Feedback Enhancement of Immune Responses by IgE, IgM, and IgG3 Antibodies

ZHOUJIE DING
Abstract

Antibodies can enhance or suppress the immune responses against their specific antigens. This phenomenon is known as antibody-mediated feedback regulation. We have studied the mechanisms underlying IgE-, IgM-, and IgG3-mediated enhancement of immune responses in mouse models using intravenous immunization. We attempted to answer the following questions: 1) Which cell type presents IgE-complexed antigens to CD4+ T cells? 2) Is complement activation required for specific IgM to enhance antibody responses? 3) Does IgM enhance CD4+ T-cell responses? 4) How are IgG3-antigen complexes transported into B-cell follicles?

We found that CD23+ B cells transporting IgE-antigen complexes into B-cell follicles were not required to prime the antigen-specific CD4+ T cells in vivo, whereas CD11c+ cells were indispensable. After examining the three most common subpopulations of CD11c+ cells in the spleen, we determined that it was CD8α- conventional dendritic cells migrating into the T-cell zone following immunization that presented IgE-complexed antigens to CD4+ T cells.

Next, we showed that specific IgM from Cµ13 mice, which is unable to activate complement, failed to enhance either antibody or germinal center responses whereas wild-type IgM enhanced both responses. Therefore, specific IgM must activate complement to enhance humoral responses. In addition, wild-type IgM did not up-regulate CD4+ T-cell responses.

Finally, we showed that IgG3-antigen complexes were transported by marginal zone B cells into B-cell follicles via binding to complement receptors 1 and 2 (CR1/2) on those cells. The immune complexes were captured by follicular dendritic cells as early as 2 h after immunization. Germinal center responses were also enhanced by IgG3. Using bone marrow chimeric mice, we found that CR1/2 expression was required on both marginal zone B cells and follicular dendritic cells to provide an optimal enhancement of antibody responses.

Keywords: IgE, IgM, IgG3, antibody responses, T-cell responses, antigen presentation, complement, complement receptors 1 and 2

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As our circle of knowledge expands, so does the circumference of darkness surrounding it.

知识的圆越大，未知的世界也越广阔。

Albert Einstein
阿尔伯特·爱因斯坦
List of Papers

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


II Ding, Z., Dahlin, J.S., and Heyman, B. CD8\(\alpha^{-}\)conventional dendritic cells are the dominant cells presenting IgE-complexed antigen to CD4\(^+\) T cells. *Manuscript*.


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Review articles (not included in the thesis)


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Abbreviations

- APCs: Antigen-presenting cells
- BCR(s): B cell receptor(s)
- BSA: Bovine serum albumin
- cDCs: Conventional dendritic cells
- CR1/2: Complement receptors 1 and 2
- DTR: Diphtheria toxin receptor
- ELISA: Enzyme-linked immunosorbent assay
- FcεRI: Fc epsilon receptor I
- FcεRII: Fc epsilon receptor II (= CD23)
- FcγRs: Fc gamma receptors
- FcµR: Fc mu receptor
- FDCs: Follicular dendritic cells
- FTY720: Fingolimod
- i.v.: Intravenously
- KLH: Keyhole limpet hemocyanin
- MBL: Mannan-binding lectin
- MHC: Major compatibility complex
- NP: (4-hydroxy-3-nitrophenyl)acetyl
- OVA: Ovalbumin
- RhD: Rhesus D antigen
- S1P: Sphingosine 1-phosphate
- SRBC: Sheep red blood cells
- T_{FH}: T follicular helper
- TNP: 2,4,6,-trinitrophenyl
Introduction

Immune responses are a series of reactions that occur after an organism encounters foreign substances or autoantigens/self-molecules. The consequences of such reactions can be either protective or pathologic depending on the type of substance recognized by the immune system. Immune responses can be divided into innate immunity and adaptive immunity. The main components of innate immunity are cellular and chemical barriers, e.g., skin, phagocytes and blood proteins such as complement. Innate immune responses occur very rapidly but have a short duration. The diversity of antigens recognized by innate immune components is limited, and no memory response is induced. In contrast, adaptive immunity can respond to a large range of antigens and has a memory response that develops against repeated exposure to antigens, although it reacts slower than innate immune responses after stimulation. Lymphoid organs containing lymphocytes and their secreted substances are the major components of adaptive immunity. Mammalian lymphoid organs comprise the bone marrow, thymus, spleen, mucosa-associated lymphoid tissue, and lymph nodes. This thesis will focus on the enhancement of adaptive immune responses via antibody-mediated feedback regulation. The model lymphoid organ studied herein is the mouse spleen.

The structure of the mouse spleen

The spleen is the largest secondary lymphoid organ in the body, in which immune responses occur primarily in response to circulating antigens. The mouse spleen is analogous to the human spleen, although there is a small difference in the marginal zone: humans have an inner and an outer marginal zone, whereas mice have an integrated one (reviewed in [1]). Therefore, the mouse is a good animal model to study spleen-based immune responses. Antigens reach the spleen via the blood. Hence, intravenous immunization was used in the present investigation to mimic blood-borne antigens. The spleen consists of two main compartments: the white pulp and the red pulp (Figure 1A). The red pulp mainly functions as a blood filter to remove foreign or damaged cells and is a storage site for iron, erythrocytes and platelets (reviewed in [1]). The white pulp initiates immune responses and is mainly composed of the marginal zone, B-cell follicle and T-cell zone (Figure 1B). Many different types of leukocytes are located in these distinct areas of the
white pulp (Figure 1C). For example, B cells residing in the marginal zone are named marginal zone B cells, while B cells that home to the follicles are called follicular B cells. Follicular B cells can recirculate between the peripheral blood and the follicles. Metallophilic macrophages, which are also located in the marginal zone, form a tight ring around follicular B cells and thus can be used as the boundary line to separate marginal zone and B-cell follicles. Follicular dendritic cells (FDCs) are another important population in the follicles. These cells are stromal cells and thus are resistant to radiation. FDCs use their dendrites to form a tight network inside B-cell follicles. T cells reside in the T-cell zone (also known as the periarteriolar lymphoid sheath) surrounded by B-cell follicles. There is a special area named the marginal zone bridging channel that extends from the T-cell zone to the red pulp (Figure 1C). One subset of CD11c⁺ dendritic cells, CD8α⁺ conventional dendritic cells (cDCs), resides therein and will be discussed later in this thesis. Germinal centers with mature B cells are generated in the centers of B-cell follicles following antigen stimulation, where somatic hypermutation and affinity maturation happens [2].

**Figure 1.** Schematic histological structure of a mouse spleen. (A) A schematic cross-section of a mouse spleen showing the white pulp and the red pulp. (B) The composition of the white pulp. (C) Examples of different cell types that reside in distinct parts of the white pulp.
Induction of immune responses in the follicles

Immune responses can be induced at different sites by distinct cell types depending on the antigen type encountered. This thesis focuses on the immune responses against T-dependent antigens occurring in splenic B-cell follicles.

Figure 2. Induction of immune responses in the follicle by T-dependent antigens. B cells and T helper cells are activated after encountering the antigen. Activated B cells (orange) and activated T cells (pink) interact at the B cell-T cell border and B cells received additional signals to proliferate and form the dark zone (DZ) of the germinal center. Some of the activated T cells differentiate into T follicular helper (TFH) cells and move into the germinal center. After somatic hypermutation in the dark zone, B cells migrate to the light zone (LZ) where they encounter FDCs displaying antigens and TFH cells. B cells with high affinity BCRs survive and either return to the dark zone for further proliferation or differentiate into memory B cells or plasma cells and exit the follicles.

Antigens can reach the follicles via different pathways (which will be discussed in the next section). There, they are recognized by specific B cells in the follicles. The B cells internalize the antigens and present the antigenic peptides on their major histocompatibility complex (MHC). Antigens are also captured by antigen-presenting cells (APCs) such as dendritic cells and
presented to T cells to activate them. The induction of immune responses in the follicles is a complicated process and has mostly been studied in lymphoid nodes rather than in the spleen (reviewed in [3]). As shown in Figure 2, activated B and T cells migrate to the B cell-T cell border to interact. Activated B cells, which have received further stimulating signals from the activated T cells, proliferate and form the dark zone of the germinal center. B cells in the dark zone undergo extensive somatic hypermutation and then migrate to the light zone of the germinal center [4]. Some of the activated T cells differentiate into T follicular helper (T\textsubscript{FH}) cells and migrate into the light zone of the germinal center. There, B cells with previously mutated B cell receptors (BCRs) are exposed to FDCs that display intact antigens on their dendrites [5]. If the BCRs have a high affinity for the antigen, the B cells will capture the antigen from FDCs and process and present antigenic peptides on their MHC to the limited numbers of T\textsubscript{FH} cells, which subsequently provide survival signals to the B cells [6,7]. B cells having BCRs with highest affinity will compete with the remaining B cells to acquire sufficient antigens and be selected. If the BCRs have a low affinity for the antigens displayed by FDCs, the B cells will be unable to generate sufficient peptide-associated MHC molecules to be selected and will undergo apoptosis and be eliminated (reviewed in [8,9]). The surviving B cells continue to undergo class-switch recombination in the light zone. Some of the class-switched B cells return to the dark zone for further proliferation and some of them differentiate into memory B cells or plasma cells that secrete antibodies with a high affinity and eventually leave the follicles. The precise mechanism underlying the preferential antigen-access by B cells in the follicles resulting in affinity maturation of antibodies is still debated (reviewed in [10-12]). However, it is likely that both FDCs and T\textsubscript{FH} cells are crucial driving forces in antibody affinity maturation.

This complicated process of inducing immune responses involves several effector cells and requires certain cell types to migrate from one region to another in the follicles. In the T-cell zone, stromal cells constantly produce high levels of CCL19 and CCL21, which are ligands for CCR7 (reviewed in [13,14]). Studies have shown that activated B cells up-regulate CCR7 and thus migrate to the B cell-T cell border to interact with T helper cells [15,16]. The organization of the germinal center requires CXCR4 to direct B cells to the dark zone and CXCR5 to direct B cells to the light zone [17]. T cells differentiating into T\textsubscript{FH} cells up-regulate CXCR5 and thus obtain access to the light zone of the B-cell follicles [18,19]. By investigating lymph nodes using multiphoton intravital microscopy, it has been shown that FDCs can recycle intact complement-opsonized antigens and thus retain them for a long period for display to the B cells [5]. This phenomenon is important for the affinity maturation of B cells, as mentioned above.
Antigen transportation to follicles

Antigens cannot cross the marginal zone of the follicles freely. There are several pathways by which antigens or immune complexes are transported into follicles to initiate immune responses. In the spleen, the splenic conduits system, a stromal network, can directly transport small blood-borne antigens to the white pulp [20]. Other pathways are dependent on cell surface receptors on B cells. Marginal zone B cells are able to shuttle between the marginal zone and the outer part of the B-cell follicle [21] due to the balance between their expression of CXCR5 and sphingosine 1-phosphate (S1P) receptors [21]. An antagonist of S1P receptor 1, fingolimod (FTY720), is able to temporarily induce the translocation of marginal zone B cells from the marginal zone into the follicles and thus has been used to study the shuttling mechanism of marginal zone B cells [22]. Several studies have reported that complement-containing immune complexes are carried from the splenic marginal zone into B-cell follicles by binding to complement receptors 1 and 2 (CR1/2) on shuttling marginal zone B cells [21,23,24]. Follicular B cells can circulate from the peripheral blood to the spleen. They express CD23, a low affinity receptor for IgE, which is also known as Fc epsilon receptor II (FceRII). Follicular B cells are able to rapidly transport IgE-antigen complexes bound to CD23 from the peripheral blood into the splenic follicles [25]. In the lymph nodes, small antigens reach the follicles via conduits [26]. Larger antigens are captured by macrophages in the subcapsular sinus [27] or by dendritic cells in the medulla and delivered to B-cell follicles [28]. In addition, B cells in the lymph node can acquire particulate antigens or immune complexes at the follicle-subcapsular sinus boundary and transport them to the border between B-cell follicles and T-cell zone [29,30].

Antibody-mediated feedback regulation

Antigen-specific antibodies can regulate immune responses to specific antigens. This phenomenon is known as antibody-mediated feedback regulation (reviewed in [31]). In the clinic, it has been applied for Rhesus prophylaxis since the early 1960s even though the underlying mechanism is not completely understood [32]. Small amounts of IgG anti-Rhesus D antigen (RhD) are administered to pregnant mothers who lack RhD on their erythrocytes within 72 h of delivery. This inhibits B cell activation and antibody responses against fetal RhD+ erythrocytes that are transferred via transplacental hemorrhage, thus preventing hemolytic disease in the RhD+ newborns (reviewed in [33]). Studies have shown that antibodies including IgG, IgE and IgM can either enhance or suppress specific antibody responses via feedback regulation by different mechanisms, depending on the class of antibody and the type of antigen that is involved.
IgG has a dual function in feedback regulation. On the one hand, specific IgG antibodies of all murine subclasses can enhance immune responses together with low doses of soluble protein antigens such as ovalbumin (OVA), bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH) [34-38]. This process occurs either via Fc receptors during IgG1-, IgG2a- and IgG2b-mediated immune enhancement [37,39] or via complement activation during IgG3-mediated immune enhancement [38,40]. Both antibody and CD4⁺ T-cell responses are enhanced by IgG2a [39], whereas only antibody responses are enhanced by IgG3 [40]. On the other hand, IgG can act as an immunosuppressor to downregulate more than 99% of the primary antibody responses to large particulate antigens [34,41-43]. This is in part likely due to epitope masking of the antigen by IgG antibodies [44]. IgE enhances both antibody and CD4⁺ T-cell responses to small soluble antigens and is dependent on CD23 expression on follicular B cells [25,45-47]. IgM can induce enhanced antibody responses to large antigens such as sheep red blood cells (SRBC) [41,48,49], KLH [34,35,50], and malaria parasites [51]. It has been reported that complement plays an important role in IgM-mediated enhancement of antibody responses [52]. This thesis focuses on the mechanisms underlying IgE-, IgM-, and IgG3-mediated immune enhancement. The key factors that have been previously established and the outstanding questions discussed in this thesis regarding the enhancement of immune responses by IgE, IgM, and IgG3 are summarized in Table 1.

Table 1. The enhancement of immune responses by IgE, IgM, and IgG3 (pre-thesis)

<table>
<thead>
<tr>
<th>Isotype</th>
<th>Enhancing antibody/CD4⁺ T cells responses</th>
<th>Antigen types regulated</th>
<th>Requiring the isotype to activate complement</th>
<th>Immune complexes transported into B-cell follicles by</th>
<th>Immune complexes presented to CD4⁺ T cells by</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgE</td>
<td>Yes/Yes</td>
<td>Small soluble proteins (OVA, BSA)</td>
<td>No</td>
<td>CD23⁺ follicular B cells</td>
<td>?</td>
</tr>
<tr>
<td>IgM</td>
<td>Yes/?</td>
<td>Particulate antigens (SRBC, malaria parasites) Large soluble proteins (KLH)</td>
<td>Yes?</td>
<td>CR1/2⁺ Marginal zone B cells</td>
<td>/</td>
</tr>
<tr>
<td>IgG3</td>
<td>Yes/No</td>
<td>Small soluble proteins (OVA, BSA)</td>
<td>Yes</td>
<td>?</td>
<td>/</td>
</tr>
</tbody>
</table>
IgE-mediated enhancement of immune responses

IgE is one of the essential modulators related to allergic diseases. Antigen crosslinking of IgE bound to a high-affinity Fc receptor for IgE, Fc epsilon receptor I (FcεRI), on mast cells or basophils can induce activation of those cells and initiate an allergic reaction locally or systemically (reviewed in [53]). When antigen-specific IgE forms a complex with small soluble antigens such as OVA-2,4,6-trinitrophenyl (TNP), it induces a several hundred-fold higher antibody response than antigen alone [45,46,54]. Specific primary responses of IgG1, IgG2a, IgA, IgM, and IgE are all enhanced following the administration of IgE-antigen complexes [45,46,55]. Thus, there is no skewing of Th1- or Th2-type antibody responses during IgE-mediated enhancement. Among the responses of all of the isotypes that are enhanced by IgE, the specific IgG response is the most pronounced and peaks as early as 6 days after priming [55]. IgE also efficiently enhances memory responses [46] and germinal center formation [25]. Moreover, a remarkably enhanced proliferation of OVA-specific T cells is observed in mice in which CD4+ T cells have been adoptively transferred from DO11.10 mice (with OVA-specific T cell receptors) and immunized with OVA-TNP together with IgE anti-TNP [25,47]. IgE-mediated immune enhancement is not affected in Fc receptor gamma chain-deficient mice lacking FcεRI and activating Fc gamma receptors (FcγRs) [37] but is completely dependent on the expression of CD23. Pre-treatment of the mice with anti-CD23 antibodies prior to immunization with IgE-antigen complexes blocks the ability of IgE to enhance antibody responses [45,46]. In addition, both the enhanced specific antibody and T-cell responses are abrogated in mice deficient in CD23 (CD23 KO) [47,54].

CD23, CD23+ B cells and the enhancement by specific IgE

CD23 (FcεRII) was first discovered on human B cells as a low-affinity receptor for IgE [56]. It does not belong to the super Ig family as do other Fc receptors but to the C-type lectin family with a C-terminal lectin head domain (Figure 3A) [57]. CD23 is expressed as membrane-bound trimers on the cell surface (Figure 3B) and can be cleaved auto-catalytically into soluble fragments. Membrane-bound murine CD23 has an affinity for IgE of $1.45 \times 10^8$ M$^{-1}$, whereas soluble murine CD23 monomer that has been cleaved from the membrane has a much lower affinity (ranging from $10^5$ to $10^6$ M$^{-1}$) [58]. Both human and murine CD23 have two isoforms, CD23a and CD23b [59-62]. In mice, CD23a is the isoform involved in IgE-mediated immune enhancement [63] and its expression is limited to B cells [64] and FDCs [65].
Although both B cells and FDCs express CD23 in mice, studies have revealed that CD23⁺ B cells are the cells that are indispensable in IgE-mediated immune enhancement. There is little enhancement of antibody responses in bone marrow chimeric mice with CD23 on FDCs but not on B cells following immunization with IgE-antigen complexes. In contrast, antibody responses are still enhanced by IgE in mice with CD23⁺ B cells but lacking CD23 expression on FDCs [66]. Moreover, the ability of IgE to enhance immune responses is abolished in CD23 KO mice [54] but can be restored by the adoptive transfer of CD23⁺ B cells [47,66]. These results demonstrate that CD23 expression on B cells is indeed required for IgE-mediated enhancement.

Transportation of IgE-complexed antigens

The mechanism by which IgE enhances immune responses via CD23⁺ B cells remains unclear. It has been shown by several in vitro studies that IgE-antigen complexes bound to CD23 on B cells from humans or mice are taken up and presented more efficiently to T cells than non-complexed antigens [67-70] and that CD23-specific monoclonal antibodies can inhibit this enhancing effect [67-69]. Such in vitro findings suggest that the up-regulation of immune responses by IgE is due to enhanced antigen presentation by CD23⁺ B cells. In vivo, IgE-antigen complexes are found on circulating CD23⁺ B cells in the blood 5 min after immunization and can be detected in splenic follicles after 30 min, together with an enhanced T cell response that peaks after 72 h (Figure 4) [25]. This result shows that CD23⁺ B cells play
an important role in the capture of IgE-antigen complexes. It also provides an alternative interpretation for the requirement of CD23⁺ B cells in IgE-mediated enhancement. Circulating CD23⁺ B cells transport IgE-antigen complexes into splenic follicles, leading to increased antigen concentrations. This process results in enhanced antibody and T-cell responses. Therefore, CD23⁺ B cells are indispensable as antigen-transporters of IgE-complexed antigens. However, whether it is those CD23⁺ B cells or other cell types that present IgE-complexed antigen to CD4⁺ T cells in vivo remains unknown, and this question will be addressed later in this thesis.

**Figure 4.** IgE-complexed antigen transportation from peripheral blood into splenic follicles. (A) IgE-antigen complexes (IgE/Antigen) bind to circulating B cells via CD23 in the blood as rapidly as 5 min after immunization. (B) Thirty minutes after immunization, circulating B cells carry the IgE-antigen complexes into splenic follicles. Activated B cells and T cells first interact at the B cell-T cell border. Next, via unknown mechanisms, antigens are efficiently presented to CD4⁺ T cells. Antigen-specific CD4⁺ T cells (dark green) then undergo extensive proliferation.

**Antigen presentation to CD4⁺ T cells**

Because IgE enhances the proliferation of specific CD4⁺ T-cells [25,47], there must be some cell type that presents the IgE-complexed antigens to T cells to activate them. As mentioned above, it has been hypothesized that B cells present IgE-complexed antigens to CD4⁺ T cells. However, other studies have suggested that B cells are unable to prime naïve T cells [71-73] and there is no evidence to date that B cells are able to present IgE-antigen complexes to CD4⁺ T cells in vivo.

However, dendritic cells, especially cDCs, are efficient antigen-presenting cells to naïve T cells. cDCs can be divided into two subtypes according to the expression of CD8α: CD8α⁺ cDCs and CD8α⁻ cDCs. In naïve mice,
CD8α⁺ cDCs reside in the T-cell zone, while CD8α⁻ cDC are located in the marginal zone bridging channel (Figure 1C). cDCs process antigens and present antigenic peptides on their major MHC to T cells. They also provide co-stimulating signals activate T cells. CD8α⁺ cDCs are able to prime CD4⁺ T cells under certain conditions [74,75] but are more efficient in cross-presentation to CD8⁺ T cells [76-81]. In contrast, CD8α⁻ cDCs are very efficient in priming CD4⁺ T cells [79,80]. They express high levels of the surface marker CD11c and thus can be depleted conditionally and systemically via the administration of diphtheria toxin in a CD11c-diphtheria toxin receptor (DTR) mouse strain, which expresses a primate DTR on CD11c⁺ cells encoded by a transgene under the control of the CD11c promoter [82]. Studies using CD11c-DTR mice have shown that CD11c⁺ cells, most likely CD11c⁺ cDCs, are involved in stimulating CD4⁺ T cell responses for defense against infectious microbes [83-85]. It is possible that those CD11c⁺ cDCs also play a role in presenting IgE-complexed antigens to CD4⁺ T cells.

Complement and antibody responses

The enhancement of immune responses by IgE does not require complement [38]. However, complement is involved in IgM- and IgG3-mediated enhancement, which will be discussed in a later section. Importantly, a functional complement system is essential for normal antibody responses against antigens alone, i.e., uncomplexed antigens (reviewed in [86]).

There are three complement-activating pathways: the classical pathway, the lectin pathway, and the alternative pathway (Figure 5). The crosstalk between complement and the immune system was first discovered in 1974 when mice treated with cobra venom factor, and thus depleted of C3, displayed severely impaired antibody responses [87]. Subsequent studies have demonstrated that normal antibody responses to antigens alone are diminished in animals that lack C2 [88], C3 [89,90] or C4 [88,89,91,92]. Importantly, mice that are deficient in the classical pathway component C1q also have significantly reduced antibody responses [93,94]. However, mice lacking factor B in the alternative pathway [95] or C5 in the late steps of complement activation (leading to formation of the membrane attack complex) [96] have normal antibody responses. Occasionally, moderately impaired antibody responses can be observed in mice that lack mannan-binding lectin (MBL) [97,98] along with occasionally increased antibody responses [99,100]. A deficiency in C1q (involved in the classical pathway only) results in markedly impaired antibody responses, whereas deficiencies in the lectin or alternative pathways have marginal effects on the antibody response. These findings indicate that complement activation through the classical pathway is most important for inducing normal antibody responses.
Figure 5. The importance of the classical pathway of complement activation for normal antibody responses. Animals lacking the complement activators or products indicated in green have normal antibody responses. Animals lacking the complement activators or products indicated in red have severely impaired antibody responses. (Adapted from Rutemark et al, 2011, Proc. Natl. Acad. Sci. U.S.A., with permission from the publisher.)

Although it is well established that the classical pathway of complement activation is critical for normal antibody responses, how the activation is triggered remains unclear. It is known that antibody-antigen complexes are the most common activators of the classical pathway. However, one study has shown that activation of the classical pathway by natural IgM (able to form complexes with many antigens with a low affinity) is not required for the induction of normal antibody responses [94]. Normal antibody responses are observed in knock-in mice (Cµ13) with natural IgM that is unable to bind C1q due to a point mutation in the µ-heavy chain [94]. However, in mice lacking natural secretory IgM (µs−/−), antibody responses against antigens such as BSA-(4-hydroxy-3-nitrophenyl)acetyl (NP), KLH-NP, bacteria, or influenza virus are impaired [101-104]. These findings indicate that natural IgM is indeed involved in the induction of antibody responses but likely functions via effector pathways other than the complement-activating pathways. Mice deficient in CR1/2 (Cr2 KO) also have severely impaired antibody responses [105-107]. In mice, CR1 and CR2 are different splice products
encoded by the same Cr2 gene. Their ligands are C3b, iC3b, C3dg, and C3d, and the expression of CR1/2 is limited to B cells and FDCs in mice (reviewed in [108]). Therefore, it is likely that the impaired antibody responses observed in animals lacking C1q, C2, C3, or C4 actually result from a lack of ligands for CR1/2 and thus an absence of CR1/2-mediated downstream effects. The CR1/2-expressing cell type that is more important for inducing normal antibody responses, B cells or FDCs, remains controversial. Some studies have reported that the most essential feature is the expression of CR1/2 on FDCs [107,109,110] and that mice with CR1/2 on FDCs have normal antibody responses to SRBC regardless of whether CR1/2 is expressed on B cells [111]. However, in other studies, the expression of CR1/2 on B cells plays an indispensable role [105,112]. A recent study has shown that CR2 is expressed mainly on B cells whereas CR1 expression is dominant on FDCs in mice [113]. Nevertheless, CR1 and CR2 are not distinguished in this thesis.

IgM-mediated enhancement of immune responses

Unlike other antibody isotypes, IgM is secreted as a pentamer. Secreted IgM is important for early defense mechanisms against microbes, in particular bacteria, with polysaccharide-rich capsules [103,114]. Only pentameric, but not monomeric, IgM can activate complement. Free pentameric IgM exists in a flat form (Figure 6A). However, it has been suggested that the binding of IgM to an antigen requires a conformational change to reveal the binding sites for C1q to activate complement (Figure 6B) [115]. An IgM-specific Fc receptor, Fc mu receptor (FcµR), was recently discovered [116,117]. Mice deficient in FcµR display reduced antibody responses against low doses of antigens as well as an increase in autoantibody production, suggesting that FcµR may be involved in IgM homeostasis and the regulation of humoral responses [116,117].

![Figure 6. Structure of IgM. (A) Free pentameric IgM exists in a flat form. (B) After binding to a multivalent antigen, pentameric IgM undergoes a conformational change into a ‘mushroom’ shape.](image-url)
Antigen-specific IgM is able to enhance antibody responses, and this phenomenon was first observed several decades ago in humans [32] and later in mice [41,48,49,118,119]. IgM can induce enhanced specific antibody responses against particulate antigens such as SRBC [41,48,49,118,119], the soluble antigen KLH [34], or malaria parasites [51]. The enhancement is more pronounced with suboptimal doses than with high doses of antigen [41,120,121]. Primary IgG, IgE and memory responses are all enhanced by IgM [48,122-124]. The enhancing effect of specific IgM is rapid and long-lasting, and it results in an increase in direct plaque-forming cells as early as 3 days after immunization [48,49] and an up-regulation of specific IgG responses for at least 3 months [122]. Both polyclonal and monoclonal specific IgM are able to enhance antibody responses [48]. IgM and antigen must be administered separately but within a few hours of each other to achieve enhanced antibody responses [118,120,125]. IgM co-administered with specific antigen also induces earlier germinal center responses than antigen administered alone [23].

Complement and enhancement by specific IgM

A few studies have suggested that complement is required for IgM-mediated enhancement. The enhancement of antibody responses by IgM is lost in C3-depleted but not in C5-deficient mice, indicating that the activation of early complement factors prior to the activation of C5 is important [52]. Moreover, a mutant monoclonal IgM anti-TNP antibody that is unable to activate complement fails to enhance antibody responses against SRBC-TNP [52]. Pentameric, but not monomeric, IgM enhances antibody responses to specific antigens, which indicates that complement must be activated by IgM during IgM-mediated enhancement [50]. In Cr2 KO mice, the enhancement by IgM is also abrogated [126]. The expression of CR1/2 on both FDCs and B cells is required for optimal enhancement of antibody responses by IgM [111]. These findings suggest an important role for CR1/2 in binding activated complement components to facilitate the induction of enhanced antibody responses by IgM. Taken together, early complement activation and ligand binding to CR1/2 appears to be essential in the enhancement of antibody responses by specific IgM. However, some doubts remain regarding these findings. First, when mutant monoclonal IgM anti-TNP was used, the enhancement was very weak [52]. Second, it is not known whether this mutant IgM is able to bind to FcμR, the receptor that has been suggested to have a role in maintaining immune responses. Third, monomeric IgM cannot bind to FcμR, which may provide an alternative explanation for the inability of monomeric IgM to enhance antibody responses [50]. Finally, a recent study has shown that normal antibody responses do not require natural IgM to activate complement. These inconsistencies raise questions concerning whether complement-activation by specific IgM is indeed necessary for the
enhancement of antibody responses, which is further investigated in this thesis.

Transportation of IgM-complexed antigens

*Figure 7.* IgM-complexed antigen transportation into B-cell follicles. Marginal zone B cells (MZ B) bind to complement-opsonized IgM-complexed antigens (IgM/Antigen) via CR1/2 and transport them into B-cell follicles. Activated B cells and T cells first interact at the B cell-T cell border. FDCs capture the complement-opsonized antigens via CR1/2 and display them on the surface. Whether the proliferation of specific CD4+ T cells (dark green) is enhanced by specific IgM is investigated in paper III.

Several studies have suggested that specific IgM is able to enhance antibody responses because higher amounts of antigen are deposited in B-cell follicles when it is administered together with specific IgM than when it is administered alone. IgM anti-SRBC induces enhanced splenic uptake of $^{51}$Cr-labeled SRBC [49]. An increased deposition of BSA-NP is observed on FDCs within 16 h of immunization when pentameric IgM anti-NP is co-administered, whereas little antigen deposition is detected when the same dose of NP-BSA is administered alone [50]. Analogously, a dimeric form of a soluble bacteriophage coat protein is detected on FDCs only when specific IgM is also administered [24]. This IgM-facilitated antigen deposition is dependent on complement. BSA-NP, which forms complexes with IgM anti-NP, is not observed on FDCs but rather remains in the splenic marginal zone in C3-depleted or Cr2 KO mice [23]. IgM-complexed BSA-NP is bound to
marginal zone B cells after immunization, and when marginal zone B cells are depleted, the localization of antigen on FDCs is severely impaired [23]. Because marginal zone B cells can shuttle between the marginal zone and B-cell follicles and they express CR1/2 [21], it is likely that IgM-complexed antigens are bound to marginal zone B cells via CR1/2 and are transported into B-cell follicles where they are deposited on CR1/2+ FDCs (Figure 7). However, none of the studies demonstrating increased antigen deposition in the follicles have investigated whether the antibody responses against those antigens are enhanced by the specific IgM.

**CD4⁺ T-cell responses and specific IgM**

In nude mice, specific IgM failed to induce enhanced antibody responses to T-dependent antigens such as SRBC [119,127], suggesting that IgM cannot function as a substitute for T helper cells. Several in vitro studies have shown that antigen opsonized with complement induces the enhancement of antigen presentation by B cells and T-cell activation compared with antigen alone [128-131]. After administration, specific IgM activates complement, and the complement factors then opsonize IgM-complexed antigen. Therefore, it is possible that enhancement of CD4⁺ T-cell proliferation can be induced by complement-opsonized IgM-antigen complexes. Whether specific IgM can enhance proliferation of CD4⁺ T cells in vivo is investigated in this thesis.

**IgG3-mediated enhancement of immune responses**

![Figure 8. IgG3 is able to activate complement (C1), and IgG3 molecules cooperatively bind to each other through Fc-Fc interactions.](image)

IgG3 constitutes only a small portion of the total IgG in mice. It is a cryoglobulin-type of antibody that is important for defense against bacterial and fungal infections [132-134]. It can self-associate through Fc-Fc interactions after binding to an antigen (Figure 8) [135-137], and likely due to this feature, it is also a potent activator of complement [138,139]. IgG3 administered together with soluble antigens such as OVA induces higher antibody responses than antigen administered alone [38,40]. FcγRI has been shown to
be the only Fc receptor that binds to IgG3 [140]. However, the enhancement of antibody responses by IgG3 does not require the expression of any of the known Fc receptors for IgG [38,40] but is dependent on complement [38]. In addition, IgG3 has only minor effects on the enhancement of CD4+ T-cell responses [40].

Complement and enhancement by specific IgG3

IgG3 can activate complement through both alternative and classical pathways [139]. The enhancement of antibody responses by specific IgG3 requires complement factors because in mice depleted of C3 and in mice deficient in CR1/2, the enhancement is severely impaired [38]. This characteristic differs from the other IgG subclasses, IgG1, IgG2a, and IgG2b, which require FcγRs but not complement to enhance antibody responses ([36,37,39,126] and reviewed in [141]). It has been hypothesized that the self-aggregating property of IgG3, which causes one IgG3 molecule to attract another after binding to an antigen and thus facilitates complement activation, explains the preference of IgG3 for the use of complement (reviewed in [86]).

Transportation of IgG3-complexed antigens

IgG3-mediated immune enhancement is more similar to IgM-mediated immune enhancement than the immune enhancement by other IgG subclasses because both IgG3- and IgM-mediated immune enhancement are severely impaired in mice depleted of C3 and in mice lacking CR1/2 [38,52,126]. As mentioned in a previous section, marginal zone B cells expressing high levels of CR1/2 are important for the immune enhancement by IgM and they transport IgM-complexed antigens from the marginal zone to B-cell follicles via CR1/2. It is likely that IgG3-complexed antigens use the same strategy to reach the B-cell follicles, and thus the concentration of antigen inside the follicles will be higher if it is administered together with specific IgG3. The hypothesis for the mechanism by which IgG3-complexed antigens are transported into B-cell follicles is shown in Figure 9. This hypothesis will be investigated in this thesis.
CD4⁺ T-cell responses and specific IgG3

Unlike IgE and IgG2a, which significantly enhance the proliferation of specific CD4⁺ T cells [25,39,47], IgG3 is a poor enhancer of CD4⁺ T-cell responses in vivo [40]. Moreover, spleen cells pulsed with IgG3-complexed OVA-TNP in vitro fail to enhance the proliferation of OVA-specific CD4⁺ T cells compared with spleen cells pulsed with OVA-TNP alone [40]. Therefore, specific IgG3 has a minor (if any) effect on CD4⁺ T-cell responses.

Figure 9. Hypothesis for the mechanism by which IgG3-complexed antigens are transported into B-cell follicles. IgG3-complexed antigens (IgG3/Antigen) are opsonized with complement factors and are likely transported into B-cell follicles via binding to CR1/2 on shuttling marginal zone B cells (MZ B). Activated B cells and T cells first interact at the B cell-T cell border, but specific CD4⁺ T cells do not undergo extensive proliferation.
Present investigation

Aims

The general aim of the studies in this thesis is to understand the mechanisms underlying antibody-mediated feedback enhancement of immune responses, in particular in response to IgE, IgM, and IgG3.

The specific aims are to answer the following questions:

I Which cells, B cells or CD11c+ cells, present IgE-complexed antigen to CD4+ T cells in vivo?

II Which subpopulation of CD11c+ cells is essential in IgE-mediated enhancement of CD4+ T cell responses? How does antigen presentation to CD4+ T cells occur?

III Is activation of complement by specific IgM required in IgM-mediated enhancement of humoral responses? Does IgM enhance CD4+ T cell responses?

IV How are IgG3-antigen complexes transported into splenic B cell follicles? Is CR1/2 on B cells or FDCs required in IgG3-mediated enhancement of antibody responses?

Experimental setup

Mouse strains

- BALB/c mice: wild-type control.
- C57BL/6 mice: donors of MHC-II (I-A^b) incompatible B cells.
- CD11c-DTR mice: with a high-affinity human DTR that is expressed under the cd11c promoter [82]. CD11c^+ cells are conditionally depleted in these mice via intraperitoneally administration of diphtheria toxin.
- CD23 KO mice: deficient in CD23 expression on all cells [54].
• DO11.10 mice: with a T cell receptor that recognizes OVA\textsubscript{323-339} on MHC-II I-A\textsuperscript{d} [142]. More than 95% of the T cells in these mice are OVA-specific.
• C\textmu{}13 mice: produce IgM with a point mutation in the \mu-heavy chain [94]. IgM in these mice is unable to activate complement.
• Cr2 KO mice: deficient in CR1/2 expression on all cells [105].

**Ex vivo CD4\textsuperscript{+} T-cell proliferation assay**
Mice were immunized and spleens were subsequently harvested at the indicated time points. Splenocytes (unfractionated or fractionated by magnetic-activated cell sorting or fluorescence-activated cell sorting) were used as APCs and co-cultured with CD4\textsuperscript{+} T cells from DO11.10 mice for 3 days. The proliferation of the CD4\textsuperscript{+} T cells was then measured. The CD4\textsuperscript{+} T cells were not labeled when \textsuperscript{3}H-thymidine incorporation into the cells was used as the readout with a beta counter. The CD4\textsuperscript{+} T cells were labeled with CFSE before adding into the cultures when the dilution of CFSE dye in cells was used as the readout with flow cytometry. No antigen was added to the in vitro cultures.

**In vivo CD4\textsuperscript{+} T-cell proliferation assay**
Mice were adoptively transferred intravenously (i.v.) with splenocytes or CD4\textsuperscript{+} cells isolated from DO11.10 mice on Day 0. On Day 1, mice were immunized i.v.. Three days later, spleens from the mice were harvested to analyze CD4\textsuperscript{+} T-cell proliferation by flow cytometry.

**Confocal microscopy**
Spleens were embedded in O.C.T. compound and frozen in liquid nitrogen. Seven to eight-micrometer non-consecutive sections were cut using a cryostat. The sections were air-dried on the slides, fixed in either acetone or 4% paraformaldehyde, and blocked with 5% horse serum. The sections were then stained with optimized concentrations of fluorescent antibodies and mounted in Fluoromount G prior to analysis.

**Enzyme-linked immunosorbent assay (ELISA)**
The concentration of mouse serum IgG specific for certain antigens was measured by ELISA. High-binding 96-well plates were coated with target antigens at 4°C overnight and blocked with 5% dry milk at room temperature for 2 h. Serum samples were then added to the plates and incubated overnight. Alkaline phosphatase-conjugated sheep anti-mouse IgG was applied for 3 h, and then the substrate solution was added to the plates before reading the absorbance at 405 nm.
Results and discussion

Paper I

IgE-mediated enhancement of CD4+ T cell responses in mice requires antigen presentation by CD11c+ cells and not by B cells

IgE-mediated enhancement of immune responses is dependent on CD23+ B cell binding IgE-complexed antigens via CD23 [25,47,54,66]. It was hypothesized that these antigen-bearing B cells present IgE-complexed antigens to CD4+ T cells because many studies have shown that B cells are able to prime naïve T cells [143-146] and, in particular, because both human and mouse B cells are able to process IgE-antigen complexes via CD23 and stimulate CD4+ T cells in vitro [67-70,147]. However, some other studies have shown that B cells cannot prime naïve T cells [71-73]. Moreover, a previous study has shown that CD23+ B cells transport IgE-antigen complexes into B-cell follicles [25]. This finding provides an alternative explanation for the role of CD23+ B cells in IgE-mediated enhancement, as antigen transporters. This inconsistency prompted us to question whether B cells are required for antigen presentation in IgE-mediated enhancement of CD4+ T-cell responses.

We first performed a series of ex vivo CD4+ T-cell proliferation assays to address the above question. Splenocytes obtained from mice 4 h after immunization with IgE-antigen complexes significantly enhanced CD4+ T-cell proliferation compared with cells from mice immunized with antigen alone. Importantly, depletion of CD19+ B cells from the splenocytes did not affect the stimulation of CD4+ T cells, whereas depletion of CD11c+ cells completely abolished the antigen-presenting capacity of the splenocytes. The antigen-presenting capacity was also lost when the splenocytes from CD11c-diphtheria toxin receptor (DTR) mice, which were depleted of CD11c+ cells by treatment with diphtheria toxin prior to immunization, were used as APCs. In addition, enriched CD11c+ cells from mice that received IgE-antigen complexes efficiently presented to CD4+ T cells.

In an alternative protocol using in vivo CD4+ T-cell proliferation assays, CD11c-DTR mice were depleted of CD11c+ cells by diphtheria toxin prior to being adoptively transferred with CD4+ T cells from DO11.10 mice. The enhancement of CD4+ T cell proliferation by IgE was again completely abolished in treated CD11c-DTR mice, whereas the enhancement was not affected in untreated CD11c-DTR mice. Both ex vivo and in vivo data showed that CD11c+ cells and not B cells were required for antigen presentation to CD4+ T cells in IgE-mediated enhancement of CD4+ T cell responses.

In addition, reconstitution of CD23 KO mice (I-Ak) with MHC-II incompatible B cells from C57BL/6 mice (I-Ab) rescued the enhancement of CD4+ T-cell proliferation by IgE in the CD23 KO mice. This result strengthened the conclusion that B cells do not present to CD4+ T cells because T cell responses are MHC-restricted.
Taken together, this study suggested a new pathway for antigen transportation and processing. IgE-antigen complexes bound to peripheral CD23+ B cells are transported into splenic follicles by recirculating follicular B cells. Subsequently, they are delivered to CD11c+ cells, which process and present antigenic peptides to CD4+ T cells and induce further proliferation. This pathway differs from that described in the in vitro studies showing that B cells take up IgE-antigen complexes via CD23 and present to CD4+ T cells [67-70,147]. In the in vitro assays, B cells were loaded directly with sufficiently high amounts of IgE-antigen complexes to stimulate T-cell proliferation. However, in our ex vivo assays, antigen-presenting cells were collected from mice 4 h after immunization. At that time, the B cells had likely already started to deliver antigens to CD11c+ cells and thus had limited amounts of antigen on their surface and were not able to prime naïve T cells. Therefore, our ex vivo assay is more physiological and probably represents the actual scenario that occurs in vivo. In parallel, depletion of CD11c+ cells in vivo in CD11c-DTR mice resulted in the loss of IgE-mediated enhancement of CD4+ T-cell proliferation, confirming that CD11c+ cells are important in this process. It is likely that the essential CD11c+ subpopulation is dendritic cells, which are known to be professional APCs and constitute the major part of the CD11c+MHC-II+ population in the mouse spleen. However, we cannot exclude the possibility that other CD11c+ cell populations, e.g., CD11c+ splenic macrophages, also play a role in this process because they are also depleted in response to diphtheria toxin in CD11c-DTR mice (reviewed in [148]). Finally, the ability of transferred MHC-II incompatible B cells to rescue the enhancement of CD4+ T-cell proliferation by IgE in CD23 KO mice further confirmed that the enhancement could not be explained by antigen presentation by B cells and suggested the presence of another cell population capable of presenting IgE-complexed antigens to T cells.

**Paper II**

**CD8α- conventional dendritic cells are the dominant cells presenting IgE-complexed antigen to CD4+ T cells**

In Paper I, we show that CD11c+ cells present IgE-complexed antigens to CD4+ T cells in vivo. Many subsets of CD11c+ cells have been identified in the mouse spleen according to their surface marker expression. Among these subsets, three are predominant: CD8α+ cDCs, CD8α- cDCs and plasmacytoid dendritic cells (pDCs) (reviewed in [149]). The aims of this study were to determine which subpopulation of CD11c+ cells plays the major role in presenting IgE-antigen complexes to CD4+ T cells and the mechanism underlying antigen presentation in the spleen.
Unlike in previous studies in which we used IgE anti-TNP and TNP-conjugated carrier proteins [25,45-47,63,66,150], in the present investigation, we established a system using OVA and monoclonal IgE anti-OVA. Similarly to IgE anti-TNP, IgE anti-OVA significantly enhanced both antibody and CD4\(^+\) T cell responses in vivo. Moreover, IgE-OVA complexes were found inside B cell follicles 0.5 h after administration and persisted until at least 4 h after immunization. Very little antigen was located in the follicles when OVA alone was administered.

Next, using fluorescence-activated cell sorting, we sorted CD8\(^{\alpha^+}\) cDCs, CD8\(^{\alpha^-}\) cDCs and pDCs from mouse splenocytes 4 h after immunization with OVA alone or with OVA together with IgE anti-OVA. These three subsets of dendritic cells were used as APCs in ex vivo CD4\(^+\) T-cell proliferation assays. CFSE-labeled OVA-specific CD4\(^+\) T cells isolated from DO11.10 mice were co-cultured with each subset of APCs for 3 days before analyzing their proliferation. We found that CD8\(^{\alpha^-}\) cDCs but not CD8\(^{\alpha^+}\) cDCs or pDCs from mice immunized with IgE-OVA complexes efficiently induced proliferation of CD4\(^+\) T cells. APCs from mice immunized with OVA alone primed CD4\(^+\) T cells poorly. 33D1 is a specific surface marker for CD8\(^{\alpha^-}\) cDCs. 33D1\(^-\)CD8\(^{\alpha^+}\) cDCs sorted from mice immunized with IgE-OVA showed remarkable stimulation of specific CD4\(^+\) T cells, whereas the same cell type sorted from mice immunized with OVA failed to achieve this effect. Therefore, CD8\(^{\alpha^-}\) cDCs and not CD8\(^{\alpha^+}\) cDCs or pDCs present IgE-complexed antigen to CD4\(^+\) T cells.

It has been reported that after stimulation, CD8\(^{\alpha^-}\) cDCs are able to migrate from the marginal zone bridging channels into the T-cell zone [151-153]. In support of this perspective, we found that CD8\(^{\alpha^+}\) cDCs migrated into the T-cell zone after immunization with IgE-OVA complexes. The migration was observed beginning at 4 h after immunization and peaked between 8 h to 24 h. Importantly, the ability to present antigens to CD4\(^+\) T cells was also highest when the splenocytes were obtained from the mice at 8 h after immunization.

In conclusion, we showed that CD8\(^{\alpha^-}\) cDCs were the dominant APCs presenting IgE-complexed antigen to CD4\(^+\) T cells. Our data indicated that CD8\(^{\alpha^-}\) cDCs migrated into the T-cell zone after immunization and might interact with B cells bearing IgE-antigen complexes at the B cell-T cell border. In that location, the CD8\(^{\alpha^-}\) cDCs captured antigens from the B cells and presented to CD4\(^+\) T cells. It is noteworthy that we found that CD8\(^{\alpha^-}\) cDCs also migrated into the T-cell zone in mice immunized with OVA alone. However, because OVA alone binds poorly to B cells and is not transported into B cell follicles, CD8\(^{\alpha^-}\) cDCs in mice that have received OVA alone are exposed to very little antigen on B cells despite migrating close to them. The migration of CD8\(^{\alpha^-}\) cDCs from the marginal zone bridging channel into the T-cell zone is probably driven by the up-regulation of CCR7 expression on their surface [153]. However, the origination of the activation signal provid-
ed to the CD8α− cDCs after immunization with OVA or IgE-OVA complexes is not understood. In addition, we showed that CD8α− cDCs did not express CD23. Hence, they must acquire IgE-antigen complexes via other pathways. There are some possible mechanisms to explain how CD8α− cDCs obtain antigens from B cells, e.g., direct phagocytosis of neighboring antigen-bearing B cells, which has been suggested to be the mechanism by which CD8α− cDCs capture antigen [74], or uptake of exosomes secreted by B cells containing antigenic peptides [154]. However, the exact mechanism by which CD8α− cDCs acquire antigens requires further investigation.

Paper III

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

As mentioned previously, complement is important for the induction of antibody responses, but normal antibody responses do not require natural IgM activation of complement [94]. However, complement is thought to be involved in the enhancement of antibody responses by specific IgM [50,52]. These inconsistent observations prompted us to re-investigate whether the enhancement of antibody responses by specific IgM is complement-dependent.

Antigen-specific wild-type IgM and Cμ13 IgM were purified from immunized BALB/c or Cμ13 mice. It has been shown that specific wild-type and Cμ13 IgM have the same molecular size and ability to agglutinate SRBC, as well as a similar half-life [94]. In addition, we found that wild-type and Cμ13 IgM bound equally well to FcμR. However, wild-type but not Cμ13 IgM induced the deposition of C3 on SRBC in peripheral blood as rapidly as 1 min after immunization. As expected, specific wild-type IgM induced a remarkable enhancement of both antibody and germinal center responses when administered together with its specific antigens (SRBC and KLH), whereas Cμ13 IgM failed to achieve this effect.

Another question is whether specific IgM can enhance CD4+ T-cell responses. Wild-type BALB/c mice were adoptively transferred with splenocytes from DO11.10 mice. After 1 day, the mice were immunized with SRBC-specific wildtype IgM together with or without SRBC-OVA conjugate. Three days later, the splenocytes obtained from the mice were analyzed for CD4+ T-cell activation and proliferation by flow cytometry. We found that specific IgM enhanced neither the proliferation nor the activation of specific CD4+ T cells compared with antigen alone, although the antibody responses against both SRBC and OVA were enhanced.

To summarize, specific IgM enhanced humoral but not CD4+ T cell responses and the enhancement required the activation of complement by the IgM. We showed that C3 was rapidly deposited on antigen after immuniza-
tion with SRBC and specific wild-type IgM but not with SRBC and specific C\textsubscript{\(\mu\)13} IgM. Because C3 fragments are ligands for CR1/2, it is likely that the enhancement was due to an increased binding of complement-opsonized antigen to CR1/2, which is highly expressed on marginal zone B cells and FDCs in mice. Using bone marrow chimeric mice, it has been demonstrated that optimal enhancement of antibody responses by specific IgM requires the expression of CR1/2 on both marginal zone B cells and FDCs [111]. Thus, our results are consistent with previous studies [23,24,50] showing that the mechanism underlying complement-dependent IgM-mediated enhancement of humoral responses is characterized by marginal zone B-cell transportation of complement-opsonized IgM-antigen complexes via CR1/2 into B-cell follicles. Subsequently, IgM-antigen-complement complexes are captured by CR1/2-expressing FDCs in the B-cell follicles and displayed to specific B cells.

FDCs can retain antigens on their surface, which is important for germinal center development and also enhances B-cell activation ([5] and reviewed in [155]). Long-term retention of antigens, recycling and repeated display of antigen on the surface of FDCs increases the chances that antigen-specific B cells will recognize the antigens and become activated, leading to enhanced antibody and germinal center responses. If the specific IgM is unable to activate complement to opsonize IgM-antigen complexes, there will be only marginal binding of the immune complexes to CR1/2 on marginal zone B cells or later on FDCs. As a consequence, there will be little enhancement of antibody or germinal center responses, as supported by our data showing that C\textsubscript{\(\mu\)13} IgM had a greatly reduced capacity to enhance antibody responses and germinal center reactions. There is direct evidence that specific IgM increases the deposition of antigens such as BSA and virus-like particles on FDCs in splenic follicles, although it has not been shown whether antibody responses against those antigens are enhanced in parallel [23,24]. We have utilized many methods to visualize the localization of SRBC and KLH after administration together with IgM, but we have failed to detect any deposition of antigen in B-cell follicles at time points ranging from 10 min to 24 h (data not shown). Nevertheless, the antibody responses were remarkably enhanced by IgM. It is likely that the labeled antigens are degraded very quickly and digested into small fragments that cannot be detected.

Our data showed that CD4\textsuperscript{+} T-cell responses were not enhanced by IgM. This finding suggested that the increased deposition of antigens on FDCs when administered together with specific IgM might concentrate sufficient antigens to allow B cells in the follicles to undergo affinity maturation and survive. Therefore, no extensive T\textsubscript{FH} cell-based selection is needed, and an equivalent number of activated CD4\textsuperscript{+} T cells in response to IgM-complexed antigen compared to those in response to antigen alone are sufficient to enhance the production of high affinity antibodies.
Paper IV

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

Specific IgG3 enhances antibody responses to soluble antigens when administered together with those antigens [38]. This enhancement is severely impaired in C3-depleted mice and in Cr2 KO mice [38] and therefore is dependent on complement activation. CR1/2 are expressed on both B cells and FDCs in mice [156]. In the present study, we investigated how CR1/2 expression on those two cell types facilitates the enhancement of antibody responses by IgG3 by comparing wild-type and Cr2 KO mice.

In contrast to IgE-antigen complexes, IgG3-antigen complexes did not bind to peripheral circulating B cells in the blood but to marginal zone B cells via CR1/2 in the spleen 2 h after immunization. IgG3 also significantly increased the localization of antigen in B-cell follicles in wild-type but not in Cr2 KO mice. In mice treated with FTY720 prior to immunization, which consequently had dislocated marginal zone B cells, the increased binding of IgG3-antigen complexes to marginal zone B cells was severely impaired and the enhancement of antigen localization in B-cell follicles by IgG3 was abolished. This finding suggested that marginal zone B cells transported IgG3-antigen complexes into B cell follicles via CR1/2. The antigens transported into the follicles co-localized with FDCs and were retained for at least 8 h after immunization. Consistent with these observations, IgG3 enhanced germinal center responses in wild-type mice; however, the enhancement was severely impaired in Cr2 KO mice.

Because both B cells and FDCs express CR1/2, whether CR1/2 expression is more important on one cell type versus the other remains unknown. Thus, four groups of bone marrow chimeric mice were created from wild-type and Cr2 KO mice with CR1/2 expression on both B cells and FDCs (WT→WT), on either B cells (WT→Cr2 KO) or FDCs (Cr2 KO→WT), or on neither cell types (Cr2 KO→Cr2 KO). As expected, IgG3 enhanced antibody responses in WT→WT mice, and the enhancement was markedly impaired in Cr2 KO→Cr2 KO mice. Interestingly, in both WT→Cr2 KO and Cr2 KO→WT mice, the enhancement was reduced compared with WT→WT mice but higher compared with Cr2 KO→Cr2 KO mice. Therefore, the expression of CR1/2 on both B cells and FDCs was important for an optimal enhancement of antibody responses by IgG3.

In summary, our data suggested that marginal zone B cells bound to complement-opsonized IgG3-antigen complexes in the marginal zone via CR1/2 and transported them into splenic follicles via their constant shuttling. The IgG3-antigen complexes were then captured by FDCs via CR1/2 and presented to germinal center B cells, which induced the ensuing enhancement of antibody responses and germinal center reactions. This mechanism is similar to the mechanism underlying IgM-mediated enhancement of antibody re-
sponses discussed in Paper III. Moreover, IgG3 shares other similarities with IgM in regulating immune responses. Neither IgM- nor IgG3-mediated enhancement of antibody responses requires Fc receptors, but both processes are dependent on complement [38,40]. Neither IgM nor IgG3 stimulates the enhancement of CD4⁺ T-cell proliferation [40,157]. However, specific IgM enhances antibody responses against particulate and large antigens, whereas IgG3 enhances antibody responses against small and soluble antigens and suppresses responses against particulate antigens [38,42,158]. A potential explanation for these observations is that IgM is more likely to undergo a conformational change from a flat pentamer to a ‘mushroom’ shape to activate complement [115], after binding to a much larger antigen such as SRBC. However, IgG3 can self-aggregate through Fc-Fc interactions [135,137], allowing it to achieve sufficient affinity to activate complement. Therefore, IgM and IgG3 may complement but not compete with one another to enhance antibody responses against different types of antigens.
Concluding remarks and future perspectives

Antibodies administered together with their specific antigen can up-regulate or down-regulate immune responses against this antigen. In this thesis, the mechanisms underlying the enhancement of immune responses by specific IgE, IgM, and IgG3 are investigated. Some outstanding questions listed in Table 1 have been addressed, and a more complete overview of IgE, IgM, and IgG3-mediated enhancement is summarized in Table 2.

Table 2. The enhancement of immune responses by IgE, IgM, and IgG3 (post-thesis)

<table>
<thead>
<tr>
<th>Isotype</th>
<th>Enhancing antibody/CD4$^+$ T cells responses</th>
<th>Antigen types regulated</th>
<th>Requiring the isotype to activate complement</th>
<th>Immune complexes transported into B-cell follicles by</th>
<th>Immune complexes presented to CD4$^+$ T cells by</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgE</td>
<td>Yes/Yes</td>
<td>Small soluble proteins (OVA, BSA)</td>
<td>No</td>
<td>CD23$^+$ follicular B cells</td>
<td>CD11c$^+$ cells (I)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Particulate antigens (SRBC, malaria parasites)</td>
<td>Yes (III)</td>
<td>CR1/2$^+$ Marginal zone B cells</td>
<td>CD8α$^+$ cDCs (II)</td>
</tr>
<tr>
<td>IgM</td>
<td>Yes/No (III)</td>
<td>Large soluble proteins (KLH)</td>
<td></td>
<td></td>
<td>/</td>
</tr>
<tr>
<td>IgG3</td>
<td>Yes/No</td>
<td>Small soluble proteins (OVA, BSA)</td>
<td>Yes</td>
<td>CR1/2$^+$ Marginal zone B cells (IV)</td>
<td>/</td>
</tr>
</tbody>
</table>

The enhancement of immune responses, antibody and/or CD4$^+$ T cells responses, by IgE, IgM, or IgG3 is indeed caused by an increase in the transportation of antibody-complexed antigens into splenic follicles. Different antibody-antigen complexes utilize distinct types of antigen-transporting cells. IgE-antigen complexes are transported by follicular B cells via CD23, whereas IgM- and IgG3-antigen complexes are transported by marginal zone B cells via CR1/2. The mechanisms underlying IgE-, IgM-, and IgG3-complexed antigens transportation into B-cell follicles in the spleen are summarized in Figures 10 and 11.
Figure 10. Transportation of IgE-complexed antigens into B-cell follicles, and antigen presentation to specific CD4\(^+\) T cells. 1) Circulating B cells bound to IgE-antigen complexes via CD23 transport antigens into B-cell follicles. 2) CD8\(\alpha\) cDCs receive an activation signal via an unknown mechanism and migrate from marginal zone bridging channels to the T-cell zone. 3) CD8\(\alpha\) cDCs acquire antigens from antigen-bearing follicular B cells at the B cell-T cell border. 4) CD8\(\alpha\) cDCs process the antigens and present them on MHC-II. 5) CD8\(\alpha\) cDCs present antigenic peptides to antigen-specific CD4\(^+\) T cells. 6) Antigen-specific CD4\(^+\) T cells become activated and proliferate.

Figure 11. Transportation of IgM- and IgG3-complexed antigens into B-cell follicles. 1) Complement-opsonized IgM- or IgG3-complexed antigens bound to marginal zone B cells (MZ B) via CR1/2 are transported into B-cell follicles via shuttling of the marginal zone B cells. 2) Complement-opsonized antigens are deposited on FDCs. 3) FDCs display antigens to follicular B cells and induce antibody responses.
IgE-antigen complexes are transported into splenic follicles by recirculating follicular B cells via binding to their CD23. IgE-antigen complexes that are bound to CD23 are likely to be protected against rapid degradation. However, in vivo, B cells do not appear to process all of the CD23-bound IgE-antigen complexes and present antigenic peptides on their MHC. This probably provides a good opportunity for CD8α− cDCs, which migrate into the T-cell zone following immunization, to capture antigens from B cells. CD8α− cDCs then present the IgE-complexed antigens to CD4+ T cells. Subsequently, the specific CD4+ T cells begin to proliferate extensively, potentially leading to an increased number of T_{FH} cells. Because T_{FH} cells are one of the limiting steps in antibody production, it is likely that additional T_{FH} cells will result in a greater number of surviving B cells and higher levels of antibody production. This may explain why both antibody and CD4+ T-cell responses are enhanced by IgE.

However, when the antibody involved is able to activate complement, e.g., IgM or IgG3, immune complexes opsonized by complement are transported into splenic follicles by marginal zone B cells via CR1/2 and then deposited on CR1/2− FDCs for display to specific follicular B cells, which later undergo positive selection and produce antibodies. It has been shown that FDCs mainly express CR1 and that marginal zone B cells mainly express CR2 [113]. It is possible that CR1 possesses a higher complement binding affinity than CR2, and thus, FDCs use CR1 to capture opsonized antigens from marginal zone B cells. CD4+ T cells are needed for IgM- and IgG3-mediated immune enhancement to T-dependent antigens, but they do not expand. There remains no conclusive explanation for why CD4+ T-cell responses are not enhanced by IgM or IgG3. However, it is likely that the long-term retention and recycling of intact antigens on the surface of FDCs provides additional opportunities for specific B cells to recognize antigens. Therefore, it may maximize the number of specific B cells generating large amounts of peptide-associated MHC molecules among the total B-cell repertoire. As a consequence, the number of surviving B cells increases after scanning by T_{FH} cells because B cells with large amounts of peptide-associated MHC molecules will receive sufficient help from T_{FH} cells and be selected. Hence, the concentration of antigens on FDCs appears to be important for the enhancement of antibody responses and does not require addition assistant from CD4+ T-cells.

Although the mechanism by which IgE, IgM, and IgG3 enhance immune responses is clearer, some outstanding questions remain. First, how CD8α− cDCs capture IgE-antigen complexes from antigen-bearing B cells is not known. It is possible that CD8α− cDCs simply endocytose part of the B-cell membrane together with antigen. Ongoing studies of adoptively transferred fluorescently-labeled CD23+ B cells into CD23 KO mice may solve this question. Second, CCR7 expression appears to play a role in the migration of CD8α− cDCs to the T-cell zone and in the distribution of B cells bearing IgE-
complexed antigens along the B cell-T cell border. It will be interesting to determine whether the blockade of CCR7 interferes with the migration of CD8α⁺ cDCs and antigen-bearing B cells, and thus interferes the enhancement of T-cell proliferation by IgE. Third, our data and those of others indicate that IgM facilitates the transportation of specific particulate and large antigens into splenic follicles, but the localization of large antigens such as SRBC in the follicles has not been directly visualized. Additional techniques could be utilized to achieve this goal, e.g., proximity ligation assays. Finally, because FDCs are able to retain IgG3-antigen complexes visibly by confocal microscopy until at least 8 h after immunization, which probably facilitates the maintenance of memory responses, it will be interesting to investigate whether memory responses are also enhanced by IgG3.

The enhancement of immune responses by specific antibodies can play important biological roles. For instance, provided that a person has encountered certain pathogens and possesses circulating pathogen-specific antibodies, a subsequent invasion by this pathogen will cause the existing specific antibodies to form immune complexes with the pathogen and start to induce elevated antibody responses. This process leads to an accelerated clearance of the pathogen because more complement-opsonized immune complexes are formed (if IgM or IgG3 is dominant) or more effector T cells are expanded (if IgE is dominant).
抗体，又称免疫球蛋白，它由 B 淋巴细胞分泌产生，是免疫系统的重要组成部分之一。抗体能够识别和中和外来物质，所以对于抵御细菌或者病毒入侵有着不可或缺的作用。这些外来物质被称作抗原，而抗原上能够被抗体识别的部分，叫做抗原表位。就像一把钥匙能开一把锁一样，每一个抗体都有自己特异性的抗原，相互特异的抗原和抗体能够非共价结合，形成免疫复合物。抗原特异性抗体能够增强或者抑制机体的免疫反应，这种现象被称作抗体介导的免疫调节作用。

抗体在结构上由重链和轻链组成，重链决定了抗体的种型。胎生的哺乳动物有五种抗体，IgA, IgD, IgE, IgG 和 IgM。其中，IgG 又存在若干种亚型。人类的 IgG 亚型分为 IgG1, IgG2, IgG3 和 IgG4 四种，而小鼠的 IgG 亚型则略有不同，分为 IgG1, IgG2a, IgG2b 和 IgG3。小鼠和人类的基因组有高度的相似性，因此是最广泛使用的实验动物模型。

脾脏是产生抗体的主要器官之一，小鼠的脾脏在生理构造上与人类的脾脏也非常相似。本论文以小鼠为模型，主要研究了在小鼠脾脏中发生的 IgE, IgM 以及 IgG3 介导的免疫增强作用。

在脾脏中，免疫反应主要产生于一种叫做滤泡的微结构中。因此，IgE, IgM 以及 IgG3 介导的免疫增强作用有一个最大的共同点，就是由于有媒介将这些抗体与抗原形成的免疫复合物高效地转运到了滤泡中，提高了滤泡中抗原的浓度，因此才增强了免疫反应。IgE 形成的免疫复合物可以结合 B 细胞表面受体 CD23，从而被这些在外周血和脾脏滤泡间循环的 B 细胞转运到脾脏滤泡中。在那里，IgE 免疫复合物被转移到了 T 细胞区中的树突状细胞捕获，递呈给 T 细胞并使其激活，因此，IgE 的免疫复合物可以使 T 细胞增殖。然后，增殖的 T 细胞又能给予脾脏内原有的滤泡 B 细胞以帮助，使它们激活并产生抗体。IgM 和 IgG3 的免疫复合物则利用了另一种细胞来达到转运的作用，这种细胞叫做脾边缘带 B 细胞。脾边缘带 B 细胞表面有补体受体，而 IgM 和 IgG3 的免疫复合物表面通常同时结合有补体，因此，脾边缘带 B 细胞能够与通过血液流到边缘带的 IgM 和 IgG3 免疫复合物结合并且把它们转运到脾脏滤泡中，从而增强免疫反应。但是，这两种抗体的免疫复合物不能使 T 细胞增殖，因为它们不会被 T 细胞区中的树突状细胞捕获，而是会被分布在滤泡 B 细胞区中的树突状细胞识别，直接递呈给周围滤泡 B 细胞，所以，IgM 和 IgG3 的免疫复合物只能使 B 细胞抗体的产生增加，而不能增强 T 细胞的反应。

研究抗体介导的免疫增强作用，能够让我们更好地理解其中的调节机制，找到制约靶点，从而合理地设计出能增强免疫反应的疫苗以及研制出能更有效地控制免疫反应发生的药物。
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