigen binding to MZ B cells or antigen deposition on FDC were observed in the C1q- or C3-deficient mice, demonstrating that the activation of the classical pathway by the IgG3-antigen complexes is sufficient for generating ligands for CR1/2 on MZ B cells and FDC. Previous findings show that the IgG3-mediated enhancement of the antibody responses is severely impaired in Cr2 KO mice, although a minor enhancement still remains[121,122,158]. There is a possibility that other receptors for the C3 sub-

fragments other than CR1/2 may be involved. Therefore, IgG3-mediated enhancement of the antibody responses was also tested in the C3 KO and C1qA KO mice. Similarly to the observations in the Cr2 KO mice[121,158], the enhancement induced by IgG3 was significantly reduced but not completely abrogated in the C3 and C1qA KO mice. There is no definitive explanation for this puzzle, but we speculate that it might occur simply because the IgG3-OVA complexes form large aggregates due to the self-aggregation tendency of IgG3, which are more immunogenic than non-aggregated OVA.

IgG3 is known to enhance the primary antibody response against small soluble antigens, such as OVA and BSA[121,122,158]. Here, we demonstrated that IgG3 could also induce an enhancement of the secondary antibody responses against OVA. The enhancement of the secondary responses could be due to either an endogenous feedback regulation by the antibodies that were produced during the priming[103,160] or to increased numbers of memory cells that were generated during the priming. To verify the existence of memory cells in the primed mice, we used the splenocyte-transplantation method and demonstrated that IgG3, together with OVA, was able to induce OVA-specific memory cells. In addition, we showed for the first time that IgG3 could enhance both the primary and secondary antibody responses against the large antigen KLH. KLH is a soluble protein with a molecular weight over 7,000 kDa, and it has been previously reported that (pentameric) IgM, IgG1, IgG2a and IgG2b can enhance responses against KLH[112,137].

In summary, the IgG3-antigen complexes captured by MZ B cells are deposited almost exclusively (50% - 90%) on FDC in splenic follicles in the presence of C1q and C3. Both the antigen localization on FDC and the optimal antibody production requires the presence of C1q and C3. This observation is consistent with the previous finding that IgG3 is a potent classical pathway activator[60], which is likely due to its unusual capacity to self-aggregate through Fc-Fc interactions[63,65,123]. This finding is also very similar to that observed in the IgM-mediated enhancement[143]. IgG3 and IgM appear to complement each other in handling different types of antigens. IgG3 enhances antibody responses against small antigens[121,122] and the large protein antigen KLH as shown here, while IgM specializes in enhancing the responses against large antigens[112,133,138]. Furthermore, IgG3 is an efficient primer for the induction of memory responses.

Paper III

Mice producing IgM unable to activate complement have impaired endogenous feedback regulation but increased antigen trapping in follicles.

IgG3, unlike the other IgG subclasses, uses the complement system to induce an enhancement in the antibody responses. Similarly, the IgM–mediated

enhancement is also dependent on the complement activation. Previous reports showed that such an enhancement was abrogated in Cr2 KO mice and mice with a low level of C3 due to a CVF treatment[117,139,142]. Pentameric IgM is a potent complement activator and is commonly believed to be the most efficient classical pathway activator due to its ability to recruit C1q. Monomeric IgM and mutant IgM that cannot activate the complement lost their ability to enhance the antibody responses[137,139,141]. However, the antibody response is unexpectedly normal in the knock-in mouse strain Cμ13, in which a point mutation in the gene encoding the IgM constant chain results in the production of IgM that cannot bind C1q and activate the complement[140]. Taking advantage of a 'physiological' immunization regime (using repeated injections of extremely low amounts of SRBC), we sought to elucidate whether endogenous IgM has the ability to induce an enhancement and whether the complement system is involved. A more thorough characterization of the Cμ13 mouse strain was also achieved.

Specific IgM was unable to enhance the antibody production in mice that were treated with CVF[139] or lack C1q and C3[143], suggesting that the classical pathway activation is sufficient for the IgM-mediated enhancement. These data made the unperturbed antibody production observed in the Cμ13 mice[140] even more puzzling. Inspired by the finding that a less marked IgM-mediated enhancement is induced against 4×10^6 SRBC (high dose) than that induced against 4×10^5 SRBC (low doses)[133], we hypothesized that amounts of SRBC that were too high were used in the previous study[140] to allow the endogenous IgM-mediated enhancement to be detected. Therefore, the immunization regime was changed to better mimic physiological conditions. WT mice were primed with a very low dose of SRBC (5×10^4), followed by an early boost with 5×10^5 SRBC three days after the priming. These mice developed more GC B cells and peanut-agglutinin positive (i.e., GC^+) follicles and exhibited higher antibody responses than mice receiving the same amount of SRBC (5.5×10^5) as a single immunization, suggesting that endogenously produced IgM also has the ability to enhance antibody responses. The same physiological immunization regime was then applied to the Cμ13 mice. Interestingly, the enhancement of the GC development and antibody production that was observed in the WT mice was impaired in the Cμ13 mice, suggesting that the endogenous feedback regulation by IgM also requires the activation of the complement.

An interesting phenomenon is that there were almost twice as many MZ B cells and slightly fewer total B cells in the Cμ13 mice compared to those in the WT mice, although no differences in other B-cell subpopulations were found[140]. Our data confirmed these observations and further showed that the MZ B cells in the Cμ13 mice exhibited higher levels of CR1/2 expression compared to those in the WT mice. The ability of the MZ B cells to capture antigens was tested using the IgG3-OVA system. As expected, more antigens bound to the MZ B cells and the FO B cells in the Cμ13 mice than in

the WT mice. However, it was the MZ B cells, not the FO B cells, that captured the vast majority of the antigens. The antigen delivery to the splenic follicles and the antigen deposition on FDC were also more marked in the Cμ13 mice compared to those in the WT mice. Strikingly, an almost 4-fold increase of the antigen deposition on FDC was observed in the Cμ13 mice. Hypothetically, a higher concentration of the antigen on splenic FDC will lead to a higher antigen-specific antibody production. Therefore, it was unexpected that the IgG3-mediated enhancement of the antibody responses against OVA in the Cμ13 mice was not higher than that in WT mice. The reason for this finding is not currently understood.

In summary, we have shown that not only passively administered IgM but also endogenous IgM have the ability to induce positive feedback regulation. Notably, the role of the endogenous complement-activating IgM in the feedback regulation is more pronounced in responses to physiological doses of antigens. Further characterization of the Cμ13 mice showed an almost double amount of MZ B cells with an up-regulated expression level of CR1/2 compared to that in WT mice. Antigen trapping in the splenic follicles of the Cμ13 mice after the immunization with the IgG3-antigen complexes was also increased, although no parallel enhancement in the antibody responses was induced.

Concluding remarks and future perspective

This work has contributed to the understanding of the role of IgG3 and IgM in the complement-mediated regulation of antibody responses (Table 4). IgM is known to enhance antibody responses through complement activation. However, this enhancing effect is limited to pentameric (not monomeric) IgM against large (not small) antigens. This is likely because IgM can only recruit complement factors after a conformational change, and this may require that IgM bind with several of its 'arms' to a large antigen. IgG3, acting in complementarity with IgM, can enhance the antibody responses against both large and small soluble antigens. Monomeric IgG3, which binds to the antigen surface, attracts other IgG3 molecules through Fc-Fc interactions, thus activating the complement system. Once opsonized by the complement, the IgM/IgG3-antigen-complexes will be recognized by CR1/2-expressing MZ B cells and captured and delivered to splenic $CR1^+$ FDC. Both $CR1^+$ FDC and $CR1/2^+$ MZ B cells are required for the optimal immune response that is mediated by IgG3 and IgM[142,158]. Possibly, $CR1^+$ FDC plays a dominant role in IgM-mediated enhancement against SRBC[142]. Furthermore, both the IgM- and IgG3-mediated enhancement of antibody responses require the presence of C1q, C3, and CR1/2 (references[117,121,139,143] and paper II). However, neither IgM nor IgG3 can enhance $CD4^+$ T cell proliferation[121,141].

Table 4. *IgG3 and IgM in the complement-mediated regulation of immune responses.*

Feedback regulation by		**Pentameric IgM (970 kDa)**	**Monomeric IgG3 (150 kDa)**
Regulation regarding antigen types	Particulate antigen (e.g., SRBC)	Enhances	Does not enhance (rather suppresses)
	Large soluble antigen (e.g., KLH > 7,000 kDa)	Enhances	Enhances
	Small soluble antigen (e.g., OVA = 45 kDa)	Does not enhance	Enhances
Complement activation capacity		yes	yes
Requirement for the presence of		CR1/2, C1q, C3	CR1/2, C1q, C3
Immune complex transported to follicles by		$CR1/2^+$ MZ B cells	$CR1/2^+$ MZ B cells
Antigen deposition on		$CR1^+$ FDC	$CR1^+$ FDC
Optimal antibody response requires the presence of		$CR1/2^+$ MZ B cells and $CR1^+$ FDC	$CR1^+$ FDC and $CR1/2^+$ MZ B cells
Enhance $CD4^+$ T cell proliferation		no	no

The highlighted conclusions drawn from the thesis are listed below.

- IgG3 facilitates OVA delivery to splenic follicles; OVA alone cannot reach the splenic follicles.
- MZ B cells capture and transport the IgG3-OVA complexes to the splenic follicles within 2 h after the administration.
- The majority of the OVA (50% - 90%) is deposited on the periphery of the FDC network 2 h after the administration and is located in the central FDC network 8 h after the administration.
- CR1/2, C1q, and C3 are crucially important for the initial steps of the antigen capture by MZ B cells and FDC.
- GC development induced by the IgG3-OVA complexes is impaired in Cr2 KO mice.
- CR1/2 expression on both B cells and FDC is required for an optimal antibody response against the IgG3-OVA immune complexes.
- IgG3 is an efficient inducer of immunological memory.
- IgG3 is able to enhance antibody responses against the large protein KLH.
- Endogenous IgM has the ability to feedback regulate SRBC-specific IgG production, and the activation of the complement by IgM is required.
- The proportion of MZ B cells in the Cμ13 mice is almost twice as high as that in the WT mice, and Cμ13 MZ B cells have a higher CR1/2-expression than WT MZ B cells.
- Compared to the WT mice, the Cμ13 mice exhibit increased trapping of the IgG3-OVA complexes by MZ B cells and FDC, but there was no increase in antibody production.

Thus far, we have obtained better knowledge regarding the crucial role of CR1/2, C1q and C3 in the IgG3-mediated upregulation of the immune response, which has been illustrated in the following two ways: (i) complement-opsonized antigen transport by $CR1/2^+$ MZ B cells and (ii) complement-opsonized antigen retention and display on $CR1^+$ FDC. However, there are always puzzles that remain to be solved.

There is a residual enhancement of the antibody response in Cr2 KO, C1q KO and C3 KO mice. This finding suggests that either (i) other cell type(s) in addition to MZ B cells (such as FDC or macrophages) can facilitate the IgG3-antigen obtaining access to splenic follicles, (ii) the increased access of the antigen to B cells in the follicles is essential but not the only explanation for the IgG3-mediated enhancement, or (iii) factors other than C are involved.

The ‘increased antigen trapping in the follicles’ but lack of ‘an enhanced primary antibody response’ in Cμ13 mice immunized with IgG3-antigen raises the question of whether there is indeed a direct correlation between the two parameters. It is possible that the antigen trapping may play a more

prominent role in the induction of memory, which is a parameter that was not analyzed in this context but can be studied in the future. Memory B cells with a specificity against certain antigens are inherently difficult to detect due to the low frequency of specific B-cell clones. In C57BL/6 mice, the antibodies produced against NP are genetically restricted and mainly comprise λ1 light chains and heavy chains with V regions encoded by the V186.2 segment of the VHJ558 gene family[161]. Therefore, NP-specific B cells can be detected using anti- λ1 antibodies and NP-fluorophore. Taking advantage of this detection ability, a panel of NP-specific monoclonal antibodies, including IgG3, can be generated to determine the NP-specific memory cells, the NP-specific GC B cells and the extra-follicular B cells.

Because FDC express receptors for various antibody isotypes, such as CR1 for complement opsonized IgG3- and IgM-antigen complexes, FcγRIIB for IgG2a, and CD23 for IgE, it would be interesting to compare the mechanisms underlying the enhancing effects induced by these different isotypes. Do all immune complexes formed by antigens together with IgG3, IgG2a, or IgE deposit on FDC *in vivo*? If yes, through what receptor(s)? Do FDC endocytose the IgG3-, IgG2a-, or IgE-antigen complexes in an *ex vivo* culture?

Research never truly ends, as there is always more to be discovered. Basic research is of great importance and should be valued and continuously supported.

Short summary in Chinese/中文摘要

抗体，又称免疫球蛋白(Immunoglobulin，简称 Ig)，是机体免疫系统在受到外来病原体(如细菌、病毒等)的刺激后由浆细胞(一种 B 细胞)产生的具有特异识别功能的 Y 形蛋白质。抗体 Y 形的两个分叉顶端各有一个特异性的抗原结合点，这些抗原结合点可以有高达数百万种的细微变化。抗体 Y 形的下部称为 Fc，能被各种效应细胞表面的 Fc 受体识别进而行驶功能。抗体可以被直接分泌到血液中，也可以依附于细胞表面成为 B 细胞感受器(B cell receptor, BCR)。小鼠的抗体共有五大型：IgM，IgD，IgA， IgG 和 IgE。IgG 又可以分为四种亚型：IgG1，IgG2a，IgG2b 和 IgG3。不同型或亚型的抗体行驶功能的方式不一。除此之外，抗体还具备正向或负向调节自我产出的能力，这一现象被称为抗体介导的免疫调节作用。本文着重研究小鼠 IgG3 及其正向调节抗体反应的机制。

IgG3 可以正向调节机体对小型可溶性蛋白(例如鸡卵白蛋白(Ovalbumin, OVA))以及大型可溶性蛋白(例如钥孔戚血蓝素(keyhole limpet hemocyanin, KLH))的体液免疫反应。IgG3 的这一功能在很大程度上依赖一些存在于血清或组织液中的活化后具有酶活性的蛋白质，又称补体(Complement, C)系统。 IgG3 单体可以通过 Fc 端吸引其他的 IgG3 单体从而形成多聚体。该多聚体与其特异性抗原形成的复合物可以激活并富集补体因子到其表面。被补体因子调和的抗原抗体复合物首先会被血液带到脾脏，然后在脾脏边缘区被边缘区 B 细胞通过补体受体(complement receptors 1 and 2，CR1/2)识别，再被边缘区 B 细胞运输进入脾脏内产生抗原特异性抗体的微结构滤泡中，最后被滤泡树突状细胞完整捕获、内吞、保存并阶段性地呈递在树突上供 B 细胞表面受体 BCR 识别。与被抗体补体调和的抗原相比，裸露的抗原靠自身很难进入脾脏滤泡内。目前的理论假设越多的抗原富集在滤泡内就能刺激机体产生更多的抗体。那么，抗原在滤泡内是如何激活 B 细胞分化并分泌抗原特异性抗体的呢? 以经典抗原 OVA 与其特异性抗体 IgG3 在小鼠体内引发的免疫反应为例，初始 B 细胞被 OVA 特异性 T 细胞激活后，先在滤泡生发中心的暗区大量克隆增殖并通过基因重排产生突变的 OVA 特异性 BCR，然后迁移到生发中心的亮区由滤泡树突状细胞和

滤泡 T 辅助细胞共同挑选表达高亲和力 BCR 的突变 B 细胞。这些 B 细胞将会回到暗区进一步克隆增殖和体细胞突变，再进入亮区被筛选出表达更高亲和力 BCR 的突变 B 细胞。在这个过程当中，突变的 B 细胞会陆续离开滤泡并分化成为分泌抗体的浆细胞或者记忆细胞 。后续研究发现，除了 CR1/2，经典补体激活通路的一些补体因子例如 C1q 和 C3 也在这一过程中起着至关重要的作用。

与 IgG3 类似，IgM 也是通过激活补体系统(complement, C)来正向调节抗体的产出。分泌型 IgM(多为五聚体)是个庞大的免疫球蛋白。IgM 跟抗原结合可以招募 C1 复合物中的 C1q 补体因子进而激活经典补体通路，富集补体因子到其表面形成超级复合物。这个超级复合物同样能被脾脏边缘区 B 细胞通过 CR1/2 识别进而转运进入脾脏滤泡细胞中，再被滤泡树突状细胞捕获并呈递。与 IgG3 不同的是，IgM 只能正向调节机体对大型抗原(例如羊血红细胞(sheep red blood cells, SRBC)，疟疾寄生虫(malaria parasite)和 KLH)的体液免疫反应。这可能与 IgM 激活补体系统的机制有关。IgM 五聚体必须先从平面构象转变成立体星型构象才能激活 C1q。

除了正反馈作用之外，IgG, IgM 和 IgE 也能负向调节机体的免疫反应。研究抗体介导的免疫调节背后的机制，有助于我们理解这些抗体在正常免疫反应以及自身免疫性疾病中的作用。通过筛选出关键的作用靶点，我们希望可以设计出合理增强免疫效应的疫苗，同时也希望能有效控制自身免疫性疾病的进程。

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