Doctoral Thesis

Virus-like particles as a model antigen: Studies of early and long-term B cell responses

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VIRUS-LIKE PARTICLES AS A MODEL ANTIGEN:
STUDIES OF EARLY AND LONG-TERM B CELL RESPONSES

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SUMMARY

The B cell compartment of the immune system is endowed with the capacity to respond to antigen encounter with rapid onset of antibody production and long-term maintenance of induced specific antibodies. This ability stems from the coexistence of distinct B cell populations, which either engage in fast, early antibody secretion or mediate more long-lasting antibody responses through the generation of effectors of humoral memory, a process which is closely regulated. Marginal zone B cells, together with B1 B cells, provide a first line of defense against pathogens accessing the body through the bloodstream or the gastrointestinal tract, while the more numerous follicular B cells are involved in germinal center reactions and in the establishment of long-term B cell memory.

B cells from each of these subsets are activated in mice by intravenous injection of virus-like particles, the model antigen used in the studies presented in this thesis. The response of marginal zone B cells to virus-like particles, which is the subject of the first two parts of the result section, only represents a minor contribution to the overall B cell response. Upon activation these B cells remain associated with the marginal zone, where they undergo rapid isotype-switching and afford early local secretion of IgG antibodies of remarkable diversity. This rapid production of a broad spectrum of IgG antibodies might be responsible for the opsonization of pathogens migrating through the spleen and thus contain infections even before follicular B cells are engaged and become the main players of the antibody response. Although the repertoire of follicular B cells responding to virus-like particles does not appear to be more diverse than that of specific B cells of the marginal zone compartment, only follicular B cells take an important part in germinal center reactions. In germinal centers, B cells can differentiate along two distinct pathways: they are either recruited to the memory B cell population or they join the long-lived bone marrow plasma cell pool, which sustains long-term antibody secretion. Some of the requirements for the induction and maintenance of these two long-term B cell populations in response to immunization with virus-like particles are addressed in the third and fourth part of the results section. The results of these studies indicate that the interaction with complement-coated antigen is essential for differentiation of germinal center B cells into the precursor population of bone marrow plasma cells. In contrast, memory B cell development is independent of signals mediated by complement receptors. However, both populations require antigen retained on follicular dendritic cells for efficient induction. At later stages, the response is largely antigen-independent and is
maintained by a long-lived but gradually declining population of antibody producing cells residing in the bone marrow. Thus, the study of early and long-term B cell responses to virus-like particles has confirmed and extended the role of marginal zone B cells early after antigen exposure and has provided new insight into the mechanisms of regulation of humoral memory.
ZUSAMMENFASSUNG


1 GENERAL INTRODUCTION

1.1 Discovery of the humoral immune system

In the 1800s, the mean life expectancy was less than half than what it is today, and infectious diseases were the most prevalent cause of early death. The fear of epidemics and infections instigated many attempts to deliberately induce immunity and led to the development of vaccination procedures. The origin of vaccination has been attributed to Edward Jenner, who in the 18th century discovered that protection against human smallpox could be obtained by inoculation with the related cowpox virus (1). His finding was extended to other diseases by Louis Pasteur, but only in 1890 were the first insights into the protective mechanism induced by vaccination provided. By studying immunity to diphtheria and tetanus, Emil von Behring and Shibasaburo Kitasato discovered that “the blood of the tetanus-immune rabbit possesses properties capable of destroying tetanus toxins”. These “properties” were further reported to be “evident in the extravascular fluid as well as in the cell-free serum of the blood” and to be “of such lasting nature that they also remain effective in the organism of other animals” (2). This serum component of immunized animals, which could transfer specific immunity to other animals, was later termed antibody. After von Behring and Kitasato’s discovery of antibodies, another 60 years passed before the cells secreting the antibodies were identified by Astrid Fagraeus as “plasma cells” (3), long known for their prominent cytoplasm and well-developed endoplasmic reticulum (ER). Since then, antibodies and the cells responsible for their production have been extensively characterized. This introduction will outline the knowledge which has been gained on the induction of antibody responses and on the effector cell populations generating these immune responses.

Because body fluids containing antibodies were once known as humors, immunity mediated by antibodies was called humoral immunity. Humoral immune responses are mediated by B lymphocytes, which are capable of generating antibody responses of remarkable diversity thereby greatly contributing to the enormous array of antigens to which the immune system is able to respond. The B cell compartment is endowed with the ability to mount a wide variety of responses which provides rapid and long-lasting protection against many different pathogenic organisms.
1.2 Generation of mature B cells

The development of B lymphocytes is a highly regulated process whereby functional peripheral subsets are produced from hematopoietic stem cells, in the fetal liver before birth and in the bone marrow afterwards. This process involves several critical stages for cells to survive and differentiate, finally establishing the mature B cell repertoire. The earliest committed B cell precursors in the bone marrow are pre-pro B cells (4), which can be recognized by expression of the B220 isoform of CD45, a marker expressed by B cells from the very early B-lineage precursors to mature B cells (5). These very early B-lineage cells have their immunoglobulin (Ig) loci in germline configuration. In their germline configuration, the variable domains of the immunoglobulin heavy chain (IgH) and κ or λ immunoglobulin light chain (IgL) are composed of clusters of gene segments encoding the variable (V), diversity (D, in heavy chain only) and joining (J) exons (6). For heavy chain variable regions, a few hundred V segments, 12 D segments and 4 J segments are thought to be encoded in the mouse genome (7). Variable region V and D genes have been grouped into families according to sequence homology (8). Developing B lineage cells in the bone marrow assemble the exons that encode IgH and IgL variable regions from V, D and J segments, through a process that is known as V(D)J recombination (6) (Figure 1.1). This site-directed, antigen-independent recombination event is initiated by the sequence-specific V(D)J recombinase, which consists of the recombination-activating gene 1 and 2 (RAG1 and RAG2) proteins. The RAG proteins recognize and bind to recombination signal sequences (RSSs) that flank the gene segments to be rearranged and introduce a DNA double-strand break (9, 10). Joining of the DNA ends is carried out by components of the non-homologous end-joining machinery (10). During the final phase of V(D)J recombination, the lymphoid-specific terminal deoxynucleotidyl transferase (TdT) increases joining diversity by adding non-template nucleotides to processed coding ends (11). The addition of these N nucleotides, together with the random combination and imprecise joining of the numerous V, D, and J segments, generates a primary antibody repertoire of considerable diversity. Hence, rearrangement of Ig genes affords the immune system with the ability to encode an enormous repertoire of antigen receptors with a relatively modest investment of genetic capacity. Rearrangement of Ig DNA begins with the junction of D and J segments of the μ heavy chain (D-Jμ) at the pro-B cell stage (4). Pro-B cells start to express CD19, a B-cell restricted surface molecule whose expression characterizes all later B lineage stages (12). Subsequently, VH-DJH rearrangement is initiated, which results in expression of the pre-B receptor, composed of
a μ heavy chain a surrogate light chain (5). Rapid cell proliferation occurs at this early pre-B cell stage. After clonal expansion, pre-B cells undergo light chain rearrangement, which leads to assembly and surface expression of the B cell antigen receptor (BCR) (13) consisting of a μ heavy chain and a κ or λ light chain. These newly formed bone marrow cells leave the bone marrow as immature B cells and continue their maturation process in peripheral lymphoid tissues.

Out of the $2 \times 10^7$ immunoglobulin M (IgM)-positive cells that develop daily in the bone marrow of the mouse, 10% are thought to reach the spleen and only 1-3% are believed to enter the mature B cell pool (15, 16). After migrating from the bone marrow to the spleen, immature B cells pass through two transitional stages, which are known as transitional type 1 (T1; newly formed B cells) and transitional type 2 (T2), before differentiating into mature B cells (17). Specific patterns of surface marker expression have been used to distinguish these stages of differentiation. As recent emigrants from the bone marrow, T1 cells show a phenotype that is closely related to that of immature bone marrow B cells: T1 cells express no or low levels of immunoglobulin D (IgD), CD21 and CD23, but high levels of membrane IgM.

Figure 1.1 Rearrangement of the Ig heavy chain locus (adapted from Chaudhuri J. et al. (14)).
GENERAL INTRODUCTION

Only a small percentage of T1 cells proceeds to the T2 stage; cells at this stage of differentiation still express high levels of mlgM, but have also upregulated mlgD, CD21 and CD23 (17). Mature B cells coexpress IgM and IgD (18); based on the relative expression levels of IgM and IgD and other phenotypic characteristics, as well as on their localization properties, mature B cells can be divided into several subpopulations.

1.3 B cell subsets

B cells reaching the mature long-lived pool are heterogeneous: some belong to the B1 B cell subset, some are enriched in the marginal zone compartment of the spleen, whereas others recirculate primarily between the B cell follicles of secondary lymphoid organs. B1 cells arise early in ontogeny, predominantly from fetal liver stem cells, and reside in the peritoneal and pleural cavity after the prenatal period, where they form a major reservoir of self-renewing B cells (19). Despite forming a major population of B lineage cells in the peritoneal cavity, B1 cells are rare in spleen, where they only represent about 2% of B cells (20). B1 cells are characterized by a unique surface phenotype (Table 1.1): they express high mlgM, low mlgD, low or no CD23 and are CD43 (20-23). B1a cells can further be distinguished by the expression of CD5, and all B1 cells in the peritoneal cavity express CD11b, in contrast to B1 cells in the spleen (20, 24, 25). B cells belonging to the B1 lineage have been reported to produce germline-encoded antibodies lacking N nucleotides, due to the absence of TdT activity in these cells, and the secreted antibodies are frequently polyreactive and of low affinity (26-28). Their repertoire is thought to be restricted and skewed towards reactivity to bacterial carbohydrate antigens and autoantigens (29). Functionally, B1 cells contribute to the production of serum IgM and are the major source of natural antibodies, which mediate immediate resistance to infection (30, 31). In addition, responses to several T cell-independent antigens are prominent among B1 cells, a feature that attributes a central role to this population in the response to gut/peritoneum and blood-borne bacterial antigens, as discussed in a later section.
Body cavities are not the only location harboring a static B cell population: after maturation, some B cells also join the sessile marginal zone B cell compartment. The marginal zone of the spleen is located at the border between the red pulp, which consists of vascular sinusoids, and the white pulp, the lymphoid region of the spleen composed of T cell areas and flanking B cell follicles. From the white pulp the marginal zone is separated by the marginal sinus (Figure 1.2) (32). Most of the blood that flows through the spleen is released at the marginal sinus, from where it can percolate through the marginal zone into the red pulp before returning to the circulation (32). Thus, cells in the marginal zone are continuously exposed to large amounts of blood and to any antigen accessing the bloodstream. The marginal zone consists of a meshwork of reticular cells. Within this reticular framework, besides B cells, dendritic cells and two distinct macrophage populations are localized. Marginal zone macrophages, which can be identified by the ER-TR9 and MARCO (macrophage receptor with collagenous structure) antibodies, are interspersed in the marginal zone with B lymphocytes, whereas metallophilic macrophages are situated at the inner side of the sinus (Figure 1.2) (33). The latter population can be recognized by the MOMA-1 antibody, a marker commonly used to identify the marginal zone region in histological sections (34). B lymphocytes of the marginal zone compartment comprise 5-10% of the splenic B cell
population. These intermediate-sized lymphocytes have a distinct phenotype characterized by high surface expression of IgM and CD21 and low levels of IgD and CD23 (Table 1.1) (22, 35-37). In addition, marginal zone B cells can be distinguished by the expression of the non-classical major histocompatibility complex (MHC) molecule CD1d (38) as well as CD9 (39). Compared to recirculating follicular B cells, marginal zone B cells also have a higher expression of the integrins LFA-1 and αβ_1 (40). The interaction between these integrins and their ligands have been shown to play an important role in the retention of marginal zone B cells in the marginal zone (40). B cell localization in the splenic marginal zone is further dependent on signaling through the sphingosine 1-phosphate receptor 1, which is expressed at high levels on marginal zone B cells as is the sphingosine 1-phosphate receptor 3.

The majority of blood-borne lymphocytes that enter the marginal sinus presumably do not possess the adhesive properties that permit lodging in the marginal zone and therefore travel through the marginal zone to the white pulp. Mature naïve B lymphocytes that possess the ability to recirculate through secondary lymphoid organs belong to the follicular B cell subset. Follicular B cells are the predominant B cell population, they inhabit lymphoid follicles of the spleen and lymph nodes and have a life-span of about 4 month (41). These small B lymphocytes can phenotypically be distinguished from B cells belonging to the marginal zone.
compartment, owing to their high expression of IgD and CD23, low levels of surface IgM and intermediate CD21 expression (Table 1.1) (22, 35-37).

Although follicular and marginal zone B cells represent two distinct subsets of B cells, it has been shown that recirculating cells collected from the thoracic duct or lymph nodes can repopulate the marginal zone (42, 43). This has led to the conclusion that the immediate precursors of marginal zone B cells are recirculating cells. However, the gradual decline of follicular B cells following the conditional knock-out of RAG-1, in contrast to virtually unchanged marginal zone and B1 B cell numbers, has suggested that marginal zone B cells, like B1 B cells, are self-renewing (41).

It is still unclear whether the B cell belonging to the different subsets are derived from a predetermined precursor in the bone marrow or whether they commit to joining a specific compartment in the periphery. There is increasing evidence, however, that the BCR plays an important role in this B lymphocyte cell-fate decision process. BCR expression is not only required for maintenance of all peripheral B cell populations (44), its specificity and signaling are also involved in commitment of B cells to the follicular, marginal zone or B1 subset. Studies in IgH chain transgenic mice have shown that transgenic B cell clones preferentially segregate into a specific B cell compartment. In addition, certain specificities have been found to be enriched in the B1 population (45). These findings have led to the suggestion that BCR composition determines commitment of B cells to a distinct subset (46-50). The role for BCR signaling in lineage commitment of B cells has been revealed by the analysis of mutant mice deficient in negative or positive regulators of B cell signaling (47, 51-53). These mice showed an altered selection of B lymphocytes into the different B cell compartments. Based on these studies, the proposition has been put forward that weak BCR signaling favors development of marginal zone B cells whereas higher BCR strength promotes generation of follicular B cells; even stronger signals derived from the antigen receptor are required for development of a B1 B cell population (54).

The phenotypic and topographic segregation of B lymphocytes into subsets is paralleled by functional differences between B cells from each compartment. As discussed in the next section, marginal zone and B1 B cells have the capacity to rapidly generate effector cells in the early stages of the immune response to T cell-independent particulate antigens. In contrast, recirculating follicular B cells provide a large repertoire from which antigen-specific cells are recruited for long-term T cell-dependent antibody responses, germinal center formation and generation of B cell memory.
1.4 Activation of B cells

According to whether antigens require the presence of T cell help to induce antibody responses, they can be assigned to two major categories: T cell-dependent or T cell-independent. The pathway leading to activation of B cells and Ig secretion, as well as the characteristics of the elicited B cell responses, differs consistently between these two classes of antigens. Most pathogenic microorganisms possess features of both types of antigens. Hence, the ability of the humoral immune system to mount rapid T cell-independent antibody responses followed by long-term, high affinity, T cell-dependent antibody production provides efficient protection against most pathogens.

**T cell-independent B cell responses**

Antigens regarded as T cell-independent fall into two groups. The first group of antigens share the characteristic of being mitogenic for B cells and therefore induce polyclonal, non-antigen-specific B cell responses (55, 56). An example of this kind of antigen is the bacterial cell wall component lipopolysaccharide (LPS). Antigens that are expressed on the surface of pathogens in an organized, highly repetitive form can also activate B cells in the absence of T cell help. This class of immunogens activates B cells by cross-linking of the BCR (57). The multivalency of such antigens enables them to induce the formation of clusters of about 10 to 20 antigen receptors (58), and this extensive cross-linking of mIg induces high levels of activation at relatively low antigen concentrations (59). This family of T cell-independent antigens comprises bacterial capsular and cell wall polysaccharides, viral glycoproteins, synthetic polymers and their haptenated counterparts (60-67). Despite their chemical diversity, these antigens have in common the highly repetitive organization of their epitopes, which are expressed in a rigid two-dimensional spacing of 5-10 nm (61, 68). In addition to the organized and repetitive polysaccharide moieties, bacteria carry a number of distinct polyclonal B cell activators, such as LPS, lipoproteins, porins and DNA containing a specific unmethylated CpG motif. These bacterial constituents, which engage Toll-like receptors on B cells, have been proposed to synergize with mIg cross-linking in T cell-independent B cell responses (69, 70). Similarly, cytokines produced by T cells, natural killer (NK) cells, macrophages and dendritic cells have been suggested to play a permissive role in activation of B cells by T cell-independent antigens (57, 70).

There is compelling evidence that the spleen plays an essential role in mounting responses to encapsulated bacteria and to T cell-independent antigens. In humans, splenectomy results in
increased susceptibility to encapsulated bacteria (71, 72). Similarly, B cell responses to various T cell-independent antigens are impaired in splenectomized mice and rats (73, 74). Marginal zone B cells have strongly been implicated in the response to these antigens. Notably, the ability to mount immune responses against polysaccharide antigens coincides with the appearance of a mature marginal zone compartment (75-77), which is delayed until the age of about two years in humans and until 2-3 weeks after birth in rodents (78, 79). More direct evidence for a role of marginal zone B cells in T cell-independent responses was obtained through the study of mice lacking marginal zone B cells, such as Pyk-2-deficient or NF-κBp50-deficient mice (80, 81). These mice were shown to be defective in the induction of B cell responses to repetitive polysaccharide antigens or in their ability to respond to encapsulated *Streptococcus pneumoniae* bacteria (80, 82).

Studies with T cell-independent particulate antigens have revealed that marginal zone and B1 B cells generate the rapid wave of IgM-producing plasmablast following exposure to blood-borne bacteria (83). Marginal zone B cells possess the unique capacity to rapidly proliferate and differentiate into plasma cells in response to T cell-independent stimuli, which permits them to react faster than the bulk of splenic follicular B cells (50, 83, 84). This ability may be due to their partially activated state, characterized by elevated resting levels of the transcriptional regulator of plasma cell differentiation Blimp-1 (83) as well as by enhanced basal expression of costimulatory B7 molecules (50). Hence, the anatomical localization of marginal zone B cells, which allows for an early contact with antigens carried by the blood, together with their ability to rapidly differentiate into antibody-secreting cells endow marginal zone B cells with the capacity to provide a first line of defense against pathogens entering the body through the bloodstream. In the initiation of T cell-independent immune responses, marginal zone B cells are assisted by blood-derived dendritic cells, whereas peritoneal macrophages provide similar support to B1 B cells (85). These interactions are crucial for B cell activation and rapid generation of plasma cells in early T cell-independent responses and have been shown to be dependent on signals delivered by the molecules of the tumor necrosis factor (TNF) superfamily BLyS and APRIL, expressed on dendritic cells and macrophages (85). Thus, marginal zone and B1 B cell activation and differentiation in response to T cell-independent antigens relies on accessory signals, mediated by dendritic cells and macrophages, in addition to mIg cross-linking.
T cell-dependent B cell activation

Soluble proteins, bacterial toxins and many common experimental antigens, such as haptenated proteins or sheep red blood cells, are dependent on T helper cells to induce B cell responses. For these T cell-dependent antigens to stimulate antibody production, they must first be taken up by dendritic cells, processed via the endocytic compartment and presented as peptides within the context of MHC class II molecules (MHCII) to naïve antigen-specific T helper cells in order to activate them (86). The recognition of peptide-MHCII complexes by the T cell receptor and the interaction with costimulatory molecules expressed on activated dendritic cells, such as B7-1, B7-2 and CD40, triggers the expression of a number of activation/adhesion molecules on T cells (87). In particular, activated T cells upregulate the ligand for CD40 (CD40L), a B cell activating surface protein, which is constitutively expressed on mature B cells (88, 89). Following clonal expansion, antigen-activated T helper cells migrate from the T cell area to the T-B borders to initiate cognate contact with activated antigen-specific B cells (90). B cells obtain help from T cells by acting as antigen-specific antigen presenting cells. In T cell-dependent B cell activation the B cell receptor has two interrelated functions. The first is to initiate signaling cascades that result in the transcription of a variety of genes associated with B cell activation (91). The second is the uptake and targeting of antigen to the MHCII processing and presentation pathway (86). As a result, B cells can efficiently engage primed T cells which are specific for the peptide-MHCII complexes displayed on their surface. The formation of T-B conjugates allows the interaction between CD40 on B cells and CD40L on activated T cells, which, with the aid of T cell-derived cytokines, activates B cells (92). This leads to the generation of extra-follicular foci of plasma cells, which initially secrete mostly IgM antibodies but can switch to IgG within a short period of time (90, 93). These early aggregates of antibody-secreting cells are located at the edge of T regions and in the red pulp and are of short-lived nature (94). As the response progresses, some activated B cells move into the follicular areas to initiate the germinal center response (95).

The CD40-CD40L interaction is critical for the outcome of T cell-dependent antibody responses: it is required for class-switch recombination and for the formation of germinal centers, where somatic hypermutation occurs and B cell memory is established (96). Germinal centers develop in the B cell follicles of secondary lymphoid organs after colonization by activated B cells (97). The formed germinal centers are oligocolonal, one to three B cell blasts have been reported to colonize each follicle (95). These blasts undergo massive clonal expansion, which displaces resting follicular B cells and induces the polarization of follicles
into a dark zone occupied by proliferating centroblasts and a light zone of non-cycling centrocytes (97). The light zone also contains a network of follicular dendritic cells (FDCs) that have the capacity to bind antigen and retain it for long periods of time (98). Antigen deposits are retained on the surface of FDCs in the form of antigen-antibody complexes, through the interaction with complement- and Fc-receptors (99). FDCs are believed to be of non-hematopoietic origin (100), but require the presence of B and T cells for generation and maintenance, as indicated by their absence in immunodeficient mice (101). TNF family cytokines produced by B cells, in particular lymphotoxin (LT) α and β, have been shown to play a central role in generation and maintenance of FDC networks (102-105).

In germinal centers the two main events of antibody maturation occur: class-switch recombination and somatic hypermutation. Class-switch recombination exchanges the initially expressed IgH constant (C) region μ (Cμ) exons for an alternative set of downstream IgH C region exons (Figure 1.1) (106). The mouse genome encodes 4 Cy (γ1, γ2a, γ2b and γ3), a Ca and a Ce region gene (107) and therefore antibodies of the IgG1, IgG2a, IgG2b, IgG3, IgA and IgE isotype are generated in addition to IgM antibodies. The process of isotype-switching allows the expression of antibodies with the same V region, and consequently the same antigen specificity, but different effector functions. In fact, the nature of the produced isotype determines its activity, notably its half-life, the ability to bind to Fc receptors and to activate complement. Although most B cells undergo class switch recombination in germinal centers, IgG-expressing B cells are also apparent in the B cell foci associated with the T region, where early isotype-switching is thought to occur (93). In addition, despite the fact that T cells are generally appreciated to be required for class switch recombination, some degree of isotype-switching, predominantly to the IgG3 isotype, also takes place with T cell-independent antigens (57).

Somatic hypermutation is a second mechanism of antibody diversification in germinal centers. This process introduces point mutations in the V region exons of IgH and IgL genes at a high rate (about $1 \times 10^{-3}$ mutations per base pair per generation) (108). These mutations are generated by an error-prone DNA polymerase (109) and are most prominent in the 3 complementarity-determining regions (CDRs) of the heavy and light chains of antibodies, the hypervariable regions composing the antigen-binding site (110). The variant BCRs arising from somatic hypermutation are expressed on the surface of centrocytes, which are selected on the basis of their high affinity for antigen (111). Selection of high affinity clones occurs in close contact with antigen-bearing FDCs and leads to the increase of the average antibody affinity, a process known as affinity maturation (97). Both, somatic hypermutation and class
switch recombination have recently been shown to be critically dependent on activation-induced cytidine deaminase (AID) activity, thereby accrediting a major role in antibody diversification to this activated B cell-specific enzyme (112).

Follicular B cells are the main players in T cell-dependent antibody responses. However, marginal zone B cells have also been shown to mount rapid and efficient responses to soluble T cell-dependent protein antigens (113). B cells of the marginal zone subset are equipped with many features suited for the presentation of protein antigens and for the activation of T helper cells. They express high basal levels of the T cell costimulatory molecules B7-1 and B7-2, which are rapidly further upregulated upon activation, and they can function as potent antigen-presenting cells (50). Marginal zone B cells have been reported to more rapidly and effectively capture and present soluble protein antigen to T cells than follicular B cells (113). In addition, the capacity of marginal zone B cells to prime naïve T helper cells was enhanced compared to their follicular counterparts in these studies (113). Hence, marginal zone B cells have been suggested to play an important role in the early phase of T cell-dependent responses to blood-borne antigens (114). Nevertheless, the fate of marginal zone B cells that interact with T cells, in particular whether they participate in germinal center reactions and undergo isotype-switching and somatic hypermutation, remains obscure.

The role of complement in B cell activation
Complement plays an important role in B cell activation and immune responses to both T cell-dependent and T cell-independent antigens. Early studies showed that activation of the complement system was essential for efficient trapping and retention of antigen within splenic follicles (115). These findings indicated that binding and localization of foreign antigens within sites important for lymphocyte responses was mediated by targeting of complement-coated antigen to complement receptor-expressing cells. As previously noted, marginal zone B cells express high levels of the complement receptor CD21 and it is believed that this population is responsible for the transport of immune complexes from the splenic marginal zone into the follicles (116-118). In follicles and in the ensuing germinal centers, complement receptors on FDCs mediate antigen retention, which promotes the antigen-driven selection of high affinity germinal center B cells and maintains B cell memory (119).

Complement-dependent follicular localization of antigen is not the only mechanism by which complement enhances B cell immunity. Bound complement enables antigens to engage the complement receptor type 2 (CD21) expressed on mature B cells. CD21 is part of a B cell surface complex that includes the signaling protein CD19 and the tetraspan molecule CD81.
Complement-coated antigen can therefore coengage the CD21/CD19/CD81 coreceptor and the BCR, which results in a lowering of the threshold of B cell activation (121, 122). Hen egg lysozyme (HEL) fused to the activated complement product C3d was demonstrated to be up to 10,000 more immunogenic than HEL alone and therefore the threshold for B cell activation was decreased by several orders of magnitude by the attachment of C3d fragments (123). The importance of complement in the induction of B cell responses was corroborated by the study of mice deficient in complement components or complement receptors. B cell responses to both T cell-dependent and T cell-independent antigens were affected in these mice, although to a varying degree depending on the amount of antigen injected and the presence of adjuvant (31, 124-128).

In addition to their role in decreasing the threshold of B cell activation, complement receptors on B cells are thought to provide survival signal to differentiating germinal center B cells. Coreceptor expression was shown to be required for survival of activated B cells in germinal centers, irrespective of their affinity for antigen (129). Thus, the induction of efficient germinal center reactions not only requires T cell help and the presence of selecting antigen on FDCs, coreceptor signaling is also critical for B cell survival in this specialized environment.

1.5 Immunological B cell memory

In germinal centers activated B cells can differentiate along two distinct pathways: they can either join the memory B cell compartment or mature into long-lived plasma cells, which take residence in the bone marrow (97). Memory B cells and bone marrow plasma cells are the main players of the B cell arm of immunological memory, the property of the immune system to mount an enhanced and augmented response upon reencounter with a pathogen or antigen. The humoral immune system contributes to protection against reinfection by maintaining high levels of specific antibody.

Memory B cells are generally described as antigen-experienced B cells, mostly displaying somatically mutated surface Igs of a downstream isotype (130). This population of B cells retain the expression of the characteristic B cell surface molecules CD19 and B220 (130), but no marker unambiguously distinguishing the memory B cell population from naïve B cells has been described, even though some have been proposed (131, 132). Memory B cells have been shown to persist as a non-secreting population of cells for long periods of time following antigen exposure (133-135), and to recirculate through secondary lymphoid organs (136).
However, a population of memory B cells, which is most prominent in humans, has been described to be associated with the marginal zone (137, 138). This population of CD21^{high}CD23^{low} B cells cannot be phenotypically distinguished from cells of the marginal zone B cell subset (139). Nevertheless, memory B cells can be identified on the basis of their expression of antibodies carrying somatic mutations, an indication that these germinal center-derived cells have undergone affinity maturation (138, 140, 141). The presence of somatic hypermutations clearly distinguishes memory B cells residing in the bone marrow from naïve B cells of the marginal zone subset, which express antibodies in their germline configuration (38, 141).

It has been debated for many years whether maintenance of B cell memory requires the presence of antigen. Memory B cells were initially shown to disappear rapidly upon transfer into unimmunized hosts, suggesting a requirement for antigen for survival of this population (142). However, labeling studies with the thymidine analog bromodeoxyuridine (BrdU) indicated that the turnover of memory B cells was low (134) and these cells were reported to survive in the absence of detectable immune complexes on FDCs (143). Further evidence against an absolute dependence of memory B cells on antigen for survival, has come from studies showing that the inducible switch of the BCR to a different specificity (for which no antigen was available), had little effect on the decay of the memory B cell population (144). Thus, memory B cells are likely to be able to survive for long periods of time in the absence of antigen.

In contrast to memory B cell survival, the generation of plasma cells through activation of memory B cells is generally believed to require antigen (145). It has been proposed that stimulation by persisting antigen causes memory B cells to continually proliferate and differentiate into short-lived plasma cells, thereby maintaining humoral memory (133, 146). As already noted, antigen is retained on the surface of FDCs through a complement- and antibody-dependent mechanism. Alternatively, in the case of living pathogens, antigen can persist in the form of low-grade chronic or latent infections (147). Repeated exposure to infectious agents also provides means of antigen reencounter, which maintains memory B cell proliferation and the generation of new plasma cells (148). Hence, if immunological memory is regarded as the protection against reinfection, which is ultimately mediated by antibodies in the serum or at mucosal surfaces, the contribution of memory B cells can be reasoned to be antigen-dependent, even if the survival of this population may not require antigen. This view has been put into question by a report supporting an antigen-independent activation of
memory B cells through polyclonal stimuli, such as CpG-containing DNA and LPS, or through bystander activation (149).

Antibody titers may also be maintained in the absence of antigen by a second mechanism: the long-term secretion of antibodies by long-lived bone marrow plasma cells. Like memory B cells, bone marrow plasma cells are generated from germinal center precursors (150). Plasma cell lodging in the bone marrow is known to be a process regulated by the chemokine receptor CXCR4 and its ligand CXCL12 (151), but it is still unclear whether the cells leaving germinal centers migrate to the bone marrow as fully differentiated plasma cells or as precursors that terminate their maturation in this organ. Not until recently has it been appreciated that plasma cells can survive for long periods of time, possibly for the life span of a mouse (152, 153). Early studies were misled by the exclusive analysis of plasma cell lifetime in spleen or lymph nodes, many of which are indeed short-lived (154-157). The finding that the bone marrow is the main source of antibody production at later stages after immunization and the study of plasma cells located at this site has led to the recognition that bone marrow plasma cells are long-lived and therefore play an important role in the long-term production of specific antibodies (152, 153, 158, 159). Support to the notion that survival of bone marrow plasma cells is independent of antigen comes from adoptive transfer experiments and from the fact that terminally differentiated plasma cells downregulate surface antigen receptors and MHCII molecules, rendering them unable to bind and present antigen (153, 160). Antibody-secreting cells in the bone marrow are not thought to be intrinsically long-lived; rather, the particular niche they inhabit supplies these cells with survival signals required for long-lasting maintenance (161-163). The interaction of the integrin VLA-4 expressed on plasma cells with ligands on stromal cells, as well as cytokines secreted by bone marrow stromal cells, such as interleukin (IL)-5, IL-6 and TNFα, have been implied in providing survival signals to bone marrow plasma cells (162, 164, 165). Competition for stromal cell-derived survival signals and for a place in the privileged site provided by the bone marrow determines access of newly generated plasma cells to the bone marrow plasma cell pool and their preservation in it. Consequently, plasma cell homeostasis in the bone marrow regulates long-term serum antibody titers. Despite the knowledge about some of the molecules promoting plasma cell survival, the factors required for the generation of long-lived plasma cells, and how they contrast to the requirements for memory B cell development, remain elusive. Bone marrow plasma cells, like all plasma cells, are critically dependent on the induction of the transcription factors Blimp-1 and XBP-1 for terminal differentiation into antibody-secreting cells (166-168). Mice deficient in these key regulators of plasma cell differentiation exhibit a
complete lack of antibody-secreting cells (166, 167). At present, it is unclear whether Blimp-1 and XBP-1 also play a role in plasma cell survival.

Similarly dependent on Blimp-1 expression is the third population which has been described to be generated upon antigen encounter and to be part of the memory B cell compartment: pre-plasma memory B cells (167). These non-secreting B cells, which originate in germinal centers and are subsequently detected in spleen and bone marrow, can been distinguished from classical recirculating B220+ memory B cells on the basis of their B220+CD138—phenotype (169, 170). Pre-plasma memory cells have a greater propensity to form plasma cells than the B220+ memory B cell subset and rapidly differentiate into plasma cells after antigen recall (170). It is unclear whether B cells identified as pre-plasma memory B cells are related to, or even coincide with, a recently described plasma cell precursor population characterized in the bone marrow (171). These post-germinal center B cells, which were reported to display a phenotype intermediate between splenic B cells and terminally differentiated plasma cells, were shown to be direct precursors to plasma cells. The presence in the bone marrow of a population of B cells representing an intermediary stage before terminal plasma cell differentiation offers an indication that germinal center-derived B cells may home to the bone marrow as plasma cell precursors before fully maturing into long-lived antibody-secreting cells.

In conclusion, in order to allow for efficient protection against reinfection, the immune system possesses various means to maintain high levels of specific antibody. From our current knowledge, B cell memory involves the combination of a population of memory B cells with the potential of long-term antigen-independent survival and the capacity to continually and rapidly differentiate into antibody-secreting cells upon antigen contact, a subset of long-lived antibody-secreting plasma cells residing in the bone marrow and, finally, a mechanism ensuring long-term antigen retention on specialized FDCs.

1.6 Virus-like particles as a model antigen: analogies and differences to other common experimental antigens

Much of the knowledge on the mechanisms and rules underlying the induction of humoral immune responses has been gained with the use of common experimental antigens such as haptens, lysozyme and sheep red blood cells. Haptens are small organic molecules which require chemical coupling to a carrier in order to be immunogenic. Examples of commonly used haptens are (4-hydroxy-3-nitrophenyl)acetyl (NP), 2,4,6 trinitrophenyl (TNP) or
2-phenyloxazalone (Ox). Depending on the carrier to which they are coupled, haptens act as T cell-dependent or T cell-independent antigens. Cross-linking to LPS or to synthetic polymers, such as ficoll or dextran, induces hapten-specific T cell-independent antibodies, as expected from a multimeric antigen (57). Although isotype-switching can occur in response to immunization with haptenated polymers, no germinal centers are formed nor is B cell memory established, analogously to purified polysaccharide antigens (57). In order to generate efficient germinal center reactions, accompanied by somatic hypermutation and induction of B cell memory, haptens must be chemically linked to a carrier protein, such as keyhole limpet hemocyanin, bovine serum albumin or chicken gamma globulin (90, 93, 172). The induction of antibody responses by haptenated proteins relies on the activation of T cells by epitopes present in the protein component of the hapten-protein conjugate (86). In addition to their dependence on T cell help, haptenated proteins, require the presence of adjuvant for efficient induction of antibody responses. In this regard, they are similar to other T cell-dependent experimental antigens, like lysozyme or ovalbumin, which are also generally injected in adjuvant in order to generate strong antibody responses.

Not dependent on T cell help or on the presence of adjuvant for delivery of inflammatory stimuli is the induction of antibody responses by many viruses. As mentioned above, viruses owe their ability to act as strong T cell-independent antigens to the highly ordered and repetitive organization of their antigenic determinants, which ensures efficient crosslinking of antigen receptors on B cells (148, 173). In addition to the generation of T cell-independent IgM responses, viruses induce T cell-dependent isotype switching, germinal center formation and B cell memory (148, 174). The influence of antigen organization and repetitiveness on the induction of B cell responses has been extensively studied using vesicular stomatitis virus (VSV). The analysis of antibody responses to the VSV glycoprotein has demonstrated that the viral glycoprotein induced efficient B cell activation when displayed in a densely packed and highly ordered array, like in the virus envelope, but not when the degree of organization was reduced by disrupting the viral particles or expressing the antigen on the surface of cells (61, 62). It was therefore proposed that the ability of B cells to distinguish between highly organized and repetitive patterns, a characteristic of the surface many pathogens, and non-organized or monomeric structures, as prevalent in the body, allows the discrimination of foreign from self antigens (174).

By expression and assembly of the capsid proteins of viruses or bacteriophages, virus-like particles (VLPs) can be generated (175-179). VLPs share with viruses the highly ordered and repetitive structure, which makes them potent immunogens in the absence of adjuvant (179-
However, in contrast to viruses, VLPs do not carry any genetic information and therefore cannot replicate in the host. Thus, they rely on efficient trapping on FDCs for long-term retention and persistence in immunized mice or humans. In the studies presented in this thesis, VLPs were chosen as a model antigen. For most of the analysis, VLPs were derived from the RNA phage Qβ. The capsid protein of bacteriophage Qβ spontaneously assembles into icosahedral particles of about 30 nm diameter, with a highly ordered and repetitive structure (Figure 1.3) (175). Thus, Qβ particles exhibit the geometry and size of a prototype virus, but, in contrast to viruses, they cannot replicate in the host, excluding the presence of significant antigen deposits other than those on FDCs, and no virus-receptor interactions are expected. Immunization with VLPs has been reported to elicit strong antibody responses even in the absence of T cell help, as observed for many viruses (180, 181). Hence, the use of VLPs as a model antigen allows for the study of B cell responses to an antigen with the structural characteristics of a virus without the complexity of viral infections.

To analyze the antibody response induced by VLPs a method to detect specific B cells and plasma cells by flow cytometry was developed at the outset of the studies presented in this thesis. This detection system provided a new tool to dissect some aspects of the mechanisms underlying the induction and regulation of early and long-term humoral immune responses to VPLs.

Figure 1.3 Repetitive structure of VLPs derived from bacteriophage Qβ: schematic representation (left) and electron microscopy image (right).
2 RESULTS

2.1 Part I: Rapid response of marginal zone B cells to viral particles

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Abbreviations used in this paper: FO, follicular; MZ, marginal zone; GC, germinal center;
PNA, peanut agglutinin
2.1.1 Abstract

Marginal zone (MZ) B cells are thought to be responsible for the first wave of antibodies against bacterial antigens. Here we assessed the in vivo response of MZ B cells in mice immunized with viral particles derived from the RNA phage Qβ. We found that both follicular (FO) and MZ B cells responded to immunization with viral particles. MZ B cells responded with slightly faster kinetics, but numerically, FO B cells dominated the response. B1 B cells responded similarly to MZ B cells. Both MZ and FO B cells underwent isotype switching, with MZ B cells again exhibiting faster kinetics. In fact, almost all Qβ-specific MZ B cells expressed surface IgG by day 5. Histological analysis demonstrated that a population of activated MZ B cells was associated with the marginal zone, probably due to the elevated integrin levels expressed by these cells. Thus, both MZ and FO B cells respond with rapid proliferation to viral infection and both populations undergo isotype switching, but MZ B cells remain in the marginal zone and may be responsible for local antibody production, opsonizing pathogens entering the spleen.
2.1.2 Introduction

B cell responses against haptenated proteins, in adjuvant, are well characterized (86). Dendritic cells in T regions stimulate naïve Th cells, which in turn proliferate and activate specific B cells. This leads to the formation of extra-follicular aggregates of antibody-forming cells at the border of the T and B region (90, 92, 95). These B cells may express the IgM or IgG isotype. From there, a few specific B cells expressing either isotype are recruited back into B cell follicles (95). Together with follicular Th cells, the germinal center (GC) reaction is subsequently initiated (97). B cell responses against bacteria follow a slightly different pattern. Bacterial antigens are eliminated from the circulation in the marginal zone (MZ), a specialized region separating the lymphocyte regions from the red pulp (32). The MZ is populated by metallophilic and MZ macrophages, which are specialized in phagocytosing particulate antigens from the circulation. In addition, bacterial antigens may be transported into the spleen by dendritic cells and granulocytes (85). Many splenic terminal arterioles end in the MZ and blood leaks through the meshwork of reticular cells into the red pulp before it is collected in venous sinuses. Therefore, the MZ is one of the best sites for blood filtration by macrophages (32). The marginal zone is also home to MZ B cells. In contrast to follicular (FO) B cells, which recirculate throughout the lymphoid system, MZ B cells are a more sessile population, which rest in the MZ for extended time periods. The mechanism of this retention has recently been elucidated, and it was shown that elevated expression of integrins (LFA-1 and α4β1) keeps MZ B cells sessile (40).

Due to their particular localization, it is not surprising that MZ B cells rather than FO B cells generate the first wave of antibodies against bacteria, especially against bacterial polysaccharides (83). A further difference between haptenated proteins in adjuvant versus bacteria is the T cell-independence of the anti-bacterial IgM response (57). Specifically, haptenated proteins in adjuvant fail to induce IgM responses in T cell deficient mice, while bacterial antigens are able to induce efficient IgM titers in the absence of Th cells. It is thought that it is the MZ B cells that are responsible for this Th cell independent IgM response and MZ B cells constitute therefore an essential part of the initial response against bacteria (57).

In contrast, the importance of the MZ B cell population for the later B cell response is less well characterized. In particular, the contribution of MZ B cells to isotype switching, GC formation and memory B cell generation upon viral or bacterial infection remains ill-defined.
Two mechanisms are responsible for the Th cell independence of anti-bacterial B cell responses. Bacteria express multiple ligands for toll-like receptors, which may result in the direct activation of B cells (182-184). LPS and non-methylated, CG-rich DNA are prominent examples of ligands binding to toll-like receptor 4 and 9, respectively (185, 186). An additional reason for T cell-independent B cell stimulation is the surface structure of bacteria, which often exhibit highly repetitive antigens. This leads to cross-linking of specific B cell receptors, which is sufficient for the induction of proliferation and IgM production (174). More recently, molecules of the TNF superfamily, such as BLys (BAFF, TALL-1, THANK or zTNF4) (187-189) and April (190) have been reported to aid in T cell-independent B cell stimulation (85, 191-194). Dendritic cells and macrophages express BLys and April whereas their receptors (TAC1, BCMA and BAFFR) (195-198) are expressed on B cells. Thus, a dendritic cell – B cell interaction may, at least in part, be able to replace the conventional CD40L–CD40 interaction between Th cells and B cells.

Is the response to viruses similar? Like bacteria, most viruses induce T cell-independent IgM responses and T cell-dependent IgG responses (174). Although some viruses have been reported to stimulate toll-like receptors (199), the dominant parameter for T cell-independent B cell responses is the repetitive surface of viruses (173). Moreover, virus-infected cells and viral particles have been reported to be filtered out by the marginal zone and also by the red pulp (136, 200). This renders it likely that MZ B cells are responsible for early Th cell-independent anti-viral B cell responses, as observed for bacteria (83). In addition, virus-specific B cells have been seen in the MZ of mice early after immunization with vesicular stomatitis virus (146) and in rhesus monkeys chronically infected with simian-human immunodeficiency virus (201). On the other hand, the highly repetitive surface of viruses may be able to directly activate FO B cells. In fact, the small size of viruses suggests that they may enter B cell follicles for direct B cell activation more easily than bacteria. Thus, an important contribution of MZ and FO B cells to the early anti-viral B cell response seems possible.

In order to study the importance of MZ B cells versus FO B cells in the early and late phase of anti-viral B cell responses, we established a method to trace B cells specific for the bacteriophage Qβ. The capsid protein of Qβ spontaneously assembles into particles with icosahedral structure of about 30 nm diameter (175). These virus-like particles exhibit the geometry and size of a prototype virus. In addition, since the mouse is not the natural host of Qβ, there are no expected virus-receptor interactions that could interfere with the site of antigen trapping. Nor will there be any viral replication which could alter the kinetics of viral elimination because Qβ particles do not carry a viral genome. Therefore, the use of Qβ-
derived particles for immunization allowed for the study of the B cell response against a particle exhibiting all the geometric features of a virus, but without potentially complicating factors, such as binding to viral receptors or viral replication. The results show that proliferation of B cells occurred rapidly and within a few days of immunization with Qβ, in both the marginal zone and the follicular B cell compartments. However, the response of MZ B cells was slightly faster, in terms of both proliferation and isotype switching. Despite rapid and efficient isotype switching, a population of MZ B cells remained localized in the marginal zone, suggesting that MZ B cells are responsible for local IgM and IgG production facilitating opsonization of pathogens entering the spleen through the blood.
2.1.3 Materials and methods

*Mice and antigens*

Female C57BL/6 mice (Harlan, Horst, The Netherlands) and mice deficient for MHC class II expression (202) (in-house breeding) were immunized at 8-12 weeks of age and kept under specific pathogen-free conditions.

Capsids of the RNA-phage Qβ were expressed using the expression vector pQβ10 and purified as previously described (203). For immunization, Qβ capsids were diluted in PBS in order to inject either 10 or 100 µg of the antigen i.v.

Serum transfer was performed with pooled immune serum from day 21 mice with 500 µl injected i.p. 1 day before analysis.

*ELISA*

10 µg Qβ in coating buffer (0.1 M NaHCO₃, pH 9.6) was coated onto ELISA plates (Nunc Immuno MaxiSorp) and ELISAs were performed according to standard protocols using HRPO-conjugated secondary antibodies (Sigma-Aldrich). Plates were developed with OPD substrate buffer (0.5 mg/ml OPD, 0.01 % H₂O₂, 0.066 M Na₂HPO₄, 0.038 M citric acid, pH 5.0; 100 µl each well) and were read at 450 nm.

All antibody titers are presented as -log₂ of 40-fold pre-diluted sera. Titers represent half maximal OD.

*Antigen uptake*

Qβ particles were labeled with the fluorochrome Alexa 488, using the Alexa Fluor 488 Protein Labeling Kit (Molecular Probes) according to the manufacturer’s instructions. 100 µg Alexa 488-conjugated Qβ was injected i.v. and spleens were removed 1 day after immunization. Individual spleens were digested twice for 30 min at 37°C in IMDM supplemented with 5% FCS and 100 µg/ml collagenase D (Boehringer Mannheim). Released cells were labeled with Cy-Chrome-conjugated anti-CD11c (HL3, BD Biosciences) and APC-conjugated anti-CD11b (M1/70, BD Biosciences) antibodies and analyzed with a FACSCalibur (Becton Dickinson).

*Immunohistochemistry*

Freshly removed organs were immersed in HBSS and snap frozen in liquid nitrogen. Tissue sections of 5 µm thickness were cut in a cryostat, placed on siliconized glass slides and fixed with acetone for 10 min. For detection of Qβ antigen, sections were incubated with rabbit
RESULTS

anti-Qß antiserum (produced by RCC Ltd., Switzerland) (diluted 1/1500), followed by alkaline phosphatase-labeled goat antibodies to rabbit immunoglobulins (Jackson ImmunoResearch Laboratories) and alkaline phosphatase-labeled donkey antibodies against goat immunoglobulins (Jackson ImmunoResearch Laboratories) (diluted 1/80). Alkaline phosphatase was visualized using naphthol AS-BI phosphate and New Fuchsin as substrate, which yielded a red precipitate. For detection of antigen-specific B cells, dehydrated tissue sections were overlaid with a solution of Qß (3.5 µg/ml) and specifically bound Qß particles were detected with rabbit anti-Qß serum followed by alkaline phosphatase-labeled secondary antibodies, as described for the detection of Qß antigen. To control for staining of Qß antigen the incubation step with Qß particles was omitted. At late time points after immunization no specific staining could be revealed when incubation with Qß was omitted, indicating that Qß-specific B cells and not persisting antigen were being detected. Few days after immunization residual follicular staining was present but specific staining was undetectable outside of follicles.

In stainings where splenic marginal metallophilic macrophages were detected together with Qß-specific B cells or IgG, metallophilic macrophages were stained using biotinylated rat anti-MOMA-1 antibodies (Biomedicals) followed by avidin-biotin-peroxidase complexes (DAKO). Detection of Qß-specific B cells was performed by incubation with Qß capsids followed by rabbit anti-Qß serum and alkaline phosphatase-labeled secondary antibodies, as described above. Alkaline phosphatase was visualized using Fast Blue and peroxidase using AEC (3-Amino-9-ethylcarbazole) as reagents.

Endogenous alkaline phosphatase was blocked by levamisole. Dilutions of secondary antibodies were made in TBS containing 5% normal mouse serum. Incubations were done at room temperature for 30 min and TBS was used for all washing steps. Color reactions were performed at room temperature for 15 min with reagents from Sigma-Aldrich. Sections were counterstained with hemalum and coverslips mounted with glycerol and gelatin.

Detection of specific B cells by flow cytometry

FACS analysis of Ag-specific B cells was based on techniques previously described by McHeyzer-Williams et al. (204).

For the detection of B cells expressing Qß-specific surface Ig, single cell suspensions of splenocytes were incubated with Qß capsids, followed by a polyclonal rabbit anti-Qß antiserum (RCC) and Cy5-conjugated donkey anti-rabbit IgG serum (Jackson ImmunoResearch Laboratories). Cells were stained with a mixture of FITC-conjugated
antibodies (anti-IgD, 11-26, eBioscience; goat anti-IgM serum, Jackson Immuno Research Laboratories; anti CD4, GK1.5; anti CD8, 53-6.7; anti-CD11b, M1/70; anti-Gr-1, RB6-8C5) and PerCP-Cy5.5-conjugated anti-CD19 (1D3) for the detection of isotype-switched B cells. B cells were further characterized with biotinylated goat anti-IgG serum (Jackson Immuno Research Laboratories) or biotinylated peanut agglutinin (PNA, Vector Laboratories), followed by streptavidin-PE. After staining, cells were resuspended in 0.005 μg/ml YO-PRO-1 (Molecular Probes) for the exclusion of dead cells. Alternatively, biotinylated antibody was omitted and dead cells were excluded by staining with 0.5 μg/ml propidium iodide (PI).

For the detection of Qβ-specific FO, MZ and B1 B cells, spleen cells were purified by magnetic cell sorting with CD19 MACS MicroBeads (Miltenyi Biotec) according to the manufacturer’s protocol. B lymphocyte purity was between 93-98%. CD19⁺ cells were stained with biotinylated goat anti-IgM serum (Jackson Immuno Research Laboratories), biotinylated goat anti-IgG serum (Jackson Immuno Research Laboratories) biotinylated PNA (Vector Laboratories), biotinylated anti-Integrin αL chain (M17/4), biotinylated anti-Integrin α4 chain (R1-2) biotinylated anti-Integrin β1 chain (Ha2/5), biotinylated anti-CD9 (KMC8), biotinylated rat IgG2b isotype control (LOU) and biotinylated rat IgG2b isotype control (eBioscience) followed by streptavidin-Tricolor (Caltag), FITC-conjugated anti-CD21 (7G6) and PE-conjugated anti-CD23 (B3B4). Alternatively, CD19⁺ cells were stained with biotinylated anti-CD5 antibody (53-7.3), followed by streptavidin-PE and FITC-conjugated goat anti-IgM serum (Jackson Immuno Research) and dead cells were excluded by the addition of 0.5 μg/ml propidium iodide. Qβ-specific cells were identified as described above.

All stainings were performed at 4°C for 30 min. Fc-receptors were blocked with anti-mouse CD16/32 (2.4G2). Antibodies were purchased from BD Biosciences, unless otherwise specified. Cells were analyzed with a FACSCalibur (Becton Dickinson).
2.1.4 Results

Qβ induces Th cell-independent IgM responses followed by Th cell-dependent IgG responses and GC formation

For the initial characterization of the B cell response against Qβ, C57BL/6 and MHC class II-deficient mice were immunized intravenously with 10 μg Qβ (Figure 2.1 A). Both mouse strains mounted efficient IgM responses by day 4. In addition, IgG antibody titers were detectable in C57BL/6 mice by day 6 and peaked at approximately day 21 before they slowly declined. In contrast, MHC class II-deficient mice generated greatly reduced (50-fold) IgG antibody titers. Thus, as expected for virally shaped particles with icosahedral structure (175), Qβ induced Th cell-independent IgM responses and a persistent and slowly declining Th cell-dependent IgG response (174).

Many T cell-independent antigens fail to induce GC formation. Nevertheless, since viruses efficiently trigger the formation of GCs (205) and since Qβ is able to induce Th cell-dependent IgG antibodies, it may be expected that immunization with Qβ results in the generation of GCs. To confirm this, mice were immunized with Qβ and spleens were isolated 3 weeks later for histology. Specific B cells were visualized with a modified method previously described for vesicular stomatitis virus (146): spleen sections were incubated with Qβ used for specific amplification and bound particles were detected with a polyclonal anti-Qβ serum. As expected from the strong Th cell-dependent IgG response, massive numbers of GCs were induced by immunization with Qβ (Figure 2.1 B). In addition to the specific B cells seen in GCs, a sizeable number of specific B cells could also be observed outside GCs. Almost all Qβ-specific GC B cells only stained on the cells surface (Figure 2.1 B, middle panel) and can be distinguished from more intensely stained cells within aggregates in the red pulp (Figure 2.1 B, lower panel, arrow). These non-GC B cells were stained in the cytoplasm suggesting that they were secreting antibody. No specific staining could be revealed when incubation with Qβ was omitted, indicating that Qβ-specific B cells, and not persisting antigen, retained in macrophages or on the surface of follicular dendritic cells, were being detected (not shown). Interestingly, as previously seen for vesicular stomatitis virus (146), GCs were rather long-lived, since high numbers of GCs could still be observed 3 weeks after immunization.

Thus, Qβ induces an early, Th cell-independent IgM response, followed by a Th cell-dependent IgG response and GC formation.
Figure 2.1: Characterisation of the B cell response against Qβ. (A) Qβ-specific IgM (squares) and IgG (triangles) titers in serum of C57BL/6 and MHC class II-deficient mice determined by ELISA. Titers represent log₂ dilutions of 40-fold prediluted sera. Data shown represent the mean ± SD of 6-12 mice. (B) Immunohistochemical detection of Qβ-specific B cells in sections of spleen 21 days post-immunization. Qβ-binding cells in GCs as well as specific cells in aggregates outside GCs (arrow) can be observed. Original magnification: x125 (upper panel), x290 (middle panel), x190 (lower panel).

**Qβ is rapidly trapped within the red pulp, marginal zone and B cell follicles**

Particulate bacterial antigens and viruses, such as vesicular stomatitis virus, are primarily trapped in the marginal zone (32, 83, 136, 200). To test whether this was also the case for Qβ, we tracked the fate of the viral particles after immunization in vivo. To be able to visualize Qβ by flow cytometry, particles were labeled with the fluorophore Alexa 488. Subsequently, the labeled particles were injected intravenously and spleens were isolated 24 hours later. Tissue was digested with collagenase and splenocytes were stained for CD11b, CD11c (Figure 2.2A) and F4/80 (not shown) to identify macrophage and dendritic cells populations, or for B220, CD4 or CD8 to stain B and T cells (not shown). Only low percentages of B or T cells bound or phagocytosed Qβ (not shown). In contrast, a larger proportion of macrophages (CD11b<sup>high</sup>/CD11c<sup>low</sup> cells in R1 and R2) and dendritic cell populations (CD11c<sup>high</sup> cells in R3 and R4, myeloid and lymphoid dendritic cells (206), respectively) ingested low amounts of the Qβ particles. The majority of particles were found in the CD11b<sup>int</sup>/CD11c<sup>low</sup>
macrophage population (R2); these cells were also positive for the F4/80 antibody (not shown), which identifies red pulp macrophages (207).

Histological detection of Qβ antigen, which was revealed by incubation of spleen sections with Qβ-specific rabbit serum, confirmed this finding and showed the red pulp and parts of the marginal zone stained brightly 24 hours after i.v. injection of 100 μg Qβ (Figure 2.2 B, upper left panel). Intense staining of macrophages in the marginal zone and red pulp indicated uptake of Qβ antigen by this population (Figure 2.2 B upper right panel, arrows). Surprisingly, a significant amount of staining was also observed in B cell follicles, where the staining had a dendritic appearance, reminiscent of the branching processes of follicular dendritic cells, suggesting that Qβ particles were localized on these cells (Figure 2.2 B, lower left panel, arrows). Control spleens from non-immunized mice did not reveal any specific staining (Figure 2.2 B lower right panel).

Hence, Qβ is not only rapidly cleared from the circulation by red pulp and marginal zone macrophages, but also efficiently reaches B cell follicles.

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**Figure 2.2:** Localization of antigen 1 day after immunization with Qβ capsids. (A) Analysis of uptake of Alexa 488-conjugated Qβ and expression of CD11b and CD11c on splenocytes from immunized (thick line) and naïve (thin line) mice. (B) Immunohistochemistry of spleen sections stained for Qβ antigen from immunized (upper and lower left panels) and from naïve (lower right panel) mice. Arrows indicate intensely stained macrophages in MZ and red pulp (upper right panel) and follicular localization of Qβ antigen (lower left panel). Original magnification: x320 (upper left), x485 (upper right), x575 (lower left), x200 (lower right).
Visualization of Qβ-specific B cells by flow cytometry

Since Qβ efficiently entered several compartments of the spleen, we set out to identify which B cell populations contributed to the Qβ-specific response. For this purpose, we developed a method to stain specific B cells for detection by flow cytometry. Essentially, we adapted a previously described method for haptenated proteins (204) and vesicular stomatitis virus (146) and applied it to Qβ. Accordingly, splenocytes from naïve or immunized mice were isolated and incubated with Qβ. B cells that specifically bound Qβ were subsequently detected using a rabbit anti-Qβ antiserum, followed by staining with Cy5-labeled secondary antibodies. Activated and isotype-switched B lymphocytes, defined as (IgM; IgD; CD4; CD8; CD11b; Gr-1; YO-PRO-1)"CD19", were gated and analyzed for binding of Qβ (Figure 2.3 A). A substantial population of Qβ-binding B cells was detected in immunized mice whereas negligible labeling was observed in spleens of naïve mice (Figure 2.3 A). No staining could be detected when omitting incubation with Qβ or using preimmune rabbit serum (not shown), confirming the absence of unspecific binding by the rabbit anti-Qβ serum. As antibody can passively bind to non-specific B cells in vivo conferring antigen binding, we transferred Qβ-immune sera into naïve mice. No change in Qβ-binding was observed (Figure 2.3 B) indicating that no passively adsorbed antibody in vivo interfered with the antigen-specific B cells detection system presented here.

Three weeks after immunization, approximately half of the isotype-switched B cells bound Qβ (Figure 2.3 A). These isotype-switched B cells expressed IgG and were to a large proportion PNAhigh, indicating that they were GC B cells (Figure 2.3 C). We subsequently examined the kinetics of the B cell response. Isotype-switched B cells became detectable 4 days after immunization and reached peak-frequencies at around day 21 (Figure 2.3 D). Frequencies declined considerably within the next 2-3 months, approximately 10-20-fold. The percentage of PNAhigh Qβ-specific B cells followed similar kinetics, but with peak frequencies slightly earlier, at around day 12 (Figure 2.3 E). This confirmed histological analysis, which indicated the presence of GCs three weeks after immunization (Figure 2.1 B). Thus, Qβ induced a more prolonged GC reaction, when compared to haptenated proteins in adjuvant, for which the GC reaction is more short-lived and usually completely gone within 21 days of immunization. However, the kinetics of the GC reaction was reminiscent of the response obtained with vesicular stomatitis virus, where GCs could be detected more than 100 days after immunization (146).
RESULTS

Figure 2.3: Induction and expansion of Qβ-specific isotype-switched B cells. (A) Representative staining of splenocytes, on day 0 and 21 after immunization, to identify Qβ-specific (IgM; IgD; CD4; CD8; CD11b; Gr-1; YO-PRO-1)CD19* lymphocytes, with mean percentages (±SEM, n=3) of Qβ-binding B cells indicated. (B) Analysis of binding of Qβ particles to isotype-switched B cells from mice injected with 500 μl Qβ-immune serum 1 day previously and from naïve controls. (C) Expression of IgG (left panel) and PNA-binding (right panel) of Qβ-specific isotype-switched B cells gated as in Figure 2.3 A, 3 weeks after immunization. Percentages indicate the mean ± SEM (n=3). Frequency of isotype-switched Qβ-specific B cells (D) and percentage of PNA* Qβ-binding cells (E) on the indicated days after immunization. Data shown represent the mean ± SEM (n≥3).

**FO, MZ and B1 B cells respond to Qβ**

Next, we assessed which B cell populations responded to Qβ. MZ B cells were distinguished from FO and transitional B cells based on CD21 and CD23 expression: MZ B cells are CD21*CD23low, whereas FO B cells are CD21*CD23high (22, 36). B1 B cells were identified by the expression of the CD5 marker (20, 29). Mice were immunized and spleen cells were isolated 5 days later. B cells were purified by magnetic cell sorting and analyzed for Qβ-binding and expression of CD21, CD23, CD5 and IgM. B cells specific for Qβ were
found in both the MZ and the FO B cell compartment (Figure 2.4 A). In addition, a population of B1 B cells also recognized Qß (Figure 2.4 B). The kinetics of the response of individual populations was assessed next. Specific B cells from all compartments expanded rapidly, but MZ and B1 B cells responded slightly faster than FO B cells (Figure 2.4 C). All subsets reached similar frequencies within their populations. However, since FO B cells by far outnumber MZ B cells, at the peak of the response the absolute number of specific FO B cells was about 5-fold higher than the absolute number of specific MZ B cells (Figure 2.4 D). The contribution of B1 B cells to the anti-Qß response was lower than for the other two cell types (Figure 2.4 D). Thus, although B cells from all the three subsets participated in the response to Qß particles, the bulk of the response was mounted by FO B cells.

The presence of Qß-specific B cells in the marginal zone was confirmed by immunohistochemistry. Spleen sections of immunized mice were stained for MOMA-1 (metallophilic macrophages) and Qß-binding. As shown in Figure 2.4 E, Qß-specific B cells could be detected in the marginal zone on day 6 after immunization, as well as at a later timepoint (day 12), when most of the specific cells, however, were found in GCs (Figure 2.1 B).
Figure 2.4: Kinetics of FO, MZ and B1 B cell responses to immunization with Qβ capsids. (A) Representative staining to identify MZ (CD21<sup>high</sup>CD23<sup>low</sup>, top panel) and FO (CD21<sup>low</sup>CD23<sup>high</sup>, bottom panel) Qβ-binding B cells 5 days after immunization. Mean percentages (±SEM) from 3 mice are shown. Values from naïve mice were 0.07±0.01 (IgM<sup>high</sup>) and 0.063±0.009 (IgM<sup>low</sup>) for FO B cells, 0.14±0.03 (IgM<sup>high</sup>) and 0.013±0.009 (IgM<sup>low</sup>) for MZ B cells. Results are representative of three experiments. (B) Identification of Qβ-specific B1 (CD5<sup>−</sup>) B cells in the spleen 5 days after immunization. Mean percentages (±SEM) from 3 mice are shown. Values from naïve mice were 0.1±0.02. Results are representative of three experiments. (C) Percentage of Qβ-specific B lymphocytes in the FO, MZ or B1 B cell compartment of the spleen at the indicated days after immunization. Results represent the mean ±SEM (n=3). One of two similar experiments is shown. (D) Absolute frequencies of FO, MZ and B1 Qβ-specific CD19<sup>+</sup> B cells in the spleen at the time points indicated after immunization. Results represent the mean ±SEM (n=3). One of two similar experiments is shown. (E) Immunohistochemical detection of Qβ-specific B cells (blue) and metallophilic macrophages (MOMA-1, brown) on spleen sections 6 (middle panel) and 12 days (right panel) after immunisation with Qβ. Control staining of naïve spleen is shown (left panel). Arrows indicate Qβ-binding B cells in the marginal zone. Original magnification: x112.5 (left and right panels), x175 (middle panel).
**FO and MZ B cells respond to Qβ in the absence of T help.**

MZ B cells are often referred to as the B cell population that responds to T cell-independent antigens, in particular to haptenated polymers or bacterial carbohydrates (208). We have shown that both FO and MZ B cells respond to Qβ in the presence of Th cells. However, it remained possible that in the absence of T cell help, only MZ B cells would respond to Qβ. To test this hypothesis, B cell responses were assessed in MHC class II-deficient mice (Figure 2.5). Both MZ and FO B cells were capable of mounting a response to Qβ in the absence of Th cells, suggesting that Th cell-independent B cell responses are not necessarily confined to the marginal zone after immunization with viral particles. MHC class II-deficient mice harbor more MZ B cells than wild type mice (not shown). This made it difficult to accurately compare the size of responding B cell populations in the presence or absence of MHC class II. Nevertheless, when the absolute number of specific MZ and FO B cells was determined, absence of MHC class II and Th cells resulted in normal numbers of specific MZ B cells, but a threefold reduction in numbers of specific B cells from the follicular compartment (Figure 2.5).

![Figure 2.5: Induction of Qβ-specific FO, MZ and B1 B cells in the absence of Th cells.](image)

Figure 2.5: Induction of Qβ-specific FO, MZ and B1 B cells in the absence of Th cells. Absolute numbers of Qβ-binding FO, MZ and B1 B cells in the spleen of MHC class II-deficient and wild type C57BL/6 mice 5 days after immunization. Results represent the mean ±SEM (n=5) after subtraction of background values from naïve mice (FO=0.10, MZ=0.01, B1=0.003 for C57BL/6 mice, and FO=0.06, MZ=0.07, B1=0.007 for MHC class II-deficient mice). One of two similar experiments is shown.
**RESULTS**

*MZ B cells undergo rapid isotype switching*

The capacity of MZ B cells to undergo isotype switching was determined next. Mice were immunized with Qβ and B cells were isolated from the spleen by magnetic cell sorting 5 and 21 days later. Purified B cells were stained for the expression of IgG, CD21, CD23 and for binding to Qβ. On day 5 after immunization, a population of IgG-expressing, Qβ-binding B cells was detectable and reached frequencies of about 1% of total CD19⁺ lymphocytes (Table 2.1). Cells with both a MZ B cell (CD21<sup>hi</sup>CD23<sup>lo</sup>) and FO B cell (CD21<sup>int</sup>CD23<sup>hi</sup>) phenotype were present in this isotype-switched Qβ-specific B cell population (Table 2.1). Qβ-specific MZ B cells were found almost exclusively in the IgG⁺ population, indicating that nearly 90% of Qβ-specific MZ B cells had undergone isotype switching at this early time point after immunization (Table 2.1). In contrast, a significant proportion (more than 30%) of Qβ-specific FO B cells could still be found in the IgG⁻ population. Three weeks after immunization, IgG-expressing MZ B cells binding Qβ were reduced about 6-fold compared to day 5 and most specific IgG⁺ B cells either exhibited a follicular phenotype (Table 2.1) or were CD21<sup>lo</sup>CD23<sup>lo</sup> (not shown).

<p>| Table 2.1: Capacity of Qβ-specific MZ and FO B cells to undergo isotype-switching* |
|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|</p>
<table>
<thead>
<tr>
<th>Day 0</th>
<th>Day 5</th>
<th>Day 21</th>
</tr>
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<tbody>
<tr>
<td>IgG⁺</td>
<td>IgG⁻</td>
<td>IgG⁺</td>
</tr>
<tr>
<td>Total Qβ⁺ (% of CD19⁺ lymphocytes)</td>
<td>0.03</td>
<td>0.04</td>
</tr>
<tr>
<td>Qβ⁺CD21&lt;sup&gt;hi&lt;/sup&gt;CD23&lt;sup&gt;lo&lt;/sup&gt; (MZ B cells) (% of CD19⁺ lymphocytes)</td>
<td>0.004</td>
<td>0.003</td>
</tr>
<tr>
<td>Qβ⁺CD21&lt;sup&gt;int&lt;/sup&gt;CD23&lt;sup&gt;hi&lt;/sup&gt; (FO B cells) (% of CD19⁺ lymphocytes)</td>
<td>0.021</td>
<td>0.031</td>
</tr>
</tbody>
</table>

*CD19⁺ cells were purified from spleens of mice 5 and 21 days after immunization. The percentage of total Qβ-binding B cells, CD21<sup>hi</sup>CD23<sup>lo</sup> Qβ-binding B cells and CD21<sup>int</sup>CD23<sup>hi</sup> Qβ-binding B cells, which were IgG⁺ or IgG⁻, was determined. Mean percentages (n=3) are shown. Results are representative of two experiments. The analysis was repeated twice at day 12 with a similar result.
A similar analysis was performed for PNA-binding. Purified B cells which displayed high (GC B cells) or low (non-GC B cells) PNA-binding and were specific for Qβ were assessed for CD21 and CD23 expression. At day 12, a population of PNA\textsuperscript{high} Qβ-binding cells was detectable, comprising 0.95% of total CD19\textsuperscript{+} lymphocytes (Table 2.2). Less than 1% of these GC B cells were of the CD21\textsuperscript{high}CD23\textsuperscript{low} phenotype of MZ B cells, but Qβ-binding GC B cells either exhibited a FO B cell phenotype (CD21\textsuperscript{int}CD23\textsuperscript{high}) or were CD21\textsuperscript{low}CD23\textsuperscript{low} (Table 2.2). Similar observations were made at a later time point (not shown). Thus, MZ B cells underwent rapid isotype switching but Qβ-specific CD21\textsuperscript{high}CD23\textsuperscript{low} B cells did not stain with the GC marker PNA.

The origin of the CD21\textsuperscript{low}CD23\textsuperscript{low} population, which constituted a significant proportion of Qβ-binding B cells at all time points analyzed, remains the subject of further investigation. Transitional B cells usually exhibit this phenotype; however, it appears unlikely although not impossible, that such transitional B cells directly differentiate into isotype-switched GC B cells.

<table>
<thead>
<tr>
<th></th>
<th>PNA\textsuperscript{high} (GC B cells)</th>
<th>PNA\textsuperscript{int/low} (non-GC B cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Qβ\textsuperscript{+} (% of CD19\textsuperscript{+} lymphocytes)</td>
<td>0.95</td>
<td>1.36</td>
</tr>
<tr>
<td>Qβ\textsuperscript{+}CD21\textsuperscript{high}CD23\textsuperscript{low} (MZ B cells) (% of CD19\textsuperscript{+} lymphocytes)</td>
<td>0.008</td>
<td>0.088</td>
</tr>
<tr>
<td>Qβ\textsuperscript{+}CD21\textsuperscript{int}CD23\textsuperscript{high} (FO B cells) (% of CD19\textsuperscript{+} lymphocytes)</td>
<td>0.352</td>
<td>0.639</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Percentages of total Qβ-binding CD19\textsuperscript{+} cells, CD21\textsuperscript{high}CD23\textsuperscript{low} Qβ-binding CD19\textsuperscript{+} cells and CD21\textsuperscript{int}CD23\textsuperscript{high} Qβ-binding CD19\textsuperscript{+} cells, which were PNA\textsuperscript{high} or PNA\textsuperscript{int/low}, were determined in spleens of mice 12 days post-immunization. Mean percentages (n=3) are shown. Results are representative of two experiments. The analysis was repeated twice at day 21 with a similar result.
Isotype-switched MZ B cells express high levels of integrins LFA-1 and αβ1 and the marker CD9

It remained possible that the isotype-switched Qβ-specific CD21^{high}CD23^{low} B cell population may not be MZ B cells but rather FO B cells with downregulated CD23 expression. To assess this possibility, we analyzed integrin αL, α4 and β1 chain levels on Qβ-specific B cells. Expression of integrins LFA-1 (αLβ2) and α4β1 has previously been reported to be upregulated about 2-fold on MZ B cells and to mediate retention of these cells in the marginal zone (40). Thus, overexpression of these integrins is responsible for the localization of MZ B cells. Consequently, increased expression of integrins LFA-1 and α4β1 is a reliable marker for MZ B cells since it determines their homing properties. As shown in Figure 2.6 A, expression of αL, α4 and β1 integrin subunits was high on isotype-switched CD21^{high}CD23^{low} B cells on day 12, confirming that they are MZ B cells. At the same time, this finding offers an explanation for the Qβ-specific B cells observed in the marginal zone 12 days after immunization: the elevated expression of integrins retains the cells in the marginal zone (40).

CD9 is an additional maker distinguishing MZ B cells from FO B cells (39). We therefore assessed the expression of this molecule on activated, isotype-switched Qβ-specific MZ and FO B cells. CD9 was expressed on Qβ-specific CD21^{high}CD23^{low} B cells (Figure 2.6 B), confirming their identity as MZ B cells, and was upregulated from resting levels, as previously shown for MZ B cells activated by LPS (39). As expected from earlier studies (39), CD9 expression was also induced on a small proportion of activated Qβ-binding FO B cells but not on specific CD21^{low}CD23^{low} cells.
Figure 2.6: Elevated expression of integrin LFA-1 and αβi and CD9 on activated MZ B cells. (A) Expression levels of integrin αL, α4 and βi subunits on Qβ-specific CD19+ MZ (thick line) and FO (thin line) B cells were determined on day 12 after immunization. Dashed lines are background staining controls for MZ (thick dash) and FO (thin dash) B cells. Representative profiles and geometric mean fluorescence intensities ±SEM (n=3-5) are shown. (B) CD9 expression levels on Qβ-specific CD19+ MZ and FO B cells and on total CD19+ MZ and FO B cells were analyzed on day 12 after immunization. Geometric mean fluorescence intensities ±SEM (n=3) are shown. Results are representative of 2-4 experiments.
2.1.5 Discussion

Antiviral B cell responses protect from many primary and most secondary viral infections. Understanding the regulation of anti-viral antibody responses is therefore of major interest, yet, most of the rules that govern B cell responses have been established with haptenated protein in adjuvant rather than viruses. While most of the underlying principles of B cell activation, proliferation and isotype switching are fundamentally similar for proteins and viruses, the details may be different. A number of factors are responsible for these differences, including viral replication, activation of the innate immune system, antigen targeting within lymphoid organs and the geometry of viral particles. In this study, we used viral particles, derived from the capsid protein of phage Qβ, to examine the B cell response at the single cell level. Since the particles do not carry genetic information and are devoid of attachment proteins, they cannot replicate in the host. Hence, the system used in this study reduced the complexity of viral infections by eliminating viral replication and virus-receptor interactions and focused on the impact of size and shape of viral particles on the B cell response.

As expected, Qβ induced massive Th cell-independent IgM and Th cell-dependent IgG responses (174). This already defined one major difference between viral particles and most other antigens, which are either Th cell-dependent and fail to induce IgM responses in the absence of T help or, alternatively, only induce Th cell-independent B cell responses but fail to trigger a T cell response. These latter antigens, such as haptenated polymers, usually do not have the capacity to efficiently induce long-lived memory IgG responses or GCs (57). Bacterial polysaccharides are an important example of this type of antigen. The inability of bacterial carbohydrates to induce Th cell-dependent IgG responses has major practical consequences, since vaccines based on such molecules fail to induce long-lived antibody-mediated protection unless conjugated to proteins (209). Viral particles combine both features. They are highly repetitive, thus inducing Th cell-independent IgM responses and consist of proteins, leading to activation of Th cells and Th cell-dependent isotype switching. Moreover, viruses efficiently induce the formation of GCs (146, 201).

Here we showed that viral particles were able to directly stimulate FO B cells, even in the absence of Th cells. In addition, consistent with the fact that Qβ is filtered out from the circulation in the red pulp and marginal zone, MZ B cells also contributed to the early IgM and IgG response. Thus, in contrast to haptenated proteins which fail to stimulate B cells in the absence of Th cells, and in contrast to bacterial antigens which primarily stimulate MZ B cells in the early phase of the response (83), viral particles triggered both B cell lineages, even
in the absence of T cell help. The difference between bacteria and viral particles may be that viral surfaces are more repetitive than bacterial carbohydrates, facilitating activation of FO B cells. Indeed, this view is supported by the finding that MZ B cells are more easily activated \textit{in vitro} than FO B cells (210). Alternatively, due to the smaller size of viral particles, they could enter B cell follicles more readily than bacteria, facilitating the direct stimulation of FO B cells. This conclusion is supported by the immunohistochemical detection of Qβ within B cell follicles. As a result, the major contribution to the anti-Qβ response was made by FO B cells, but MZ B cells were clearly involved in the antibody response to this antigen. Injection of another virus-like particle, derived from the bacteriophage AP205, confirmed the participation of MZ B cells in the response to viral particles (not shown). In fact, B cells from both the marginal zone and follicular compartment responded to immunization with AP205 particles; however, as observed for Qβ, FO B cells accounted for the major part of the response (not shown).

Non-viral exogenous protein antigens have also recently been reported to induce activation of MZ B cells (113, 211), but the contribution of MZ B cells to the overall antibody response to these T cell-dependent protein antigens, as compared to viral particles, remains ill-defined.

Surprisingly, MZ B cells were able to undergo isotype switching more rapidly than FO B cells. This was particularly interesting since the general expectation was that MZ B cells were mainly responsible for the early IgM response. Nevertheless, such rapid production of IgG antibodies is consistent with an early and local role of defense of MZ B cells. Specifically, IgM antibodies exhibit a high avidity due to their pentameric structure. Thus, IgM antibodies may bind efficiently and with higher avidity to pathogens even if they exhibit a lower affinity. However, the ability of IgM to recruit the weapons of the innate immune system is limited. In fact, IgM antibodies can essentially only activate the classical pathway of complement (212).

In marked contrast to IgM antibodies, IgG antibodies only bind if they have a comparably high affinity for the antigen. However, once bound, they not only facilitate the classical and alternative pathway of complement, but also are able to recruit and activate a pool of destructive cell types, including natural killer cells, macrophages and granulocytes (213). Thus, rapid production of IgM antibodies followed by an early wave of IgG antibodies seems optimal for the rapid elimination of pathogens. However, due to the highly destructive potential of IgG antibodies, it is necessary to keep their production under tight control. This may give a physiological explanation for the observation that isotype-switched MZ B cells are not recruited efficiently to the GC reaction, where long-lived B cell memory is established.
The data of the present study do not indicate an important role of MZ B cells in the GC reaction. Specifically, almost no CD21$^{high}$CD23$^{low}$ cells expressed the GC B cell marker PNA. However, it cannot be excluded that a fraction of MZ B cells may modulate their expression of CD21 and CD23 upon activation and may not be recognized as MZ B cells in our study. Nevertheless, with respect to integrin expression GC B cells appeared as a rather homogenous population of B cells and no subpopulation of GC B cells exhibiting the characteristic integrin expression of MZ B cells could be identified. A recent study has suggested that MZ B cells respond to immunization with haptenated proteins with isotype switching and GC formation (211). In this study, MZ B cells were transferred into scid mice before immunization. However, the spleen of scid mice does not have a marginal zone where MZ B cells could have homed to, rendering the interpretation of the immune response mounted by such B cells difficult. In addition, lymph node-derived B cells can differentiate into MZ B cells upon adoptive transfer into RAG-deficient mice (43), indicating that transferring B cells into a new host may alter their characteristics.

How are MZ B cells receiving signals for isotype switching? Th cells are rarely encountered in the marginal zone; however, they can travel through the blood and marginal zone before entering or leaving the T regions. In addition, receptors for BLys may also be present in the marginal zone (85, 193, 194). Thus, MZ B cells may receive signals delivered by BLys/April on dendritic cells or CD40L on passing Th cells, which could drive local isotype switching (85, 193, 194). With this respect, it is interesting to note that most of the larger specific MZ B cell aggregates were observed in a specialized region of the MZ, namely at a site where the MZ zone meets the T cell zone. Thus, isotype switching of MZ B cells may not occur at the interface of the T and B areas but rather at the contact site of the T cell area with the marginal zone. However, since Qß particles are rapidly and efficiently trapped on follicular dendritic cells, it cannot be excluded that follicular or early GC B cells are initially activated and induced to switch to IgG and subsequently migrate from developing GC to the marginal zone and acquire a MZ B cell phenotype. This would be consistent with previous studies showing that the expression of the RNA editing enzyme activation-induced cytidine deaminase, whose activity is required for class switch recombination (112), is restricted to GC B cells (214). Nevertheless, this enzyme has been reported to be induced in MZ B cells after in vitro activation (215), giving support to the concept of AID expression in extra-follicular sites.

Retention of activated MZ B cells in the marginal zone could have an important physiological role. Local production of antibodies by MZ B cells may help in opsonizing viral particles and bacteria entering the MZ, facilitating their removal. Thus, activated MZ B cells may be
responsible for local production of antibodies in order to efficiently rid the body of blood-borne pathogens. In contrast, the role of FO B cells may be to produce antibodies for systemic protection and generate B cell memory. This view is supported by the fact that FO B cells differentiate into GC B cells, which are precursors for the systemic B cell response consisting of plasma cells in the bone marrow and memory B cells recirculating throughout the lymphatic system.

In conclusion, the present study shows that viral particles trigger activation and proliferation of FO and MZ B cells. Both FO and MZ B cells undergo isotype switching, but only the former efficiently contribute to the GC reaction. This suggests that MZ B cells may be responsible for local antibody production leading to opsonization of pathogens migrating through the spleen, while FO B cells may play a role in systemic immunity by differentiating into memory B cells and plasma cells residing in the bone marrow.

**Acknowledgments**

We are grateful to G. Lipowsky for supplying purified Qβ and to A. Fuluriya for critically reading the manuscript.
2.2 Part II: Heterogeneous repertoire of marginal zone B cells specific for virus-like particles

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Running title: Diverse response of marginal zone B cells to virus-like particles
Key words: B cells/Antibodies/Repertoire Development
Abbreviations used in this paper: FO, follicular; MZ, marginal zone; VLP, virus-like particle
2.2.1 Abstract

Marginal zone (MZ) B cells differ from follicular (FO) B cells in their functional, phenotypic and localization properties. It is still unclear whether B cells from the MZ compartment also have distinct or biased BCR specificities. To address this question, we analyzed the antibody repertoire of murine MZ and FO B cells induced by immunization with two different virus-like particles (VLPs). Antibody sequences isolated from sorted VLP-specific MZ and FO B cells were similar in heavy chain V, D and J gene segment usage. Sequence analysis of CDR3 regions of antibodies from MZ and FO B cells also revealed no consistent difference in N nucleotide additions or CDR3 length. In contrast, somatic hypermutations were reduced in CDR regions of antibodies from MZ B cells compared to those from FO B cells. These results indicate that the response of MZ B cells to VLPs is clonotypically heterogeneous and suggest that the BCR repertoire of the mouse MZ B cell compartment is not significantly restricted.
2.2.2 Introduction

The B cell compartment has evolved to provide rapid and long-lasting protection against a large variety of pathogens. This is achieved by the coexistence of functionally distinct subsets of B cells. Recirculating, follicular (FO) B cells are the predominant B cell population and are the main subset involved in T cell dependent antibody responses and germinal center reactions, in which memory B cells and BM plasma cells are generated and long-term immunity is established (90, 93, 97). However, protective immune responses not only require long-lived, high avidity IgG antibodies, an early and rapid wave of IgM-producing plasma cells is also beneficial. For many blood-borne antigens, in particular bacteria, this task is achieved by a special B cell compartment residing in the marginal zone (MZ) of the spleen (32, 83). The anatomical localization of MZ B cells, which allows for an early contact with antigens carried by the blood, and their ability to rapidly differentiate into antibody-secreting cells endow MZ B cells with the capacity to provide a first line of defense against pathogens entering the body through the bloodstream (50, 83, 84). In addition to these functional and topographic differences, MZ B cells can phenotypically be distinguished from B cells of the FO B subset: MZ B cells are IgM<sup>high</sup>IgD<sup>low</sup>CD<sub>21</sub><sup>high</sup>CD<sub>23</sub><sup>low</sup> whereas FO B cells have an IgM<sup>low</sup>IgD<sup>high</sup>CD<sub>21</sub><sup>int</sup>CD<sub>23</sub><sup>high</sup> phenotype (22, 35-37). In their early antibody response to blood-borne antigens MZ B cells are supported by B<sub>1</sub> B cells (83). This population of B cells is also the major source of natural antibodies mediating immediate resistance to infection (30, 31).

Although some of the factors involved in the generation and maintenance of these distinct B cell populations have been defined, the parameters that determine whether an immature B cell is recruited into the FO or MZ B cell compartment remain unclear. The analysis of mutant mice deficient in negative or positive regulators of B cell signaling and the study of transgenic mice expressing different levels of the Epstein-Barr virus protein LMP2A as a BCR surrogate have revealed a role for BCR signaling in lineage commitment of B cells (47, 51-53, 216). These studies have led to the proposition that weak BCR signaling favors the development of MZ B cells whereas higher BCR strength promotes the generation of FO B cells; even stronger BCR signals are required for development of a B<sub>1</sub> B cell population (54). However, there is also evidence that B cells are selected into the MZ, FO and B<sub>1</sub> compartment on the basis of their specificity. In IgH chain transgenic mice, transgenic B cell clones preferentially segregate either into the MZ, FO or B<sub>1</sub> compartments. This has led to the suggestion that
BCR composition determines commitment to a distinct B cell subset (46-50, 217). In addition, certain specificities have been found to be enriched in the B1 population (45). The fact that different V\textsubscript{H} genes are preferentially expressed in FO B cells and B1 B cells (218), provides further evidence that B cells are selected into these compartments on the basis of their antigen receptor.

Although it is generally appreciated that a repertoire selection step in the development of the MZ B cell subset occurs (48), an extensive repertoire analysis of murine MZ B cells is still missing. A comparative analysis of the antibody repertoire of MZ and B1 B cells in L chain transgenic mice has revealed that IgH chain sequences of MZ B cells in these mice are heterogeneous (219). In contrast, studies in rats and humans have suggested a bias in the V\textsubscript{H} gene usage of MZ B cells (140, 141). To address the complexity of the MZ B cell repertoire, we compared heavy chain variable region sequences from MZ and FO B cells in a polyclonal immune response induced by virus-like particles (VLPs) in wild-type mice. For this purpose we used VLPs derived from bacteriophages Q\textbeta and AP205. Q\textbeta capsids form icosahedral particles of about 30 nm diameter (175) exhibiting a highly ordered repetitive structure. Immunization with VLPs derived from the bacteriophage Q\textbeta induces early, T cell-independent IgM responses, followed by slowly declining T cell-dependent IgG responses (180, 220). We have previously shown that both FO and MZ B cells respond to immunization with Q\textbeta, even in the absence of T cell help, and that a population of Q\textbeta-specific B cells remains localized in the MZ (220). VLPs of the RNA phage AP205 have a similar structure and size as Q\textbeta particles (221) and, comparably to Q\textbeta, strong, long-lasting antibody responses are elicited by immunization with AP205 (unpublished observations).

The analysis of the IgH chain repertoire of MZ and FO B cell induced by these two VLPs showed similar V, D and J gene segment usage and no consistent difference in N nucleotide additions or CDR3 length of the antibodies expressed by these two subsets. However, a reduction of somatic hypermutations was observed in CDR regions of VLP-specific antibodies from MZ B cells compared to those from FO B cells. These results indicate that MZ B cells are capable of generating a highly variable antibody response to immunization with VLPs and argue against a significant restriction in the diversity of the MZ repertoire.
2.2.3 Materials and methods

Mice and antigens
Female C57BL/6 mice (Harlan, Horst, The Netherlands) were immunized at 8-12 weeks of age and kept under specific pathogen-free conditions.

Capsids of the RNA phage Qβ were expressed using the expression vector pQβ10 and purified as previously described (203). For immunization, Qβ capsids were diluted in PBS in order to inject 10 μg of the antigen i.v.. AP205 coat protein (221) was cloned into pQβ10 vector (175). VLPs were isolated from E. coli expressing the coat protein gene by a combination of ammonium sulphate precipitation and gel filtration steps. For immunization, AP205 particles were diluted in PBS in order to inject 25 μg of the antigen i.v..

Detection of specific B cells by flow cytometry
Single cell suspensions of spleen or lymph node cells were purified by magnetic cell sorting with CD19 MACS MicroBeads (Miltenyi Biotec) according to the manufacturer’s protocol. B lymphocyte purity was between 93-98%. Detection of B cells expressing Qβ-specific surface Ig was performed by incubation with Qβ, followed by a polyclonal rabbit anti-Qβ serum (produced by RCC Ltd., Switzerland) and Cy5-conjugated donkey anti-rabbit IgG serum (Jackson ImmunoResearch Laboratories), FITC-conjugated anti-CD21 (7G6), PE-conjugated anti-CD23 (B3B4) and PE-TxR-conjugated anti-B220 (RA3-6B2). For the analysis of MZ B cell-specific markers, biotinylated anti-Integrin αγ chain (M17/4), biotinylated anti-Integrin α4 chain (R1-2), biotinylated anti-Integrin β1 chain (Ha2/5) or biotinylated anti-CD9 (KMC8) followed by streptavidin-Tricolor (Caltag) were used. AP205-specific CD19+ B cells, purified by magnetic cell sorting, were identified using Alexa 647-conjugated AP205 particles, which had been labeled with the Alexa Fluor 647 Protein Labeling Kit (Molecular Probes) according to the manufacturer’s instructions. FITC-conjugated anti-CD21 (7G6) and PE-conjugated anti-CD23 (B3B4) were used to identify marginal zone and follicular B cells. Dead cells were excluded by the addition of 0.5 μg/ml propidium iodide.

All stainings were performed at 4°C for 30 min. Fc-receptors were blocked with anti-mouse CD16/32 (2.4G2). Antibodies were purchased from BD Biosciences, unless otherwise specified. Cells were analyzed with a FACSCalibur (Becton Dickinson).
**RESULTS**

**PCR amplification of heavy chain variable regions, sequencing and sequence analysis**

Splenicocytes from 10 immunized mice were pooled and stained for detection of VLP-specific MZ and FO B cells. 1-7×10⁴ specific B cells were sorted into TRI Reagent (Molecular Research Center) with a FACSVantage (Becton Dickinson). Total RNA was extracted from sorted cells according to the manufacturer's instructions. First strand cDNA was synthesized from total RNA using random nonamer primers and SuperScript II reverse transcriptase (Invitrogen). Heavy chain variable regions were amplified with a modified version of a previously described set of primers (222). The following primers were used: HF1, 5'-GAGGAAACCGTGACCGTGGT-3'; HF2, 5'-GAGGAGACTGTGAGAGTGGT-3'; HF3, 5'GCAGAGACAGTGCACCAGAGT-3'; HF4, 5'-GAGGAGACGGTGACTGAGGT-3' (forward primers) and HB1, 5'-GAGGTCAGCTBCAGCAGTGC-3'; HB2, 5'-GAGGTCAGCTBCAGCAGTGC-3'; HB3, 5'-CAGGGTGCAGCTGAAGSASTC-3'; HB4, 5'-GAGGTCAGCTBCAGCAGTGC-3'; HB5, 5'-CAGGGTGCAGCTGAAGSASTC-3'; HB6, 5'-CAGGTTCAGCTBCAGCAGTGC-3'; HB7, 5'-CAGGTTCAGCTBCAGCAGTGC-3'; HB8, 5'-GAGGTCAGCTBCAGCAGTGC-3'; HB9, 5'-GAVGTGAWGYTGTTGGAGTGC-3'; HB10, 5'-GAGGTCAGSKGTTGGAGTGC-3'; HB11, 5'-GAKGTCAMCTGGTAGTGGAGTGC-3'; HB12, 5'-GAGGTCAGCTBCAGCAGTGC-3'; HB13, 5'-GAGGTCAGCTBCAGCAGTGC-3'; HB14, 5'-GARGTRAAGCTTCTCAGTGC-3'; HB15, 5'-GAGGTGAARCTTCTCAGTGC-3'; HB16, 5'-CAGGTTACTCTCTGATGGTGC-3'; HB17, 5'-CAGGTTACTCTCTGATGGTGC-3'; HB18, 5'-GATGTGAACTTGGAAGTGTC-3'; HB19, 5'-GAGGTCAGCTBCAGCAGTGC-3' (reverse primers). For amplification the Advantage 2 PCR Kit (Clontech) was used following the manufacturer's instructions. Purified PCR products were cloned into the pCRII-TOPO vector using the TOPO TA Cloning Kit (Invitrogen). Plasmid DNA was isolated from single colonies and clones containing a 400 basepair insert were sequenced using the BigDye Terminator v3.1 kit (Applied Biosystems) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Cloned sequences were matched against the international ImMunoGeneTics database (IMGT) (http://imgt.cines.fr) (223-226).

**Quantitative RT-PCR analysis**

Quantitative RT-PCR analysis of S1P₃ mRNA levels was performed on the same cDNA samples used for amplification of heavy chain variable regions. Quantitative real-time PCRs were performed on an iCycler Thermal Cycler (Bio-Rad) using 3.2 μl of diluted cDNA, 0.3 μM of each of the two primers, 5 nM fluorescein (Bio-Rad) and Brilliant SYBR Green QPCR
RESULTS

Master mix (Stratagene) in a final reaction volume of 20 µl. The cycling conditions were 10 min at 95°C, followed by 45 cycles of 20 s at 95 °C and 1 min at 58 °C. The following primers were used for amplification: 5’-GGAGCCCTAGACGGGAGT-3’ (forward primer) and 5’-CCGACTGCAGAAGGAGTGT-3’ (reverse primer). Quantification of β-actin cDNA by real-time PCR was performed for each sample to allow for normalization between samples. All reactions were performed in triplicates. Dissociation curve analysis was performed to verify the presence of a single PCR product. Quantification of the transcripts was determined with the iCycler iQ Optical System Software (Bio-Rad) using the comparative threshold cycle method.

Statistical analysis

Levels of statistical significance between means were determined using the Student’s t test.
2.2.4 Results

Detection of MZ and FO B cells specific for VLPs Qβ and AP205

To isolate MZ and FO B cells induced by immunization with VLPs we made use of a previously described antigen-specific B cell detection system relying on visualization of bound VLPs to specific B cells by flow cytometry (220). Purified CD19⁺ B cells were stained with anti-CD21 and anti-CD23 antibodies, to distinguish CD21<sup>high</sup>CD23<sup>low</sup> MZ B cells and CD21<sup>int</sup>CD23<sup>high</sup> FO B cells, and binding to Qβ or AP205 particles was determined. On day 12 after immunization, antigen-specific MZ and FO B cells could be identified in the spleen of mice immunized with Qβ (Figure 2.7 A) or AP205 (Figure 2.7 B). Thus, immunization with VLPs induced a response by B cells from both the MZ and FO compartment.

![Figure 2.7: Detection of Qβ- and AP205-specific FO and MZ B cells. (A) Representative staining to identify FO (CD21<sup>high</sup>CD23<sup>high</sup>) and MZ (CD21<sup>high</sup>CD23<sup>int</sup>) Qβ-binding B cells 12 days after immunization. CD19⁺ cells were purified by magnetic cells sorting before staining. Mean percentages of Qβ-binding B cells are shown. Mean values from naïve mice were 0.14 for FO B cells and 0.28 for MZ B cells. (B) Representative staining to identify FO and MZ AP205-binding B cells 12 days post-immunization. CD19⁺ cells were purified by magnetic cells sorting before staining. Mean percentages of AP205-binding B cells are shown. Mean values from naïve mice were 0.18 for FO B cells and 0.26 for MZ B cells.](image-url)
**RESULTS**

**Qβ-specific CD21\textsuperscript{high}CD23\textsuperscript{low} B cells exhibit markers of MZ B cells and are absent in lymph nodes**

Upon activation, FO B cells may alter their CD21 and CD23 expression and acquire a MZ phenotype. To confirm that CD21\textsuperscript{high}CD23\textsuperscript{low} B cells induced by immunization with VLPs were MZ B cells, we assessed on Qβ-specific CD21\textsuperscript{high}CD23\textsuperscript{low} and CD21\textsuperscript{int}CD23\textsuperscript{high} B cells the expression of surface molecules distinguishing MZ B cells. MZ B cells have been reported to exhibit elevated levels of the integrins LFA-1 (α\textsubscript{L}β\textsubscript{2}) and α\textsubscript{4}β\textsubscript{1}, which mediates their retention in the MZ (40), as well as the surface marker CD9 (39). In addition, MZ B cells have higher expression of sphingosine-1 phosphate receptor 3 (SIP3) than FO B cells (227). As we have previously shown (220), Qβ-binding cells with a CD21\textsuperscript{high}CD23\textsuperscript{low} phenotype displayed increased surface expression of integrin α\textsubscript{L}, α\textsubscript{4} and β\textsubscript{1} chains and of CD9 when compared to specific cells with a FO phenotype (Figure 2.8 A). SIP3 mRNA levels, determined by quantitative PCR analysis, were also notably increased in sorted Qβ- and AP205-binding B cells with a CD21\textsuperscript{high}CD23\textsuperscript{low} phenotype compared to CD21\textsuperscript{int}CD23\textsuperscript{high} B cells (Figure 2.8 B). Thus, VLP-specific B cells identified as MZ B cells in this study display markers characteristic of MZ B cells in addition to their CD21\textsuperscript{high}CD23\textsuperscript{low} phenotype.

MZ B cells constitute a sessile, non-recirculating compartment of the spleen (32). In murine lymph nodes, B cells with a CD21\textsuperscript{high}CD23\textsuperscript{low} phenotype of MZ B cells are missing or very poorly represented (Figure 2.8 C) (210). To further address the possibility that activated FO B cells modulated their expression of CD21 and CD23, we analyzed the phenotype of Qβ-specific B cells in lymph nodes. As shown in Figure 2.8 C, B cells binding Qβ had either a FO phenotype (CD21\textsuperscript{int}CD23\textsuperscript{high}) or were CD21\textsuperscript{low}CD23\textsuperscript{low}. No specific cells with CD21\textsuperscript{high}CD23\textsuperscript{low} expression levels characteristic of MZ B cells could be detected, essentially excluding that FO B cells acquired a MZ phenotype upon activation.
Figure 2.8: Qß-specific CD21^{high}CD23^{low} MZ B cells express elevated levels of markers of MZ B cells and are absent in lymph nodes. (A) Expression levels of integrin α₄, α₆ and β₇ subunits and of CD9 were determined on Qß-specific CD19^{+} MZ and FO B cells and on total CD19^{+} MZ and FO B cells 12 days after immunization. Geometric mean fluorescence intensities ±SEM (n=3) are shown. (B) Qß- and AP205-specific MZ and FO B cells, identified as shown in Figure 2.7, were purified by FACS from pooled spleens 12 days after immunization and S1P3 receptor mRNA levels were determined by quantitative RT-PCR. Expression levels are depicted in relation to ß-actin expression. Quantitative RT-PCR of each sample was performed in triplicates; results are represented as the mean ± SD. (C) Expression of CD21 and CD23 was analyzed on Qß-binding and total B cells isolated from spleen and lymph nodes 12 days after i.v. immunization with Qß. Similar results were obtained in draining lymph nodes after s.c. injections. CD19^{+} cells were purified by magnetic cells sorting before staining. Mean percentages of cells with a CD21^{high}CD23^{low} and CD21^{int}CD23^{high} phenotype are indicated.
For the comparative analysis of the antibody repertoire of MZ and FO B cells induced by immunization with VLPs, Qß- or AP205-specific B cells were stained as shown in Figure 2.7 and purified by FACS from spleens of mice immunized 12 days previously. Amplification of heavy chain variable regions was performed using a previously described set of primers incorporating all mouse heavy chain variable domain sequences collected in the Kabat database (222). Cloned \( V_H \) sequences were matched against the international ImMunoGeneTics database (IMGT) (http://imgt.cines.fr) (223-226) in order to determine the most closely related immunoglobulin sequence. The analysis comprised 50 unique, in-frame VDJ rearrangements for each B cell subset and antigen.

We first classified the cloned immunoglobulin sequences according to their \( V, D \) and \( J \) gene segments. As shown in Figure 2.9 A, sequences isolated from Qß- and AP205-specific MZ and FO B cells displayed a comparable variability in \( V_H \) family usage. The distribution of \( V_H \) genes expressed by MZ and FO B cells was also similar and exhibited no obvious bias towards any member of a particular gene family (Figure 2.9 A). Similar results were obtained for \( D \) and \( J \) gene segments (Figure 2.9 B and C). Thus, the response of MZ B cell to VLPs was heterogeneous with respect to \( V, D \) and \( J \) gene segment usage and did not show signs of restricted clonotype expression.
Figure 2.9: Comparison of VH, DH, and JH gene segment usage by MZ and FO B cells specific for Qß or AP205 particles. Qß- and AP205-specific MZ and FO B cells, identified as shown in Figure 2.7, were purified by FACS from spleens of immunized mice 12 days after injection of Qß- or AP205 VLPs. Heavy chain variable regions were cloned and matched against the international ImMunoGeneTics database of murine immunoglobulin sequences. The pattern of Vn gene families (A, upper panels), VH genes (A, lower panels), DH families (B) and Jh genes (C) expressed in MZ and FO B cells specific for the VLPs is shown.

Antibody sequences from MZ B cells display N nucleotide additions and CDR3 length comparable to those from FO B cells

It has been reported that MZ B cells express antibodies with shorter CDR3 regions and reduced N nucleotide additions compared to recirculating B cells (141, 219), which would result in a lower degree of CDR3 diversity. We addressed this question for MZ B cells specific for VLPs and compared CDR3 regions of MZ and FO B cells. The distribution of the length of CDR3 regions expressed by MZ and FO B cells specific for Qß and AP205 showed...
RESULTS

a similar pattern (Figure 2.10 A). The mean length of CDR3 sequences from Qβ-specific MZ B cells was slightly reduced compared to those from FO B cells (12.5 ± 0.4 and 13.3 ± 0.3 amino acids, respectively). However, no significant difference in the length of CDR3 regions was observed between MZ B cells and FO B cells specific for AP205 particles (11.7 ± 0.4 vs. 11.6 ± 0.4 amino acids), arguing against consistently shorter CDR3 regions in VLP-binding antibodies expressed by MZ B cells. N nucleotide additions were present at Vh-D and D-Jh junctions of immunoglobulin sequences of both subsets of B cells (Figure 2.10 B). The total number of inserted nucleotides was in a similar range (0-18 nucleotides) and only very few sequences were devoid of N nucleotide additions (1/100 for MZ sequences and 4/100 for FO B cells). In accordance with slightly shorter CDR3 regions, the mean number of N nucleotide additions at the D-Jh junctions, but not that at Vh-D junctions, was reduced in sequences from Qβ-specific MZ B cells compared to those from FO B cells (Figure 2.10 B). In contrast, AP205-specific MZ and FO B cells expressed antibodies with a similar number of inserted N nucleotides at both the Vh-D and D-Jh borders, again arguing against consistently reduced N nucleotide additions and CDR3 lengths in immunoglobulins expressed by MZ B cells. These results suggest that antibodies with a high degree of CDR3 diversity are produced by MZ B cells in response to immunization with VLPs.
Somatic hypermutations are reduced in antibody sequences from MZ B cells

Most MZ B cells in rats and mice have been shown to express germline encoded antibodies (38, 141). However, somatic hypermutations have been reported in immunoglobulin sequences isolated from human and rat MZ B cells, supporting the idea that the MZ contains a reservoir of memory B cells (138, 140, 141, 228). We investigated the presence of somatic hypermutations in IgH sequences from MZ B cells specific for VLPs. For this purpose, each sequenced VDJ region was assigned to the most homologous published germline sequence and frequencies of mutated nucleotides in antibodies expressed by MZ and FO B cells were compared. To avoid artifacts arising from strain differences, only sequences matching database entries from C57BL mice were considered (29 and 30 sequences for Qβ-specific MZ and FO B cells, respectively; 34 and 35 sequences for AP205-specific MZ and FO B cells, respectively). As shown in Figure 2.11 A, the average number of mutations in CDR1 and
RESULTS

CDR2 regions of Qß-specific antibodies and in CDR1 regions of AP205-specific antibodies was significantly lower for MZ B cells compared to FO B cells. Most of the mutations in CDR regions of both MZ and FO B cells lead to amino acid changes. These replacement mutations were reduced in CDR regions of antibodies from MZ B cells compared to FO B cells (Figure 2.11 B). In contrast, framework regions of antibodies expressed by MZ and FO B cells were found to be similarly mutated. Furthermore, the proportion of antibodies expressed in germline configuration was higher for MZ B cells than FO B cells (10% vs. 3% of sequences for Qß-specific MZ and FO B cells; 47% vs. 17% of sequences for AP205-specific MZ and FO B cells, p<0.05). Hence, MZ B cells induced by immunization with VLPs generally expressed antibodies with fewer mutations than FO B cells, which may suggest reduced affinity maturation of this B cell compartment.

**Figure 2.11:** Comparison of the number of somatic hypermutations in IgH chain sequences from MZ and FO B cells specific for Qß or AP205. To avoid artifacts arising from strain differences, only sequences matching database entries from C57BL mice were considered (Qß-specific MZ and FO B cells 29 and 30 sequences respectively; AP205-specific MZ and FO B cells 34 and 35 sequences respectively). (A) Number of mutated nucleotides in the indicated regions of the V\textsubscript{H} gene. (B) Number of replacement (R) and silent (S) mutations in CDR1 and CDR2 of the V\textsubscript{H} gene segments. Mean values ± SEM are shown; statistically significant differences are indicated by asterisks (p<0.05).
2.2.5 Discussion

While the BCR has clearly been shown to be essential for maintenance of all B cells (44), to what extent it determines peripheral B cell fate remains ill-defined. There is increasing evidence that BCR specificity as well as BCR signaling are involved in the commitment process of B cell to the FO, MZ and B1 compartment and therefore that the repertoire of these distinct B cell subsets is the result of a selection process (54, 114, 229). A bias in specificity and \(V_H\) gene usage have been demonstrated for B1 B cells, and a similar restriction in the repertoire has been postulated for MZ B cells (45, 48). However, although the analysis of \(V_H\) gene usage and CDR3 diversity of MZ B cells in rats and humans suggest an enrichment of certain clones in the MZ (140, 141), the BCR repertoire of murine MZ B cells has been reported to be highly heterogeneous (219). In this study, we directly compared the repertoire of MZ and FO B cells responding to immunization with VLPs derived from bacteriophages Q\(\beta\) and AP205. For either VLPs, the response of both subsets was highly polyclonal and similarly diverse, as assessed by V, D, and J gene segment usage and by CDR3 length and composition. In contrast to previous studies (141, 219), we did not observe consistently shorter CDR3 regions or reduced N nucleotide additions in IgH chain sequences from MZ B cells. This suggests that, in the response to VLPs, CDR3 diversity of antibodies expressed by MZ and FO B cells was comparable and indicates that B cells with similar CDR3 length are recruited into the MZ and FO compartment. This finding, together with the absence of an obvious bias in heavy chain gene segment usage of VLP-specific MZ B cells, argues against an important restriction of the antibody repertoire of this subset of B cells. Our results are in agreement with a previous report showing that the response to the hapten (4-hydroxy-3-nitrophenyl)acetyl (NP) of adoptively transferred MZ B cells is clonotypically heterogeneous (211).

Besides being the main B cell population in the spleen participating in the early immune response to blood-borne T cell-independent antigens (83), MZ B cells have also been shown to be involved in T cell-dependent antibody responses (113). Q\(\beta\) particles induce early T cell-independent IgM responses followed by T cell-dependent IgG responses (220). In a previous study we have shown that most Q\(\beta\)-specific MZ B cells expressed IgG by day 5; FO B cells underwent isotype-switching with a slight delay but most Q\(\beta\)-specific B cells expressed IgG on day 12 (220). We therefore assume that most of the MZ and FO B cells analyzed in this study have switched to IgG and that these supposedly IgG-expressing B cells are derived from a T cell-dependent response.
Immunoglobulin sequences isolated from VLP-specific MZ B cells displayed a reduction in somatic hypermutations compared to those derived from FO B cells. In mice and rats a predominance of germline encoded sequences or sequences with few mutations has been reported (38, 141). However, this kind of analysis is complicated by the fact that the MZ contains a reservoir of memory B cells whose antigen receptors have undergone somatic hypermutation and affinity maturation (137). These antigen-experienced memory B cells localizing in the MZ cannot phenotypically be distinguished from B cells belonging to the MZ B cell lineage, since they also possess a CD21<sup>high</sup>CD23<sup>low</sup> phenotype (139). Nevertheless, the fact that Qß-binding CD21<sup>high</sup>CD23<sup>low</sup> B cells identified as MZ B cells in this study expressed additional makers of MZ B cells, such as the integrins LFA-1 and α<sub>4</sub>β<sub>1</sub>, CD9 and S1P<sub>3</sub> and the absence of Qß-specific cells with a CD21<sup>high</sup>CD23<sup>low</sup> phenotype in lymph nodes support that these cells are MZ B cells and not activated FO B cells that have modulated their CD21 and CD23 expression. Yet, we cannot formally exclude that some MZ B cells may change their CD21/CD23 phenotype upon activation and may not have been recognized in this study. However, as FO B cells by far outnumber MZ B cells, the contribution of MZ with an altered phenotype to the FO B cell repertoire may be assumed to be minimal. In addition, since our analysis was performed early after immunization (day 12), few VLP-specific memory B cells are likely to have left germinal centers, migrated to the MZ and acquired a MZ phenotype. Thus, cross-contamination of the two populations is expected to be minimal.

The early time point of the analysis may also account for the generally very small number of hypermutations found in the cloned antibody sequences. Alternatively, highly repetitive antigens, such as VLPs may be able to select high affinity clones from early on, explaining the lack of somatic hypermutations. This notion is consistent with the early high affinity IgG responses observed after immunization with vesicular stomatitis virus (230) and with the reduced accumulation of somatic mutations reported in high affinity B cells compared to low affinity B cells (139). Finally, the reduction of somatic hypermutations observed in antibodies expressed by MZ B cells might also be an indication of reduced affinity maturation and antigen-driven selection of this population of B cells. This result is in accordance with our previous observation that MZ B cells are not efficiently recruited to germinal centers (220), where somatic hypermutation is thought to occur.

The notable IgH chain diversity of the MZ B cell clones responding to VLPs suggest that MZ B cells are a heterogeneous population whose repertoire is not substantially restricted. The maintenance of a MZ B cell compartment with an essentially diverse and flexible repertoire, together with the enrichment in the MZ of those antigen specificities most likely to be
encountered, might provide the most efficient first line of defense against many blood-borne pathogens.

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2.3 Part III: Complement receptors regulate differentiation of bone marrow plasma cell precursors expressing transcription factors Blimp-1 and XBP-1

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Running title: Complement regulates plasma cell differentiation

Abbreviations used in this paper: ASC, antibody-secreting cell; BCR, B cell Ag receptor; FDC, follicular dendritic cell; GC, germinal center; NP, (4-hydroxy-3-nitrophenyl)acetyl; PNA, peanut agglutinin
2.3.1 Abstract

Humoral immune responses are thought to be enhanced by complement-mediated recruitment of the CD21/CD19/CD81 coreceptor complex into the B cell antigen receptor (BCR) complex, which lowers the threshold of B cell activation and increases the survival and proliferative capacity of responding B cells. To investigate the role of the CD21/CD35 complement receptors in the generation of B cell memory, we analyzed the response against viral particles derived from the bacteriophage Qβ in mice deficient in CD21/CD35 (Cr2−/−). Despite highly efficient induction of early antibody responses and germinal center (GC) reactions to immunization with Qβ, Cr2−/− mice exhibited impaired antibody persistence paralleled by a strongly reduced development of bone marrow plasma cells. Surprisingly, antigen-specific memory B cells were essentially normal in these mice. In the absence of CD21-mediated costimulation, Qβ-specific post-GC B cells failed to induce the transcriptional regulators Blimp-1 and XBP-1 driving plasma cell differentiation, and the anti-apoptotic protein Bcl-2, which resulted in failure to generate the precursor population of long-lived plasma cells residing in the bone marrow. These results suggest that complement receptors maintain antibody responses by delivery of differentiation and survival signals to precursors of bone marrow plasma cells.
2.3.2 Introduction

Protective immunological memory against reinfection with most viruses largely depends on the induction of long-lasting antibody responses. This concept provides the basis of all successful vaccines used to date (231). B cell memory is characterized by increased frequencies of long-lived memory B cells and elevated levels of specific antibodies (232). Both memory B cells and BM antibody-secreting cells (ASCs), which sustain long-term antibody production (158, 159), are thought to originate in germinal centers (GCs) (97). However, the mechanisms underlying recruitment of GC B cells into the memory B cell or BM plasma cell compartment remain ill defined. Selective accumulation of high affinity ASCs in the BM has suggested that high antigen affinity of the B cell Ag receptor (BCR) favors differentiation of GC B cells into plasma cells (233, 234). While a minimal threshold of signal strength is required for differentiation into a long-lived plasma cell, selection into the memory B cell population appears to be less stringent (233, 234). Additional signals have been reported to drive these two pathways; for instance CD40L, IL-4 or ligation of CD27 direct differentiation of GC B cells towards a memory phenotype (235-237) whereas commitment to a plasma cell fate is promoted by IL-10 and requires IL-6 (161, 236, 238). Signals determining plasma cell fate decision are dependent on the induction of the transcription factors Blimp-1 and XBP-1 for formation of Ig-producing cells (166, 167). Together these regulators drive terminal differentiation of B cells into ASCs, by promoting a plasma cell phenotype and extinguishing gene expression programs involved in proliferation and GC function (239).

Survival of B cells in GCs during the antigen-driven selection process leading to high-affinity memory B cells and plasma cells is dependent on signaling through the CD21/CD19 complex (129). The interaction of CD21 with complement-coated antigen appears to provide a selective advantage to GC B cells. Two additional mechanisms have been proposed by which CD21/CD35 enhances humoral immunity (240-242). Firstly, recruitment of the CD21/CD19/CD81 complex into the BCR complex lowers the threshold of B cell activation. Secondly, complement receptors CD21/CD35 enhance trapping of antigen on follicular dendritic cells (FDCs) thereby driving the GC reaction and maintaining B cell memory.

Insight into the role of complement receptors in humoral responses has been gained through the study of mice with a genetically disrupted Cr2 locus, deficient for the expression of CD21 (complement receptor 2) and CD35 (complement receptor 1). These mice have been reported
to have impaired antibody responses and defective GC formation in response to T cell-dependent and T cell-independent antigens (124, 126, 127). However, antibody responses were affected to a varying degree dependent on the nature and amount of antigen used in these studies. The role of CD21/CD35 in the generation of immunological memory also remains controversial. While Cr2−/− mice infected with vesicular stomatitis virus maintained memory antibody titers comparably to controls (243), accelerated loss of serum antibody was reported in responses to the hapten (4-hydroxy-3-nitrophenyl)acetyl (NP) (244). Furthermore, expression of CD21/CD35 was essential for generation of memory B cells to carrier-coupled NP in the absence but not in the presence of adjuvants (128).

To dissect the role of complement receptors in the induction of immunological B cell memory to a highly repetitive antigen capable of efficient crosslinking of surface Ig on B cells, virus-like particles from the RNA phage Qβ were used as a model antigen. Qβ capsids form icosahedral particles of about 30 nm diameter (175) with a highly ordered repetitive structure, which makes them potent B cell immunogens in the absence of adjuvant (180, 181). Therefore, Qβ particles exhibit the geometry and size of a prototype virus without displaying potentially complicating factors such as viral replication. Immunization with Qβ induces an early, T cell-independent IgM response, followed by a persistent and slowly declining T cell-dependent IgG response (220). Qβ particles efficiently induce GC formation, with antigen-specific GC B cells peaking around day 12 and being still detectable at late stages after immunization (220). Immunization of Cr2−/− mice with Qβ showed that short-term primary responses, induction of GCs and memory B cell formation were independent of complement receptors. In contrast, maintenance of long-lasting antibody titers by BM plasma cells required CD21/CD35. CD21 promoted differentiation of a plasma cell precursor population expressing the plasma cell-specific transcription factors Blimp-1 and XBP-1 as well as the anti-apoptotic protein Bcl-2. These results suggest that engagement of complement receptors on B cells by complement-coated antigen is critical for generation of long-lived plasma cells in the BM responsible for maintenance of memory antibody titers.
2.3.3 Results

Maintenance of antibody titers is impaired in Cr2/− mice

Immunization with a single dose of virus-like particles derived from the bacteriophage Qβ elicits strong, long-lasting IgG responses (220). To assess the role of complement receptors in the induction and maintenance of antibody responses to this antigen, Cr2/− and WT mice were immunized i.v. with 10 μg Qβ and antibody titers were measured at several time points after immunization. Anti-Qβ IgG antibody levels were similar in Cr2/− mice and WT mice early after immunization (Figure 2.12 A). However, whereas in WT mice serum anti-Qβ IgG antibodies increased and reached a peak around day 21, antibody production was not sustained in Cr2/− mice. After the third week post-immunization, antibody titers in Cr2/− mice were substantially reduced compared to WT littermates and exhibited nearly a 12-fold reduction 9 wk after immunization. Thus, Cr2/− mice generate normal short-term anti-Qβ responses but fail to maintain antibody titers in the later phase of the response.

![Figure 2.12](image)

Figure 2.12: Maintenance of anti-Qβ antibody titers and generation of BM ASCs are impaired in Cr2/− mice. (A) C57BL/6 and Cr2/− mice were immunized i.v. with 10 μg Qβ and Qβ-specific serum IgG titers were determined by ELISA. (B) Frequencies of Qβ-specific IgG ASCs in spleen and BM of Cr2/− and WT mice were determined by ELISPOT assay. Values are given as the mean ± SEM, with significant differences between means indicated by asterisks (*, p<0.01; **, p<0.05). The analysis was repeated twice on day 8, 21 and 100 with a similar result.

BM plasma cells but not memory B cells are reduced in Cr2/− mice at late stages after immunization

Elevated levels of IgG antibodies are thought to be maintained by long-lived plasma cells residing in the BM (245) as well as by memory B cells continually differentiating into ASCs through activation by persisting antigen (147). We therefore followed the kinetics of ASCs
RESULTS

and memory B cells in Cr2\(^{-/}\) and WT mice (Figure 2.12 B). As expected from serum antibody levels, up to 12 d after immunization numbers of Qβ-specific ASCs were similar or slightly increased in spleen and BM of Cr2\(^{-/}\) mice compared to WT controls. At later stages after immunization, the number of cells secreting anti-Qβ IgG antibodies, especially those in the BM, were reduced in Cr2\(^{-/}\) mice and the frequency of Qβ-specific cells in the BM plasma cell pool was more than 10-fold lower in Cr2\(^{-/}\) mice than in WT controls 9 wk after immunization (Figure 2.12 B). Therefore, generation of presumably short-lived plasma cells in the spleen was normal in Cr2\(^{-/}\) mice but the formation of long-lived BM plasma cells was strongly reduced.

To quantify Qβ-specific memory B cells we used an antigen-specific B cell detection system relying on detection of bound Qβ to specific isotype-switched B cells by flow cytometry (Figure 2.13 A) (220). Activated and isotype-switched B lymphocytes, defined as (IgM; IgD; CD4; CD8; CD11b; Gr-1; YO-PRO-1)\(^{-/}\)B220\(^{+}\), were gated and analyzed for Qβ-binding on several days after immunization. Qβ-specific B cells were increased in Cr2\(^{-/}\) mice early in the immune response, but antigen-specific memory B cells reached similar frequencies in Cr2\(^{-/}\) and WT mice 9 wk post-immunization (Figure 2.13 B). Therefore, generation and persistence of Qβ-specific memory B cells was normal in Cr2\(^{-/}\) mice. Frequencies of Qβ-specific B cells in LNs reflected those found in the spleen, while no Qβ-binding memory B cells could be detected in the BM (not shown). To confirm that the Qβ-binding (IgM; IgD; CD4; CD8; CD11b; Gr-1; YO-PRO-1)\(^{-/}\)B220\(^{+}\) cells identified at late stages after immunization were memory B cells, we adoptively transferred sorted cells into irradiated recipients and assessed whether they mounted an anamnestic response. Six days after adoptive transfer and immunization, relative antibody titers (titer per transferred cells) were significantly higher in recipient mice that had received Qβ-binding IgM\(^{low}\)IgD\(^{low}\) B cells than in mice transferred with naïve IgM\(^{high}\)IgD\(^{high}\) B cells or total isotype-switched B cells (Figure 2.13 C). Thus, cells identified as (IgM; IgD; CD4; CD8; CD11b; Gr-1; YO-PRO-1)\(^{-/}\)B220\(^{+}\) binding Qβ are bona fide memory cells.

Since normal frequencies of Qβ-specific memory B cells were observed in Cr2\(^{-/}\) mice, we tested their ability to mount an efficient recall response late after immunization. Cr2\(^{-/}\) and WT mice were primed and challenged 6 mo later with Qβ; the increase in antibody titer was analyzed 6 d after secondary immunization. Anti-Qβ titers increased substantially in both strains, more than 70-fold in Cr2\(^{-/}\) mice and nearly 50-fold in WT mice (Figure 2.13 D), indicating that recall responses were normal in Cr2\(^{-/}\) mice.
Similar results were obtained for a second virus-like particle, derived from the bacteriophage AP205. Cr2' mice immunized with AP205 exhibited reduced maintenance of antibody titers and generation of BM plasma cells compared to controls 9 and 13 wk after immunization (Figure 2.13 E), despite normal induction of early antibody responses (not shown). In contrast, as observed for Qß, frequencies of AP205-specific memory B cells and the capacity to mount efficient recall responses to AP205 particles was comparable in Cr2' and WT mice (Figure 2.13 E).

Thus, in the absence of complement receptors normal antigen-specific memory B cells were induced by immunization with virus-like particles, but complement receptors were required for generation and/or maintenance of long-lived BM plasma cells.

**Figure 2.13:** Induction of memory B cells is normal in Cr2' mice. (A) Representative staining of splenocytes from naïve and immunized mice to identify Qß-specific (IgM; IgD; CD4; CD8; CD11b; Gr-1; YO-PRO-1)' B220' cells. PNA-binding on Qß-specific isotype-switched B cells is shown. (B) Frequency of Qß-specific isotype-switched B cells in spleens of Cr2' and WT mice. The analysis was repeated twice on day 8, 21 and 100.
with a similar result. (C) Anti-Qβ antibody levels induced in irradiated WT mice 6 d after immunization and adoptive transfer of Qβ-binding (IgM; IgD; CD4; CD8; CD11b; Gr-1; YO-PRO-1)B220+ B cells, total (IgM; IgD; CD4; CD8; CD11b; Gr-1; YO-PRO-1)B220+ B cells or B220+IgM+IgD+ B cells. Results are expressed as serum IgG ELISA titers per transferred cells. (D) Qβ-specific serum IgG levels in Cr2−/− and WT mice 6 mo after primary immunization and 6 d after secondary challenge with Qβ. (E) B cell responses induced in Cr2−/− and WT mice by injection of 25 μg AP205. Frequencies of AP205-specific IgG ASCs in spleen and BM and of AP205-specific memory B cells in spleen were determined 9 wk after immunization. AP205-specific serum IgG was measured 13 wk after primary immunization and 6 d after antigen recall. All data represent the mean ± SEM, mean values statistically different from WT levels are indicated by asterisks (*, p<0.01; **, p<0.05).

**GCs are efficiently induced in Cr2−/− mice by immunization with Qβ**

As long-lived plasma cells, along with memory B cells are generated in the GC reaction (97) we assessed the induction of Qβ-specific GC B cells in Cr2−/− and WT mice. For this purpose, isotype-switched Qβ-specific B cells were analyzed for binding to the GC marker PNA (Figure 2.13 A) and the frequency of PNAhigh Qβ-specific B cells in spleens of Cr2−/− and WT mice was determined at several time points after immunization (Figure 2.14 A). In both groups of mice high frequencies of PNAhigh Qβ-specific B cells were observed (Figure 2.14 A) and similar frequencies of specific GC B cells were reached. Surprisingly, Qβ-specific GC B cells were induced earlier in Cr2−/− mice than in WT mice, but reduced numbers of GC cells were observed at later stages after immunization. The frequency of Qβ-specific PNAlow B cells was comparable in the spleen of Cr2−/− and WT mice at all time points analyzed (not shown).

These results were confirmed by immunohistochemistry (Figure 2.14 B). Staining of serial spleen sections for Qβ-specific B cells (220) and PNA-binding revealed no significant difference in the number and size of Qβ-specific GCs generated in Cr2−/− and WT mice 12 and 21 d after immunization. Thus, Cr2−/− mice exhibited no obvious deficiency in size or architecture of GCs induced by immunization with Qβ particles.
**Figure 2.14:** Immunization with Qβ induces efficient GC formation in Cr2⁻/⁻ mice. (A) Frequency of Qβ-specific PNA⁺⁺ B cells in spleens of Cr2⁻/⁻ and WT mice. Isotype-switched Qβ-binding PNA⁺⁺ B cells were identified as shown in Figure 2.13 A. Data are expressed as the mean ± SEM, mean values statistically different from WT levels are indicated by asterisks (*, p<0.01; **, p<0.05). (B) Immunohistochemical detection of Qβ-specific B cells and PNA-binding cells in serial sections of the spleen of Cr2⁻/⁻ and WT mice 12 and 21 d after immunization. Original magnification: x62.5 (day 12), x75 (day 21).

**Blimp-1, XBP-1 and Bel-2 fail to be induced in isotype-switched PNA⁺⁺ B cells specific for Qβ in Cr2⁻/⁻ mice**

The induction of normal numbers of GC B cells in Cr2⁻/⁻ mice by immunization with Qβ suggested a role for complement receptors in the differentiation process following antigen-driven B cell expansion. Terminal differentiation of plasma cells has been shown to require the transcription factors Blimp-1 (167) and XBP-1 (166). We therefore analyzed the induction of these two regulators of plasmactic differentiation in B220⁺⁺IgM⁺⁺IgD⁺⁺ Qβ-binding PNA⁺⁺ GC B cells and in B220⁺⁺IgM⁺⁺IgD⁺⁺ Qβ-specific B cells with a PNA⁺⁺ phenotype. Antigen-specific PNA⁺⁺ and PNA⁺⁺ B220⁺⁺ cells were gated as shown in Figure 2.13 A and purified by FACS from spleens of Cr2⁻/⁻ and WT mice 12 d after injection of Qβ.
RESULTS

Blimp-1 and XBP-1 mRNA levels were determined by quantitative RT-PCR. As apparent in Figure 2.15 A, Blimp-1 and XBP-1 mRNA was upregulated 12-13-fold in WT mice in the PNA low but not in the PNA high Qβ-specific B cell population. In contrast, in Cr2-/- mice significant levels of Blimp-1 and XBP-1 failed to be induced in PNA low Qβ-specific B cells. Expression of Blimp-1 and XBP-1 in antigen-specific GC B cells from WT mice was comparable to background levels found in purified T cells (not shown). The spliced form of XBP-1, which has been reported to appear late in plasma cell differentiation and to be associated with increased Ig synthesis (246), could not be detected in any of the samples (not shown).

To dissect the role of CD21/CD35 on B cells vs. FDCs in the induction of Blimp-1 and XBP-1, we analyzed the expression of these transcription factors in Qβ-specific B cells from chimeric mice having normal FDCs but Cr2-/- B cells. For this purpose we transferred splenocytes derived from Cr2-/- or WT mice into sublethally irradiated C57BL/6-CD45.1 recipients. On day 8 and 12 after immunization anti-Qβ antibodies were present at comparable levels in both groups of chimeras, but not in irradiated control mice which had not received any B cells (not shown). As observed for Cr2-/- mice, sorted Qβ-specific isotype-switched CD45.1+B220+PNA low B cells from chimeric mice having Cr2-/- B cells and WT FDCs displayed strongly reduced Blimp-1 and XBP-1 mRNA levels when compared to cells from control chimeras (Figure 2.15 B). Hence, in the absence of stimulation through complement receptors, Qβ-specific isotype-switched PNA low B cells were unable to induce sufficient levels of the key transcription factors driving plasma cell differentiation. This indicates that the failure of Cr2-/- mice to generate long-lived plasma cells is a B cell-intrinsic defect and is not related to the absence of CD21/CD35 on FDCs.

The reduction of Blimp-1 and XBP-1 levels in PNA low Qβ-specific B cells from Cr2-/- mice was concomitant to a reduced expression of the anti-apoptotic protein Bcl-2 (Figure 2.15 C). This observation is consistent with in vitro studies showing that recruitment of the B cell coreceptor during antigen-dependent B cell activation induced Bcl-2 expression (247). Thus, absence of survival mechanisms regulated by Bcl-2 may further explain the loss of BM plasma cells in Cr2-/- mice.
RESULTS

Figure 2.15: Qβ-specific isotype-switched PNA\textsuperscript{low} B cells from Cr2⁻/⁻ mice exhibit reduced levels of Blimp-1, XBP-1 and Bel-2 mRNA. Qβ-binding isotype-switched PNA\textsuperscript{high} and PNA\textsuperscript{low} B cells, identified as shown in Figure 2.13 A, were purified by FACS from 3-4 pooled spleens 12 d after immunization. Blimp-1, XBP-1 and Bel-2 mRNA levels were determined by quantitative RT-PCR. (A) Expression of Blimp-1 and XBP-1 in purified cells from Cr2⁻/⁻ and WT mice. (B) Blimp-1 and XBP-1 expression in Qβ-specific B cells from chimeric mice having Cr2⁻/⁻ B cells and WT FDCs and from control chimeras. 5x10⁷ splenocytes from Cr2⁻/⁻ or C57BL/6 mice were adoptively transferred into sublethally irradiated C57BL/6-CD45.1 recipients and mRNA levels were determined in CD45.1⁺ B cells from immunized recipient mice. (C) Bcl-2 mRNA levels in sorted cells from Cr2⁻/⁻ and WT mice. Expression levels are depicted in relation to β-actin expression. Quantitative RT-PCR of each sample was performed in triplicates. Results are represented as the mean ± SD. One of two similar experiments is shown.

Qβ-specific isotype-switched B220\textsuperscript{high}PNA\textsuperscript{low} cells are not secreting antibody

We next set out to characterize further the Qβ-specific isotype-switched B220\textsuperscript{high}PNA\textsuperscript{low} B cell population which expressed the transcriptional regulators Blimp-1 and XBP-1 and the anti-apoptotic protein Bel-2 and which was absent in Cr2⁻/⁻ mice. As Blimp-1 and XBP-1 are expressed in plasma cells, we assessed whether Qβ-specific PNA\textsuperscript{low} B cells were secreting antibody and determined the phenotype of splenic plasma cells 12 d after immunization. For detection of Qβ-specific plasma cells, splenocytes were permeabilized and intracellular binding of fluorescently labeled Qβ particles to (CD4; CD8; CD11b)B220\textsuperscript{high} and B220\textsuperscript{low} B cells was determined by flow cytometry. Surface staining was blocked by preincubation with unlabelled Qβ. As shown in Figure 2.16 A, Qβ-specific plasma cells, expressing high levels of cytoplasmic antibodies, had exclusively a B220\textsuperscript{low}PNA\textsuperscript{low} phenotype. These bright
intracellularly stained cells could only be detected after permeabilization, consistent with the fact that terminally differentiated plasma cells downregulate surface Ig expression. Cells expressing Qβ-specific cytoplasmic antibodies were absent when B220<sup>high</sup> B cells were gated, therefore excluding that the Blimp-1 and XBP-1-expressing PNA<sup>low</sup> B cells, which displayed a B220<sup>high</sup> phenotype, were terminally differentiated plasma cells. A population of B220<sup>low</sup>PNA<sup>low</sup> cells exhibiting cytoplasmic Igs specific for Qβ could also be detected in Cr2<sup>-/+</sup> mice. The frequency of these cells was comparable in the spleen of Cr2<sup>−/−</sup> and WT mice (Figure 2.16 B). This is consistent with the normal ASC numbers detected on day 12 in Cr2<sup>−/−</sup> mice by ELISPOT assay, which were in the same range as those obtained by flow cytometry. These results suggest that the Qβ-binding isotype-switched B220<sup>high</sup>PNA<sup>low</sup> population which expresses the transcription factors Blimp-1 and XBP-1 and fails to develop in Cr2<sup>−/−</sup> mice, are antigen-experienced B cells which have left GCs and are committed to a plasma cell fate, but have not yet fully differentiated into ASCs.

![Figure 2.16](image_url)

**Figure 2.16:** B220<sup>high</sup>PNA<sup>low</sup> B cells specific for Qβ are not secreting antibody. (A) Splenocytes from naïve and immunized (day 12) WT mice were permeabilized and intracellular expression of Qβ-specific antibodies was detected with Alexa 647-labeled Qβ. (CD4; CD8; CD11b)B220<sup>high</sup> and B220<sup>low</sup> cells were gated and analyzed for PNA-binding. Mean percentages of Qβ-specific plasma cells are indicated. Surface staining was blocked by preincubation with unlabelled Qβ; the specificity of the staining was controlled with Alexa 647-conjugated...
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AP205. (B) Frequency of Qβ-specific B220^lowPNA^low plasma cells in spleen of Cr2^−/− and WT mice on day 12 after immunization. Values are given as the mean ± SEM.

Qβ-specific GC-derived BM plasma cell precursors are absent in Cr2^−/− mice

A population of B cells representing an intermediary stage before terminal plasma cell differentiation has been described recently (171). These post-GC B cells, which are direct precursors to plasma cells, were identified in the BM and display a phenotype intermediate between splenic B cells and terminally differentiated plasma cells. Such plasma cell precursors were shown to retain expression of the BCR, B220 and MHCII, albeit lower levels than splenic B cells, and to express the plasma cell marker CD138 as well as receptors capable of interacting with BM stroma, such as VLA-4, LFA-1 and CD44 (171). To confirm the identity of Qβ-specific PNA^low Blimp-1/XBP-1-expressing B cells as precursors of plasma cells, the expression of these surface markers identifying post-GC plasma cell precursors was determined on isotype-switched PNA^lowB220^high B cells binding Qβ 12 d after immunization. As shown in Figure 2.17 A, CD138 was induced on a proportion of Qβ-binding PNA^low B cells but not on specific GC B cells. The integrins VLA-4 and LFA-1 as well as CD44 were also upregulated on Qβ-specific PNA^low B cells compared to the PNA^high B cell population. Therefore, cell surface markers which are known to be upregulated in the differentiation process of post-GC B cells to BM plasma cells were induced in isotype-switched Qβ-specific PNA^low B cells. The presence of cells with this plasma cell precursor phenotype was also determined in immunized Cr2^−/− mice (Figure 2.17 B). Consistent with the fact that Blimp-1/XBP-1-positive isotype-switched B cells binding Qβ were absent in these mice, a population of cells with upregulated CD138, VLA-4, LFA-1 and CD44 expression failed to be induced. These results indicate that generation of post-GC precursors to plasma cells requires the interaction of complement-coated antigen with its receptors.

In support of the hypothesis that the identified population represented plasma cell precursors destined to migrate to the BM, Qβ-specific B cells identified in the blood of immunized mice had a B220^highPNA^low phenotype and did not express cytoplasmic IgS (Figure 2.17 C). This suggests that Qβ-specific precursors of plasma cells homed to the BM before terminal differentiation, in accordance with the presence of a plasma cell precursor population in this organ (171). However, cells with surface Ig specific for Qβ could not be detected in the BM at any time point. This is an indication that upon arrival in the BM, precursors of plasma cells
rapidly lose surface Ig expression and acquire cytoplasmic Ig expression as required for antibody secretion.

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Figure 2.17: Qβ-specific isotype-switched PNA\textsuperscript{low} B cells exhibiting a partial plasma cell phenotype are absent in Cr2\textsuperscript{−} mice. (A) Expression of CD138, VLA-4, LFA-1 and CD44 on Qβ-specific PNA\textsuperscript{low} and PNA\textsuperscript{high} B cells from WT mice. B220\textsuperscript{+} splenocytes were purified by magnetic cell sorting; IgM\textsuperscript{low}IgD\textsuperscript{low} B cells binding Qβ and low or high levels of PNA were gated and analyzed for expression of the indicated surface markers. One of three similar experiments is shown. (B) Comparison of CD138, VLA-4, LFA-1 and CD44 expression on Qβ-specific PNA\textsuperscript{low} and PNA\textsuperscript{high} isotype-switched B220\textsuperscript{+} splenocytes from Cr2\textsuperscript{+} and WT mice on day 12 after immunization. (C) Phenotype of Qβ-specific B cells in the blood of WT mice on day 12 after immunization. PNA-binding on Qβ-specific (IgM; IgD; CD4; CD8; CD11b; Gr-1; YO-PRO-1)B220\textsuperscript{+} cells was determined. Expression of cytoplasmic Ig in Qβ-specific PNA\textsuperscript{low} B cells was assessed by analysis of binding of Alexa 647-labeled Qβ to permeabilized (CD4; CD8; CD11b) B220\textsuperscript{low} cells.
Pre-plasma memory B cells are formed normally in Cr2−/− mice

A population of memory B cells, which originate in GCs and can be distinguished from classical recirculating B220+ memory B cells on the basis of their B220−CD138− phenotype, has been described (169, 170). These cells have a greater propensity to form plasma cells than the B220+ memory B cell subset and rapidly differentiate into plasma cells after antigen recall (170). Non-secreting B220−CD138− B cells have been referred to as pre-plasma memory B cells and their formation has been shown to require Blimp-1 expression (167). Since complement receptors played a role in the induction of Blimp-1, we analyzed the generation of pre-plasma memory B cells in Cr2−/− mice immunized with Qβ. As shown in Figure 2.18 A, a population of Qβ-binding (IgD; CD4; CD8; YO-PRO-1)− cells with a B220−CD138− phenotype could be detected in the spleen of immunized Cr2−/− and WT mice. The frequency of these cells on day 12 and 21 was comparable in Cr2−/− and WT controls (Figure 2.18 B). Thus, generation of pre-plasma memory B cells was not dependent on complement receptors, despite their formation reportedly requiring Blimp-1 expression. As already mentioned, cells with surface Ig receptors specific for Qβ could not be detected in the BM and therefore no Qβ-specific pre-plasma memory B cells could be identified in this organ.

Figure 2.18: Pre-plasma memory B cells are induced normally in Cr2−/− mice. (A) Analysis of B220 and CD138 expression on Qβ-binding (IgD; CD4; CD8; YO-PRO-1)− splenocytes to identify B220−CD138− pre-plasma
memory B cells. Mean percentages of B220\(^-\)CD138\(^-\), B220\(^+\)CD138\(^-\) and CD138\(^+\) cells in Cr2\(^-\) and WT mice are indicated. (B) Frequency of Q\(\beta\)-specific pre-plasma memory B cells in spleens of Cr2\(^-\) and WT mice 12 and 21 d after immunization. Results are expressed as the mean ± SEM.

**Generation of isotype-switched Q\(\beta\)-specific PNA\(^{low}\) B cells is GC-dependent**

The fact that short-term anti-Q\(\beta\) antibody responses were normal in Cr2\(^-\) mice suggested that complement receptors were required mainly for differentiation of GC-derived plasma cells. Consequently, if isotype-switched PNA\(^{low}\) B cells, which required complement receptors for induction of Blimp-1 and XBP-1, are post-GC plasma cell precursors, this population should not be induced in the absence of GCs. To address this question we analyzed the generation of isotype-switched B220\(^+\)PNA\(^{high}\) and PNA\(^{low}\) B cells in TNFR1\(^-\) mice, which lack mature FDC networks and do not form GCs (248). As expected, on day 12 after immunization, Q\(\beta\)-specific PNA\(^{high}\) GC B cells were absent in spleens of TNFR1\(^-\) mice (Figure 2.19 A). Q\(\beta\)-specific B cells with a PNA\(^{low}\) phenotype were also more than 10-fold reduced, indicating that these cells are generated in GCs (Figure 2.19 A). Further, lower frequencies of splenic B220\(^{low}\)PNA\(^{low}\) cells expressing cytoplasmic Igs specific for Q\(\beta\) were detected in TNFR1\(^-\) mice (Figure 2.19 B), suggesting that a proportion of these antibody-producing cells derived from GC B cells. Cells with this phenotype were present in normal numbers in Cr2\(^-\) mice (Figure 2.16 B). Hence, some of the plasma cells in the spleen of Cr2\(^-\) mice did arise from the GC reaction and are likely to represent splenic short-lived plasma cells.

The lack of GC formation resulted in reduced anti-Q\(\beta\) IgG antibody levels in TNFR1\(^-\) mice compared to WT controls (Figure 2.19 C). However, early antibody production was normal, similarly to what observed in Cr2\(^-\) mice. Not affected by the absence of GCs was also the ability of TNFR1\(^-\) mice to mount efficient recall responses. These results are consistent with earlier studies showing normal induction of memory B cells and reduced persistence of antibody titers in TNFR1\(^-\) mice after immunization with vesicular stomatitis virus (249). The fact that in the absence of GCs the course of the anti-Q\(\beta\) antibody response was remarkably similar to the response observed in Cr2\(^-\) mice, suggests that Cr2\(^-\) mice have a defect in the generation of GC-derived plasma cells. The reduction of Q\(\beta\)-specific isotype-switched PNA\(^{low}\) B cells in the absence of GCs provides further evidence that this population, which requires complement receptors for upregulation of Blimp-1 and XBP-1, originates in GCs.
RESULTS

Figure 2.19: Isoform-switched Qβ-specific PNA low B cells are reduced in the absence of GC formation. (A) Frequency of total, PNA high and PNA low Qβ-binding isotype-switched B220+ cells in spleens of TNFR1−/− and WT mice 12 d post-immunization. (B) Frequency of splenic B220 low plasma cells with cytoplasmic Ig specific for Qβ in immunized TNFR1−/− and WT mice. (C) Levels of anti-Qβ serum IgG antibodies induced in TNFR1−/− and WT mice by immunization with Qβ. Mice were boosted on day 55 after primary immunization for analysis of recall responses. All data represent the mean ± SEM, mean values statistically different from WT levels are indicated by asterisks (*, p<0.01; **, p<0.05).

Short-term antigen trapping is efficient but long-term antigen persistence is reduced in Cr2−/− mice

Complement receptors together with Fcγ receptors mediate antigen trapping on FDCs thereby sustaining humoral immunity. Therefore, we analyzed antigen retention in the spleen of Cr2−/− and WT mice 12 and 21 d after injection of 100 μg Qβ; at these time points deposits of Qβ particles are found exclusively in B cell follicles (Figure 2.20). Histological staining for Qβ antigen showed that antigen was efficiently trapped in the spleen of Cr2−/− mice at day 12 (Figure 2.20); note that at this time point Blimp-1 and XBP-1 expression in the PNA low B cell population was already dramatically different between Cr2−/− and WT mice. However, 3 wk after immunization, deposits of Qβ antigen were reduced in Cr2−/− mice compared to controls (Figure 2.20). This suggests that binding of Ag-IgG complexes to Fcγ receptors was sufficient to ensure short-term antigen trapping but complement receptors were required for long-term antigen persistence. The reduced time span of Qβ trapping in Cr2−/− mice may be responsible for the faster decline of GC reactions in these mice.
Figure 2.20: Short-term trapping of Qβ particles is efficient but long-term antigen retention is reduced in Cr2⁻/⁻ mice. Histological staining of Qβ antigen on spleen sections from WT and Cr2⁻/⁻ mice on day 0, 12 and 21 after immunization with 100 μg Qβ. Original magnification: x50 (B6, day 0), x75 (Cr2⁻/⁻, day 0), x69 (day 12), x82.5 (day 21).
2.3.4 Discussion

Complement receptors provide an important link between innate and acquired immunity and their role in the induction of humoral immune responses has been clearly demonstrated (124, 126, 250). In this study, we dissected the role of CD21/CD35 complement receptors in the induction of different effectors of humoral memory. We showed that in response to repetitive antigens, such as virus-like particles derived from bacteriophages Qß and AP205, generation of antigen-specific memory B cells was normal, but differentiation of GC B cells into long-lived plasma cells residing in the BM was substantially reduced. As a consequence, Cr2−/− mice were unable to maintain persistent antibody titers, a hallmark of protective, long-lasting humoral immunity. Considering that both memory B cells and BM plasma cells are end products of the GC reaction, it was surprising that only generation of BM plasma cells was affected by absence of complement receptors. Distinct differentiation pathways have been postulated for the selection of these two compartments in GCs. Whereas high affinity for antigen is required for commitment to a plasma cell fate, the memory B cell population appears to be more heterogeneous with respect to affinity and its survival in GCs relies on antigen-dependent signals preventing apoptosis (233, 234). Our results suggest that not only does coligation of the CD21/CD19/CD81 coreceptor and the BCR by complement-coated antigen decrease the affinity threshold needed for B cell activation (122), but it also increases the avidity of the interaction of B cells with antigen as required for promoting differentiation of GC B cells into BM plasma cells. In the absence of CD21, the threshold of signal strength required for differentiation into long-lived plasma cells may not be reached by most GC B cells, but signaling may still be sufficient for differentiation and survival of memory B cells. We therefore speculate that those few BM plasma cells that develop in Cr2−/− mice (Figure 2.12 B) are clones with high affinity BCR, which compensates for the lack of CD21. This would be consistent with increased affinity maturation in Cr2−/− mice, which has been reported but is subject to debate (128, 244). Short-term antibody responses induced by immunization with Qß were normal in Cr2+/− mice. This indicates that different mechanisms underlie regulation of short-lived, non GC-derived plasma cells participating to the early phase of the antibody response and BM plasma cells responsible for long-term maintenance of memory titers.

The degree of antigen organization is crucial to the activation of B cells (62) as well as to the requirement for costimulatory molecules (251). Highly repetitive antigens such as viral
particles are capable of efficient crosslinking of BCRs, which induces potent antibody responses even in the absence of T cells (174) or CD21/CD35 (181). Qβ capsids display a highly ordered structure comparable to that of viruses, conferring on them the ability to efficiently crosslink surface Ig on B cells. This may explain why antibody responses and GC reactions were efficiently induced by immunization with Qβ particles in Cr2-/- mice and is consistent with previous reports of normal antibody responses to vesicular stomatitis virus (243) and influenza virus (252) in these mice. In contrast, efficient antibody responses to other experimental antigens lacking the structural feature repetitiveness seem to be more dependent on CD21 (126, 127, 244). However, although antigen repetitiveness was able to compensate for the absence of CD21 to some extent, generation of persistent serum antibody was not achieved by immunization with Qβ. Similarly, an increase of antigen load or administration of antigen in inflammatory adjuvants has been shown to mitigate the defects in humoral responses of Cr2-/- mice (124, 128, 244); nevertheless antibody persistence was impaired even with optimal antigen doses in adjuvant (244).

Reduction of Qβ-specific BM plasma cells in Cr2-/- mice correlated with a failure to induce Blimp-1 and XBP-1 expression in post-GC B cells, from which long-lived BM ASCs are thought to arise (253). This observation suggests that complement receptors are essential for Blimp-1/XBP-1-mediated induction of plasmacytic differentiation in GCs. Blimp-1 has been described to be expressed in a small subset of GC B cells (254, 255). Owing to their partial plasma phenotype these Blimp-1+ GC cells were assumed to be committed to exit GCs and to differentiate into plasma cells. In contrast to previous reports we did not detect Blimp-1 expression in Qβ-specific GC B cells, but transcription of Blimp-1 was present in isotype-switched B cells with a PNA low phenotype and surface Ig specific for Qβ. Consistent with the expression of transcriptional regulators driving plasma cell differentiation, these cells displayed a partial plasma cell phenotype, characterized by the upregulation of CD138, VLA-4, LFA-1 and CD44. A population of Qβ-specific B cells with this phenotype was absent in Cr2-/- mice. This is in accordance with a failure of post-GC B cells to induce Blimp-1 and XBP-1 in the absence of complement-mediated stimulation. Despite expression of Blimp-1 and XBP-1, Qβ-specific PNA low B220 high B cells did not stain for intracellular Ig, indicating that BM plasma cell precursors may leave the spleen before they secrete antibodies.

A clear role for complement receptors on FDCs in the maintenance of B cell memory has been demonstrated in chimeric mice with Cr2-/- FDC stroma and normal B cells (256). FDCs are thought to mediate long-term antigen retention, which may continually stimulate
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differentiation of memory B cell into ASCs (147, 257). The reduced persistence of Qβ particles on FDCs observed in Cr2^+ mice in this study, confirmed the role of complement receptors in long-term antigen retention. However, the presence of substantial Qβ depots in Cr2^-/- mice on day 12 suggests that Ag-IgG complexes on FDCs were efficiently trapped through Fcγ receptors for short periods after immunization. Despite normal antigen trapping at this time point, Cr2^-/- mice failed to induce Blimp-1, XBP-1 and Bcl-2 expression and to upregulate surface molecules characteristic of plasma cell precursors. This indicates that reduced antigen retention was not responsible for the observed phenotype and is in agreement with previous studies reporting a direct role for CD21/CD35 on B cells for induction of long-lasting antibody responses (119, 258). Nevertheless, a lack of complement receptors on FDCs and consequent reduced long-term antigen trapping may contribute to the inability of Cr2^-/- mice to maintain long-term ASCs and is compatible with our observation that GC reactions decayed more rapidly.

In conclusion, our results suggest that induction of long-lasting antibody production, which is mediated primarily by BM plasma cells, requires more than BCR signaling, even with antigens that are capable of efficient BCR crosslinking, such as viral particles. A complement-mediated signal, revealing the activation of the innate immune system, is essential. This allows focusing long-term antibody production on pathogens and keeps the induction of plasma cells capable of secreting specific IgG antibodies over extended periods of time under tight control of both the adaptive and the innate immune system.
2.3.5 Materials and methods

Mice and antigens
C57BL/6 mice (Harlan, Horst, The Netherlands), C52rA- mice (124), TNFR1−/− mice (259) and C57BL/6-CD45.1 mice were immunized i.v. with 10 or 100 µg Qβ or 25 µg AP205. Animal experiments were conducted in accordance with protocols approved by the Swiss Federal Veterinary Office.

Qβ capsids were expressed using the vector pQβ10 and purified as described (203). AP205 coat protein (221) was cloned into the pQb10 vector (175) and expressed and purified similarly as Qβ.

ELISA
ELISAs were performed as described (220). Titers represent log2 dilutions of 40-fold prediluted sera at half maximal OD.

ELISPOT assay
Qβ/AP205-specific ASC frequencies were determined as described (260). Briefly, 24-well plates were coated with 10µg/ml Qβ or AP205. Spleen or BM cells were added in MEM containing 2% FCS and incubated for 5 h at 37°C. Cells were washed off and plates were incubated successively with goat anti-mouse IgG (EY Labs) and alkaline phosphatase-conjugated donkey anti-goat IgG antibodies (Jackson ImmunoResearch) before development of alkaline phosphatase color reactions.

Flow cytometry
Detection of B cells expressing Qβ-specific surface Ig was performed by incubation with Qβ, followed by a polyclonal rabbit anti-Qβ serum (produced by RCC Ltd., Switzerland) and Cy5-conjugated donkey anti-rabbit IgG serum (Jackson ImmunoResearch Laboratories). AP205-specific B cells were identified similarly, using a polyclonal rabbit anti-AP205 serum (generated in the laboratory of Dr. P. Pumpens, University of Latvia, Riga).

Isotype-switched B cells were detected with a mixture of FITC-conjugated antibodies (anti-IgD, 11-26c.2a; goat anti-IgM serum, JacksonImmuno Research Laboratories; anti CD4, GK1.5; anti CD8, 53-6.7; anti-CD11b, M1/70; anti-Gr-1, RB6-8C5) and PE-TxR-conjugated anti-B220 (RA3-6B2). Biotinylated peanut agglutinin (PNA; Vector Laboratories) and streptavidin-PE were used to assess PNA-binding. Pre-plasma memory B cells were detected
with biotinylated anti-CD138 (281-2), streptavidin-Tricolor, PE-conjugated anti-B220 (RA3-6B2) and FITC-conjugated antibodies to IgD (11-26.c.2a), CD4 (GK1.5) and CD8 (53-6.7). Dead cells were excluded by staining with 0.005 μg/ml YO-PRO-1 (Molecular Probes).

To characterize PNA\textsuperscript{low} Qβ-specific B cells, B220\textsuperscript{+} splenocytes, purified by magnetic cell sorting with B220 MicroBeads (Miltenyi Biotec), were stained with biotinylated antibodies (anti-CD138, 281-2; anti-CD11a, M17/4; anti-CD49d, R1-2; anti-CD44, IM7) followed by streptavidin-Tricolor (Caltag) and PE-conjugated goat anti-mouse IgM F(\text{ab}')\textsubscript{2} (SouthernBiotech), PE-conjugated rat anti-mouse IgD (11-26, e-Bioscience), FITC-conjugated PNA.

Qβ-specific plasma cells were detected by incubation with unlabeled Qβ, to block binding to surface IgG, and biotinylated PNA followed by streptavidin-PE, PE-TxR-conjugated anti-B220 (RA3-6B2) and FITC-conjugated antibodies to CD4 (GK1.5), CD8 (53-6.7) and CD11b (M1/70). After permeabilization, cells were incubated at room temperature with Qβ particles labeled with the fluorochrome Alexa 647, using the Alexa Fluor 647 Protein Labeling Kit (Molecular Probes).

Fc-receptors were blocked with anti-mouse CD16/32 (2.4G2). Antibodies were purchased from BD Biosciences, unless otherwise specified.

**Adoptive transfer experiments**

5x10\textsuperscript{7} splenocytes from naïve Cr2\textsuperscript{-/-} and C57BL/6 mice were transferred with 10 μg Qβ into sublethally irradiated (450 Rad) C57BL/6-CD45.1 recipient mice. Irradiated control mice were given antigen but no cells.

For adoptive transfer of memory B cells, Qβ-binding IgM\textsuperscript{low}IgD\textsuperscript{low} and total IgM\textsuperscript{low}IgD\textsuperscript{low} splenocytes were purified by FACS from C57BL/6 mice immunized 6 wk previously. Control naïve B cells (IgM\textsuperscript{+}IgD\textsuperscript{+}) were sorted from unimmunized mice. Single cell suspensions of 10\textsuperscript{4} Qβ-binding IgM\textsuperscript{low}IgD\textsuperscript{low}, 10\textsuperscript{5} IgM\textsuperscript{low}IgD\textsuperscript{low} or 10\textsuperscript{5} IgM\textsuperscript{+}IgD\textsuperscript{+} B cells were injected together with 10\textsuperscript{7} purified CD4\textsuperscript{+} cells into sublethally irradiated recipients, which were immunized with 10 μg Qβ.

**Immunohistochemistry**

Freshly removed organs were snap frozen in liquid nitrogen. Tissue sections of 5 μm thickness were cut in a cryostat and fixed with acetone. For detection of Qβ antigen, sections were incubated with rabbit anti-Qβ serum (RCC), followed by biotinylated sheep anti-rabbit Iggs (The Binding Site) and alkaline phosphatase-labeled streptavidin (Roche). Alkaline
phosphatase was visualized using the Vector Blue substrate (Vector Laboratories). Sections were counterstained with Vector Nuclear Fast Red (Vector Laboratories). For detection of Qβ-specific B cells, spleen sections were incubated with Qβ and bound particles were detected with a polyclonal anti-Qβ serum as described (220). PNA-binding cells were stained with biotinylated PNA (Vector Laboratories) followed by avidin-biotin-peroxidase complexes (DAKO) before alkaline phosphatase was visualized.

Quantitative RT-PCR

0.5-1×10⁵ specific B cells were sorted into TRI Reagent (Molecular Research Center) and total RNA was extracted according to the manufacturer's instructions. First strand cDNA was synthesized using random nonamer primers and SuperScript II reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed on an iCycler Thermal Cycler (Bio-Rad) using the following primers for amplification (sense primer is given first): for Blimp-1 ATGGAGGACGCTGATATGAC and GATGCCTCGGCTTGAAC; for XBP-1 CGTAGACGTTCTGGCTATG and GGACCGGGTACCATAATG; for Bcl-2 TCGTGACCTCAGAGATG and AACTCAAAGAAGGCCACAATC; for β-actin TCACCATGGATGATGATATCGC and TGAAGGTCTCAAACATGATCTGG. Quantification of β-actin cDNA was performed for each sample to allow for normalization between samples. Dissociation curve analysis was performed to verify the presence of a single PCR product. Quantification of the transcripts was determined with the iCycler iQ Optical System Software (Bio-Rad) using the comparative threshold cycle method.

Statistical analysis

Levels of statistical significance between means were determined using the Student’s t test.

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2.4 Part IV: Regulation of memory antibody levels: key role for duration of antigen presentation on follicular dendritic cells

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Abbreviations used in this paper: AFC, antibody-forming cell; FDC, follicular dendritic cell; GC, germinal center; LT, lymphotoxin; PNA, peanut agglutinin; VLP, virus-like particle
2.4.1 Abstract

Protective antibody levels can be maintained for years upon infection or vaccination. In this report, we studied the role of persisting antigen associated with follicular dendritic cells (FDCs) in maintaining B cell memory. As a model antigen, virus-like particles (VLPs) derived from bacteriophage Qβ were used. This form of antigen cannot replicate in the host and does not need to be administered in adjuvant for induction of strong and long-lasting antibody responses. Thus, VLPs can only persist as “natural depot” on FDCs. We addressed the question of the importance of antigen retained on FDCs at distinct stages of the B cell response by depleting the FDC population with a lymphotoxin β receptor (LTβR)-Ig fusion protein at different time points after immunization. Our results demonstrate that the induction of germinal center (GC) reactions and the establishment of humoral memory were dependent on FDC-associated antigen. Generation of bone marrow plasma cell precursors and memory B cells required the presence of FDC-associated antigen early after immunization but the contribution of the ongoing GC reaction to the maintenance of antibody levels was negligible at later time points. These findings indicate an important role for antigen persisting on FDCs in the first weeks after immunization, when long-term effectors of humoral memory are induced. At later stages after immunization, the response is largely antigen-independent and maintained by a gradually declining population of antibody-secreting plasma cells in the bone marrow.
2.4.2 Introduction

Antigen can persist in the spleen and lymph nodes of immunized animals for extended periods of time following immunization (99). It is sequestered in the form of immune complexes on the surface of follicular dendritic cells (FDCs), which reside in the primary B cell follicles and germinal centers (GCs) (99). Deposits of unprocessed antigen retained on FDCs are believed to play a role in the development of GC reactions and to be important for affinity maturation. However, FDCs have also been proposed to provide non-specific antigen-independent support for B cell proliferation and differentiation, questioning the function of FDC-associated immune complexes (261, 262). Antigen deposits on FDC are further appreciated to play a role in the maintenance of B cell memory, but the general requirement for antigen persistence in long-term humoral responses is still subject to debate. Although memory B cells have been demonstrated to possess the potential to survive in absence of specific antigen and detectable immune complexes (143, 144), they have been shown to proliferate in association with persisting antigen and to require stimulation with antigen to differentiate into antibody secreting cells (133, 146). The continued proliferation and differentiation of memory B cells into short-lived plasma cells has been proposed to maintain humoral memory (99, 147). However, this model has been called into question by the observation that plasma cells residing in the bone marrow are long-lived and can secrete antibody for extended periods of time without requiring antigen (152, 153, 160). Based on these findings a major role for bone marrow plasma cells in preserving serum antibody titers has been suggested. Thus, the relative contribution of long-lived bone marrow plasma cells and continually differentiating memory B cells to the maintenance of humoral memory remains controversial. To address the role of persisting antigen in regulating B cell memory, we depleted the FDC population and, as a consequence, FDC-associated antigen, using a lymphotoxin β receptor (LTβR)-Ig fusion protein. LTα/β signaling is required for maintenance of mature FDC networks and inhibition of the interaction of FDCs with LTα/β expressing B cells by injection of LTβR-Ig fusion proteins has been shown to cause a rapid disappearance of functional FDCs and the markers specific of this population, such as FDC-M1, FDC-M2 and CR1 (263, 264). Furthermore, LTβR-Ig treatment has been demonstrated not only to prevent trapping of newly formed immune complexes, but also to eliminate previously sequestered antigens (263). Previous studies have reported that injection of LTβR-Ig before immunization abolished GC formation and led to impaired antibody responses to sheep red blood cells (264). However, the effect of
LTβR-Ig treatment after the B cell response has been induced and, in particular, at late time points after immunization has not yet been addressed. Here we report that depletion of FDCs and the associated antigen had a major impact on the induction of B cell responses to virus-like particles (VLPs) when performed during the early phase of the response, but was largely irrelevant once the antibody response had been established. Our findings suggest that antigen trapped on FDCs is required for establishing memory B cells and long-lived plasma cells, but plays a negligible role at later time points after immunization with VLPs, when persisting antibody levels are mainly maintained by a very slowly declining plasma cell population residing in the bone marrow.
2.4.3 Materials and methods

Mice and antigens
C57BL/6 mice (Harlan, Horst, The Netherlands) were immunized i.v. with 10 µg Qβ. Qβ capsids were expressed using the vector pQβ10 and purified as described (203).

Depletion of FDCs and retained antigen
LTβR-Ig was prepared by fusing the coding region of the extracellular domain of the LTβR to the constant region of human IgG1. The construct was transfected into EBNA cells (Invitrogen) using Lipofectamin Plus (Invitrogen) and cultured in serum-free medium. The LTβR-Ig fusion protein was purified using Protein G Sepharose columns (Pharmacia). Mice were injected i.p. with 300 µg LTβR-Ig on days 9 and 11 (early FDC-depletion), 39 and 41 (late FDC-depletion) and 100 and 102 (very late FDC-depletion) after immunization with Qβ particles.

ELISA
ELISAs were performed as described (220). Titers represent log2 dilutions of 40-fold prediluted sera at half maximal OD.

ELISPOT assay
Qβ-specific antibody-forming cell frequencies were determined as described (260). Briefly, 24-well plates were coated with 10µg/ml Qβ. Spleen or BM cells were added in MEM containing 2% FCS and incubated for 5 h at 37°C. Cells were washed off and plates were incubated successively with goat anti-mouse IgG (EY Labs) and alkaline phosphatase-conjugated donkey anti-goat IgG antibodies (Jackson ImmunoResearch) before development of alkaline phosphatase color reactions.

Flow cytometry
Detection of B cells expressing Qβ-specific surface Ig was performed by incubation with Qβ, followed by a polyclonal rabbit anti-Qβ serum (produced by RCC Ltd., Switzerland) and Cy5-conjugated donkey anti-rabbit IgG serum (Jackson ImmunoResearch Laboratories) as previously described (220). Isotype-switched B cells were detected with a mixture of FITC-conjugated antibodies (anti-IgD, 11-26c.2a; goat anti-IgM serum, JacksonImmuno Research Laboratories; anti CD4, GK1.5; anti CD8, 53-6.7; anti-CD11b, M1/70; anti-Gr-1, RB6-8C5)
and PerCP-Cy5.5-conjugated anti-CD19 (1D3). Biotinylated peanut agglutinin (PNA; Vector Laboratories) and streptavidin-PE were used to assess PNA-binding. Dead cells were excluded by staining with 0.005 μg/ml YO-PRO-1 (Molecular Probes). Fc-receptors were blocked with anti-mouse CD16/32 (2.4G2). Antibodies were purchased from BD Biosciences, unless otherwise specified.

**Immunohistochemistry**

Freshly removed organs were snap frozen in liquid nitrogen. Tissue sections of 5 μm thickness were cut in a cryostat and fixed with acetone. For detection of Qβ antigen, sections were incubated with rabbit anti-Qβ serum (RCC), followed by biotinylated sheep anti-rabbit Iggs (The Binding Site) and alkaline phosphatase-labeled streptavidin (Roche). FDCs were detected using the rat FDC-M1 (BD Biosciences) or FDC-M2 (ImmunoKontact) antibodies followed by biotinylated rabbit anti-rat antibodies (Jackson Immuno Research Laboratories) and alkaline phosphatase-labeled streptavidin (Roche). IgD-expressing B cells were stained with sheep anti-mouse IgD antibodies (The Binding Site) and horse radish peroxidase-labeled rabbit anti-sheep antibodies (Jackson Immuno Research Laboratories). Alkaline phosphatase was visualized using the Vector Blue substrate (Vector Laboratories) and horse reddish peroxidase with the substrate diaminobenzidine (Sigma).
2.4.4 Results and discussion

We have previously shown that a single injection of VLPs derived from bacteriophage Qβ elicits strong, long-lasting IgG responses (220). Immunization with Qβ induces efficient GC reactions, which peak around day 12 and are maintained at low levels at least up to 100 days post-immunization (220). We further observed that Qβ particles are rapidly transported into B cell follicles after intravenous immunization (220), where the antigen could be exclusively detected on FDCs later after injection, and persisted in high amounts up to 3 weeks after immunization (265) and even longer (unpublished data). Indeed, since VLPs cannot replicate in the host and do not require administration in adjuvant, they are only expected to persist as immune complexes on the surface of FDCs.

To reveal the role of FDC-associated antigens in driving B cell memory, we attempted to deplete FDCs using a LTβR-Ig fusion protein. In order to evaluate the efficacy of this treatment in depleting FDCs and the associated antigen, mice were immunized with Qβ-derived VLPs and, on day 9 and 11 after immunization, LTβR-Ig was injected. Disappearance of FDCs and trapped Qβ particles was assessed by immunohistochemistry one week after LTβR-Ig treatment. As shown in Figure 2.21 A, injection of LTβR-Ig led to the loss of FDCs, as revealed by an absence of staining for the FDC-specific markers FDC-M1 and FDC-M2. Consistent with the disappearance of FDCs, no Qβ antigen could be detected in mice treated with LTβR-Ig, in contrast to the readily visible deposits present in untreated control mice (Figure 2.21 B). The activity of the LTβR fusion protein was confirmed by the reduction in frequencies of marginal zone B cells observed in treated mice (not shown), which is in agreement with the reported alteration of the marginal zone organization caused by inhibition of LTα/β signaling (40, 264). Thus, a substantial depletion of FDC-associated antigen was achieved by treatment with LTβR-Ig.
We studied the effect of a depletion of FDCs and the related role of antigen persisting on this cell population at three different stages of the B cell response: early (−d10), late (−d40) and very late (−d100) after immunization. For these analyses anti-Qβ serum IgG titers, numbers of Qβ-specific IgG antibody-forming cell in spleen and bone marrow and frequencies of Qβ-binding total isotype-switched B cells and GC B cells in the spleen were determined as previously described (220, 265). As shown in Figure 2.22, substantial frequencies of Qβ-specific B cells and plasma cells were induced by immunization with the VLPs, consistent with earlier observations (220, 265). In the first set of experiments, FDCs and retained Qβ antigen were depleted on days 9 and 11 after immunization, i.e. shortly before GC reactions reach the peak, and the impact on the B cell response was assessed 10 and 45 days after the last LTβR-Ig treatment (corresponding to days 21 and 56 after immunization). As shown in Figure 2.22 A, isotype-switched B cells specific for Qβ were 10-fold reduced in spleens of treated animals compared to controls 10 days after depletion of FDCs and the associated antigen. Qβ-specific GC B cells were also drastically (13-fold) reduced and they accounted for most of the loss of specific cells, since 80-90% of Qβ-binding B cells were PNA^high in at this time point (Figure 2.22 A). No reduction in Qβ-specific total or GC B cells was observed in mice injected with control Ig protein (not shown). Frequencies of anti-Qβ IgG antibody-forming cells in the spleen, but not those in the bone marrow, were also slightly lower in mice
injected with LTβR-Ig compared to untreated controls (Figure 2.22 B). However, no short-
term effect of the treatment on specific serum antibody titers was observed (Figure 2.22 C).
The analysis of mice 45 days after FDC-associated antigen had been depleted further
highlighted a major impact of an early LTβR-Ig treatment on the long-term antibody response
induced by VLPs. At this later time point, treated mice displayed a 3-fold reduction in anti-Qβ
serum antibody levels (Figure 2.22 C), which was the result of a corresponding reduction of
Qβ-specific antibody-forming cells in both the spleen and bone marrow (Figure 2.22 B).
Similarly to what observed at the earlier time point of analysis, frequencies of Qβ-specific
isotype-switched B cells were also lower in spleens of mice which had been depleted of FDC-
associated antigen (Figure 2.22 A). The reduction of bone marrow plasma cells became
obvious only at this later time point after mice had been treated with LTβR-Ig and the GC
reaction prematurely stopped. We conclude that massive numbers of bone marrow plasma cell
precursors are generated in GCs in the first month after immunization and subsequently
localize to the bone marrow. Early administration of LTβR fusion protein aborted GC
reactions, as evidenced by drastically reduced Qβ-specific PNA^high B cells (Figure 2.22 A),
and resulted in a cessation of bone marrow plasma cell generation, as this antibody-secreting
population originates in GCs. Thus, in the early stages of B cell responses, FDCs and the
antigen retained on their processes play a crucial role in the development of GCs and in
establishing long-term antibody production. However, an absolute need for persisting antigen
cannot be asserted from this analysis, since the impact of an absence of antigen deposits on
FDCs cannot be distinguished from an effect deriving from perturbed GC reactions. While
formation of GCs is indisputably important for induction of normal IgG responses, a
requirement for the presence of antigen on FDCs for their development has recently been
questioned. In fact, studies in transgenic mice deficient in secreted antibody have reported
normal GC responses in the absence detectable immune complexes (143, 266), arguing
against an essential role for FDC-bound antigen in GCs development and suggesting that
deposition of immune complexes on FDCs might be dispensable for generation of normal
humoral immune responses.
RESULTS

Figure 2.22: Early depletion of FDCs and associated antigen inhibits normal induction of B cell responses to immunization with Qβ. Mice immunized with Qβ particles were treated with LTβR-Ig on day 9 and 11 post-immunization. Total isotype-switched Qβ-binding B cells and Qβ-binding PNA$^{high}$ germinal center B cells in the spleen (A), Qβ-specific IgG antibody-forming cells in spleen and bone marrow (B) and anti-Qβ IgG serum antibody titers (C) were determined for LTβR-Ig treated and control untreated mice on day 21 and 56 after immunization.

We next addressed the importance of antigen retained on FDCs after the bulk of the GC reaction had terminated and most GC B cells had differentiated into memory B cells or bone marrow plasma cells. For this purpose, mice were treated with LTβR-Ig on days 39 and 41 after immunization with Qβ; the effect of the treatment was subsequently analyzed 21, 50 and
106 days after the last injection of the fusion protein (corresponding to day 60, 89 and 145 after immunization). Depletion of FDCs and the associated antigen resulted in a short-term reduction of isotype-switched Qβ-specific B cells (Figure 2.23 A), as expected from the fact that GC reactions were still ongoing, albeit at lower levels, at this later time of treatment. However, generation and maintenance of memory B cells was not significantly affected by this late injection of the LTβR-Ig, as demonstrated by comparable frequencies of Qβ-binding B cells present in spleens of treated and control mice 145 days after immunization (Figure 2.23 A). This suggests that most Qβ-specific memory B cells had been generated by the time of depletion of FDCs and the antigen retained on their surface and that the maintenance of this Qβ-specific memory B cell population was independent of persisting antigen. In agreement with the experiments in which LTβR-Ig was injected early, existing GCs were eliminated, as indicated by the reduction of Qβ-specific PNA<sup>high</sup> cells observed in spleens of treated mice compared to controls (Figure 2.23 A). However, in contrast to the dramatic effect of an early abrogation of GC reactions, termination of GC reactions at this later time point after immunization had no impact on the number of Qβ-specific spleen and bone marrow antibody-forming cells (Figure 2.23 B). Thus, the contribution of GCs to the long-lived bone marrow plasma cell pool late after immunization was negligible and a stable population of plasma cells could be maintained independently of the presence of antigen. Consistently, no significant difference in anti-Qβ serum antibody titers was observed between mice in which FDC-associated antigen had been eliminated by LTβR-Ig treatment and untreated control mice (Figure 2.23 C). These findings indicate that humoral memory had largely been established by the 5-6 week post-immunization and could be maintained without detectable antigen.
Figure 2.23: Late depletion of FDC-associated antigen has no impact on the generation and maintenance of long-term humoral responses to Qβ particles. Mice were injected with LTβR-Ig 39 and 41 days after immunization with Qβ. On days 60, 89 and 145 after immunization total isotype-switched Qβ-binding B cells and Qβ-binding PNA<sup>high</sup> germinal center B cells in the spleen (A), Qβ-specific IgG antibody-forming cells in spleen and bone marrow (B) and anti-Qβ IgG serum antibody (C) titers were determined.

In accordance with the previous results, depletion of persisting antigen very late after immunization (on day 100 and 102) had no noticeable effect on the frequency of Qβ-specific memory B cells or on the number of bone marrow plasma cells secreting anti-Qβ antibodies (Figure 2.24). Notably, at this late time point after immunization a large proportion of the plasma cells secreting antibodies specific for Qβ was localized in the bone marrow, consistent
with the notion that the bone marrow is a major site of antibody production at late stages of humoral responses (158, 245). Thus, the maintenance of Qβ-specific long-term B cell effector populations, bone marrow plasma cells and memory B cells, did not require the presence of the antigen persisting on FDCs.

**Figure 2.24:** Maintenance of memory B cells and bone marrow plasma cells is independent of antigen persisting on FDCs. Mice immunized with Qβ were treated with LTβR-Ig on day 100 and 102 after immunization. Frequencies of total isotype-switched Qβ-binding memory B cells in the spleen (A) and of Qβ-specific IgG antibody-forming cells in spleen and bone marrow (B) were assessed 8 weeks after depletion of FDC-associated antigen (i.e. 158 days after immunization).

Taken together our results demonstrate that antigen deposits on FDCs are required in the early phase of B cell responses to VLPs, when GCs afford a significant output of cells destined to differentiate into memory B cells and long-term antibody-secreting plasma cells. At later time points after immunization, although some B cells continue to proliferate in GCs, their overall contribution to the pool of memory B cells and bone marrow plasma cells is limited. This finding is consistent with previous studies showing that abrogation of GC reactions by injection of anti-CD40L antibody early after immunization with haptinated proteins had a more dramatic impact on the generation of high affinity bone marrow antibody-forming cells than a late disruption of GCs (267). Similarly, treatment with anti-CD40 after well formed GCs were present in the spleen has been reported not affect short-term serum antibody levels (268).

Furthermore, our experiments indicate that antibody production late after immunization with VLPs could be maintained in the absence of antigen retained on FDCs and suggest a major role for long-lived plasma cells residing in the bone marrow in preserving long-term elevated serum antibodies. This argues against an important contribution of memory B cells
continually differentiating into short-lived plasma cells through stimulation with antigen to the maintenance of antibody titers to VLPs in the late phase of the B cell response. However, our results confirm earlier studies showing that the memory B cell population can survive in the absence of detectable antigen. We conclude that, in the response to VLPs, antigen persisting on FDCs is required during the first few weeks of the response for establishing memory B cells and long-lived plasma cells, but appears to be largely dispensable for maintaining memory antibody levels.
3 GENERAL DISCUSSION

In light of the great number and diversity of pathogenic microorganisms encountered by the immune system throughout life, it is conceivable that distinct B cell populations have evolved to ensure rapid and long-lasting protection. B cells of the marginal zone and B1 B cell subsets mediate rapid protection in the early phase of humoral immune responses. These two populations of B cells are not only strategically located at the ports of entry of gut/peritoneum and blood-born pathogens, they are also capable of responding to antigen contact with rapid proliferation and differentiation into antibody-secreting cells (83). Thus, effectors from the marginal zone and B1 B cell compartments generate an early wave of antibodies, before the numerically dominating follicular B cells are activated and take over the response. Efficient protection against infectious organisms not only requires rapid antibody production, to hinder the fast multiplication and spreading of pathogens, it must also be of long-lasting nature, in order to avoid reinfection with the same pathogen. The induction of B cell memory is a competence of the follicular B cell population. Besides generating highly diverse and specific antibodies to combat established infections, follicular B cells differentiate into memory B cells or bone marrow plasma cells (130). These two effector populations of the late phase of the B cell response are responsible for maintaining high levels of specific antibodies over extended periods of time. However, the immune system keeps long-lasting antibody production under tight control. Hence, it has evolved distinct mechanisms, involving antigen as well as signals from the innate arm, to regulate the generation and maintenance of the populations of B cells responsible of preserving long-term antibody secretion.

3.1 Antibody responses to VLPs: analogies and differences to other common experimental antigens

Immunologists have made use of many different experimental antigens to study antibody responses and the rules by which they are governed. The analysis of the B cell responses induced by these antigens has afforded a broad knowledge of the regulation of antibody production but has also led to many conflicting findings. Much information has been derived from studying B cell responses elicited by haptenated proteins, which have constituted an important tool to investigate B cell immunity. As previously discussed, hapten-protein conjugates are dependent on the presence of T cell help for induction of IgM and IgG
antibodies. After injection of haptenated proteins, hapten-specific germinal centers are formed; these germinal center reactions peak early (around day 7-8), then rapidly decline and have usually terminated three weeks after immunization (90, 267). B cell memory is also efficiently induced by these antigens, as demonstrated by efficient recall responses, increased frequencies of hapten-binding memory B cells, and the accumulation of specific antibody-forming cells in the bone marrow (90, 204, 267). Generation of strong antibody responses by haptenated proteins requires the presence of adjuvant, which aids in their presentation and ensures long-term antigen release. However, although extensive analysis of specific antibody responses to haptenated proteins have contributed substantially to the understanding of how B cell responses are regulated, the antigenic properties of hapten-protein conjugates in adjuvant may differ substantially in many respects from those of naturally occurring pathogens. In addition, it has been shown that the frequencies of hapten-specific naïve B cells are much higher than those of B cells specific for biologically relevant antigens, such as neutralizing epitopes on viruses or bacterial toxins (148). These increased B cell frequencies could have an influence on the outcome of some of the studies. Thus, the use of viruses or bacteria as immunogens may prove more reliable when trying to dissect the mechanisms underlying humoral immunity to pathogens. A virus that has been widely used to study B cell responses is VSV. In contrast to haptenated proteins, VSV is capable of activating B cells in the absence of T cell help (269). T cell-independent IgM responses are followed by strictly T cell-dependent IgG responses (270), which only slowly decline with time (146). Germinal centers are efficiently induced by immunization with VSV, reach maximal numbers in the second and third week after infection and can be still observed at very late time-points after immunization (146). This marks a clear difference to the germinal center reactions triggered by haptenated proteins, which are fully established earlier but are of more limited duration. Similarly to hapten-protein conjugates and other T cell-dependent antigens, generation of B cell memory is a hallmark of the response induced by immunization with VSV (136).

VLPs lie somewhere between “real world” antigens, such as viruses and bacteria, and commonly used artificial antigens, such as haptenated or soluble proteins. When compared to humoral immune responses directed against naturally occurring infectious microorganisms, B cell responses induced by VLPs represent a simplification. In particular, the replication of pathogens and their interactions with receptors on cells, as well as the activation of the innate immune system caused by infectious microorganisms, may have an influence on the B cell response. Nevertheless, the organization and presentation of antigenic epitopes on VLPs closely resembles that of viruses and therefore VLPs possess the structural features of
naturally occurring pathogens, in contrast to haptenated proteins or other experimental
antigens. Furthermore, as opposed to hapten-protein conjugates, adjuvant is not required for
the induction of antibody responses with VLPs, allowing a more physiological presentation of
the antigen. The common structural properties of VLPs and viruses are likely to be the reason
for the many similar characteristics of the B cell responses elicited by these two groups of
antigens. The studies presented in this thesis showed that Qβ particles were capable of
activating B cells in the absence of T cell help (220), as observed for viruses like VSV.
Moreover, the induction of IgG antibodies required MHCII-restricted T cell help (220), again
similarly to what observed for VSV. Not surprisingly, as for VSV (271), the CD40-CD40L
interaction was critical for the induction of IgG responses to Qβ (unpublished data). As
expected from the strong IgG response, efficient germinal center formation was detected after
immunization with Qβ (220, 265). Germinal center B cells reached maximal numbers
between the second and third week post-immunization and could still be detected at very late
time points after immunization (220). Thus, Qβ-specific germinal centers reactions peaked
later and were prolonged compared to those induced by haptenated proteins (90, 267) but
were reminiscent of those observed after injection of VSV (146). Like for haptenated proteins
and VSV, memory B cells and long-lived plasma cells residing in the bone marrow were the
output of the germinal center reaction induced by Qβ particles and provided long-lasting
antibody production (220, 265, 272).

Efficient antibody responses to most antigens fail to be induced in the absence of organized
lymphoid tissue. This has been demonstrated by the study of mice deficient in TNF family
molecules or their receptors, which lack a normal splenic architecture (273, 274). As reported
for non-viral T cell-dependent antigens, organized primary B cell follicles and FDC networks
were required for induction of normal antibody responses to Qβ particles. This was
demonstrated by the reduced anti-Qβ IgG response mounted by TNFR1−/− mice (265). From
this point of view Qβ clearly differs from live VSV, which has been reported to induce strong
neutralizing antibody in the absence of mature FDC networks and germinal center formation
(249). Induction of pro-inflammatory cytokines by infection with live VSV is a likely
explanation for the observed difference. Indeed, UV-inactivated VSV particles also induced
reduced antibody responses in TNFR1−/− mice (249).

The efficiency of antigen trapping is another parameter influencing B cell responses. Shortly
after intravenous injection, Qβ particles were localized in the marginal zone and red pulp of
the spleen, and were present at high levels in macrophages (220). Antigen was also rapidly
transported into B cell follicles, the only site where antigen deposits could be detected at later
time points after immunization (220, 265). In follicles and germinal centers Qß particles were retained on the surface of FDCs where they persisted at detectable levels at least up to day 30 (265). Clearance of these VLPs from the circulation therefore occurred similarly to what reported for VSV, which was also detected in the marginal zone and red pulp few hours after intravenous injection, rapidly localized to B cell follicles and remained associated with FDCs for long periods of time (136, 146, 249). Since VSV does not significantly replicate extraneuronally in mice (275), both antigens rely on efficient trapping on FDCs for long-term persistence in immunized mice, and therefore memory B cell responses might be expected to be similar. The mechanism of transport of VLPs from the marginal zone and red pulp onto the surface of FDCs remains the subject of further investigation. Antibodies, both the naturally occurring as well as those secreted locally very early after immunization, are likely to play an important role. Activated complement components may also be involved in the targeting of Qß antigen to the surface of FDCs. However, the efficient short-term trapping of Qß particles observed by us in Cr2-/- mice argues against an absolute requirement for complement in the transport of the VLPs to follicles (265).

3.2 Early responses to VLPs: the role of the marginal zone B cell compartment

Rapid activation of marginal zone B cells

B cells of the marginal zone compartment are poised to combat infectious threats reaching the blood, an ability they owe to their unique location as well as their capacity to rapidly secrete antibodies. The rapid onset of antibody production by marginal zone B cells upon antigen encounter has evidenced that, despite being by definition naïve and expressing IgM, B cells of the marginal zone subset are functionally similar to memory B cells. For this reason, they have been proposed to embody a “natural immune memory” compartment of the spleen (48). Although the very early participation of marginal zone B cells in responses to blood-borne T cell-independent bacterial antigens is believed to be the most important function of marginal zone B cells, they have recently been demonstrated to be involved in the early stages of T cell activation and in antibody responses to T cell-dependent protein antigens (113). The functional versatility of the marginal zone B cell population was confirmed and extended in the studies presented in this thesis: marginal zone B cells were shown to respond to immunization with viral particles and to engage in early local secretion of IgG antibodies (220).
The importance of marginal zone B cells in T cell-independent antibody responses has been emphasized by studies with mutant mice in which this population of B cells is absent or reduced. Loss of marginal zone B cells in Pyk-2\(^{-/-}\) mice was shown to lead to defective humoral responses to the T cell-independent antigens TNP-Ficoll and TNP-LPS. (80). Similarly, impaired antibody responses to TNP-Ficoll were observed in Lsc\(^{-/-}\) and CD22\(^{-/-}\) mice, which display a reduced marginal zone B cell compartment (52, 276). Contrary to expectations, absence of marginal zone B cells in RBP-J\(^{-/-}\) mice had no impact on the antibody response to TNP coupled to Ficoll or LPS (277). However, these mice exhibited increased susceptibility to blood-borne *Staphylococcus aureus* (277). Compromised control of encapsulated bacteria has also been reported in NF-\(\kappa\)Bp50\(^{-/-}\) mice, another mouse strain with a defect in marginal zone B cell development, which were shown to be susceptible to *Streptococcus pneumoniae* (81, 82). These findings in mice deficient in a normal marginal zone B cell compartment confirm earlier observations in splenectomized or newborn rodents and humans, from which the essential role of marginal zone B cells in the induction of efficient antibody responses to T cell-independent antigens and in protection against encapsulated bacteria and was first inferred (72-77).

Immunity to extracellular bacteria is mediated largely by antibodies specific for bacterial capsular and cell wall polysaccharides (70). Antibody responses to polysaccharide antigens on bacteria, in which B cells of the marginal zone subset are preferentially engaged, display some notable features, as the study of B cell responses to *Streptococcus pneumoniae* has demonstrated. Specifically, mice systemically immunized with intact bacteria have been shown to mount T cell-independent anti-polysaccharide IgM responses, whereas induction of IgG antibodies was dependent on T cell help and CD40-CD40L interactions (278, 279). Coexpression of protein with polysaccharides in the intact bacteria is believed to serve to recruit cognate T cell help for polysaccharide-specific B cells, similar to what has been demonstrated for soluble protein-polysaccharide conjugate vaccines (209, 280). Surprisingly, an accelerated kinetics of induction of anti-polysaccharide IgG antibodies compared to those directed against the protein components of the bacteria has been observed in response to injection of *Streptococcus pneumoniae* (278, 279). In addition, the T cell help for optimal responses against the bacterial capsular and cell wall polysaccharides was demonstrated to be delivered more rapidly than that required for optimal induction of antibodies to bacterial proteins (278, 281). In contrast to enhanced secondary responses to bacterial protein components, no or only modest boosting of anti-polysaccharide antibodies was observed upon secondary immunization with *Streptococcus pneumoniae*, indicating that no apparent
polysaccharide-specific memory had been generated despite engagement of T helper cells (278, 279). Thus, an early induction of T cell-dependent IgG responses, rapid delivery of T cell help and a relative inability to generate immunological memory characterize the IgG response to capsular and cell wall polysaccharide antigens on Streptococcus pneumoniae. Since marginal zone B cells have been implicated in the response to the polysaccharide antigens displayed by these bacteria (83), it is tempting to speculate that the functional properties of this population of B cells underlie the outcome of the IgG responses to the bacterial-associated polysaccharide antigens. This notion is supported by our observation that marginal zone B cells fail to be recruited to germinal centers, where the memory B cell pool is established.

The nature of the responding B cell population might explain the similarity between early antibody responses elicited by VLPs and those directed against bacterial polysaccharides. While the involvement of marginal zone B cells in the response to bacterial antigens has clearly been stated, the role of this B cell compartment in responses to viral particles has not yet been clearly elucidated. Mutant mice lacking the spleen have been reported to exhibit a delay in the IgM response induced by intravenous infection with VSV (282), which may be due to the absence of a rapidly responding marginal zone B cell population. Our studies with bacteriophage-derived VLPs have indicated that B cells of the marginal zone B cell compartment participated in the IgM and IgG response to this type of antigens (220). Marginal zone B cells responded to immunization with VLPs with rapid proliferation and isotype switching and remained localized in the marginal zone (220). Thus, as expected from a T cell-independent antigen administered intravenously, VLPs induced activation of marginal zone B cells. Surprisingly, B cells from the marginal zone subset were able to undergo isotype switching more rapidly than the follicular counterparts (220). Since IgG responses were drastically reduced in the absence of MHCII-restricted T cell help (220), it is likely that T cells delivered help to Qß-specific marginal zone B cells for induction of isotype-switching.

The early isotype-switching of marginal zone B cells after immunization with VLPs is reminiscent of the accelerated kinetics of induction of IgG antibodies specific for bacterial polysaccharides when compared to those elicited by bacterial proteins (278, 279). The inference that the different kinetics of the IgG response to polysaccharide or protein components of bacteria is based on a differential involvement of marginal zone B cells and follicular B cells in these responses follows this recognition. From our studies, marginal zone B cells do not appear to be recruited to germinal center reactions, as demonstrated by an absence of Qß-specific PNA^hi B cells with a marginal zone phenotype as well as the reduced
somatic hypermutations present in antibody sequences isolated from specific marginal zone B cells (220, 283). Thus, since B cell memory is appreciated to be established in germinal centers, it is unlikely that activated marginal zone B cells differentiate into memory B cells. This may offer an explanation for the inability of bacterial polysaccharide antigens to generate efficient B cell memory (278, 279), if the marginal zone B cell compartment accounts for most of the anti-polysaccharide response. Retention of activated marginal zone B cells in the marginal zone allows for early local production of IgM and IgG antibodies, which might be important for opsonization of pathogens migrating through the spleen. This early local antibody production may therefore help to contain blood-borne infections before follicular B cells are activated and give rise to more long-lived systemic IgG production.

**Trapping and transport of antigen by marginal zone B cells**

The notion of a static population of antibody-secreting marginal zone B cells conflicts with reports showing that marginal zone B cells leave their original location upon activation with particulate blood borne bacteria or LPS (48, 284). In T cell-independent responses to bacterial antigens, marginal zone B cells have been reported to migrate to the T-B border and bridging channels and to associate with dendritic cells, which support their differentiation into plasmablasts (85). In response to soluble protein antigen, a similar migration of marginal zone B cells to T cell areas has been observed; at this site marginal zone B cells are thought to present antigen and activate T cells (113). The relocalization of activated marginal zone B cells has also been associated with their function to capture antigen and transport it into follicular regions in order to propagate immune responses. Injection of *Escherichia Coli* bacteria into rats has been reported to result in a loss of marginal zone B cells and a concomitant increase of cells with a IgM⁺IgD⁺ phenotype in the splenic follicles (285). This intra-splenic migration of marginal zone B cells was shown to be independent of complement; however, complement depletion completely inhibited trapping of immune complexes on FDCs (285). Studies in mice have provided further evidence for a role of marginal zone B cells in transport and deposition of immune complexes onto FDCs (116). Marginal zone B cells have been demonstrated to bind high levels of IgM-containing immune complexes in a complement-dependent process and depletion this population has been shown to abrogate the formation of antigen deposits in follicles (116). The impact of marginal zone B cell deficiency on antigen trapping has been highlighted in Pyk-2⁻ mice, which are devoid of this B cell subset. The distribution of blood-borne polysaccharide antigens in these mice was clearly altered and has indicated that B cells of the marginal zone compartment play an important role
in the trapping of antigen in the early stages of the immune response (80). A similar defect in local concentration of antigen in the marginal zone has been observed in mice deficient in complement components or complement receptors (80). In these mutant mouse strains TNP-Ficoll failed to localize to marginal zone B cells, suggesting that antigen capture by these cells relies on activation of the complement pathway (80). These findings attest a clear function of B cells of the marginal zone compartment in trapping antigen carried by the bloodstream. To what extent marginal zone B cells subsequently traffic into follicles and are involved in the deposition of antigen on FDCs remains to be elucidated.

The mechanisms underlying the migratory response of marginal zone B cells are also still being explored. It has been proposed that the interaction of marginal zone B cells with marginal zone macrophages is required to retain the B cells in the marginal zone (117). This association was shown to be perturbed upon migration of marginal zone macrophages to the red pulp in response to phagocytosis of blood-borne pathogens (117). The absence of retention signals delivered by macrophages, which were mediated by the MARCO scavenger receptor, resulted in trafficking of marginal zone B cells to follicular regions and possibly in antigen delivery to these sites (117). Relocalization of marginal zone B cells into lymphoid follicles has further been shown to involve reduced responsiveness to lysophospholipid sphingosine 1-phosphate (SIP) (227). Upon exposure to LPS or cognate antigen marginal zone B cells were reported to downregulate SIP receptors 1 and 3 and to migrate into the splenic white pulp possibly in response to the follicular chemokine CXCL13 or CCR7 ligands (227). Migration to lymphoid follicles after exposure to LPS has also been associated with decreased integrin activity in marginal zone B cells and the consequent reduced adhesiveness to the LFA-1 and α4β1 ligands expressed by resident stromal cells (40).

As stated above, upon immunization with Qβ particles a population of activated Qβ-specific B cells remained localized in the marginal zone (220), where we believe they were responsible for early local secretion of IgG antibodies. This notion was confirmed by the presence of IgG+ plasmablasts in close association with MOMA-1+ marginal zone macrophages after immunization with Qβ-derived VLPs (unpublished observations). Thus, although we cannot exclude early migratory events of marginal zone B cells to B cell follicles or T cell zones in response to exposure to Qβ particles, a mass migration of Qβ-specific B cells out of the marginal zone seems unlikely. This is consistent with observations reported for gene-targeted mice expressing the heavy chain variable region of a neutralizing anti-VSV antibody. In these mice, B cells specific for VSV, which localized in the marginal zone in naïve mice, remained and accumulated in the marginal zone after immunization (286).
Complement-dependent localization of antigen in the marginal zone has been described for polysaccharide and viral antigens (31, 80). Although Qβ particles were detected in the marginal zone after intravenous immunization, predominantly associated with macrophages, the pattern of antigen staining in the spleen of mice immunized with Qβ was more diffuse than the one observed after injection of TNP-Ficoll or VSV antigen (80, 220, 287). In addition, we did not note high levels of Alexa-labeled Qβ bound to B cells 24 hours after injection (unpublished data), as opposed to polysaccharide antigens, which efficiently target B cells in the marginal zone (80). Similar low levels of antigen binding to B220+ cells have been reported in studies using parovirus-derived VLPs (288). The reason for these divergent results may lie in a lower efficiency of VLPs in activating the alternative complement pathway and in the consequent reduced capacity of antigen to bind to marginal zone B cells via their highly expressed complement receptors. These observations are consistent with the Cr2-independent follicular localization of Qβ particles (265), which contrasts to the well-documented requirement for complement in the deposition of other antigens on FDCs (115, 118, 256). Due to their small size, Qβ-derived VLPs might enter B cell follicles more readily than antigens such as bacteria. Notwithstanding, marginal zone B cells might be implicated in the transport of Qβ particles to B cell follicles, in a process not involving complement.

**Marginal zone B cells: a population with a restricted repertoire?**

It is still unclear whether the special functional characteristics of the marginal zone B cell subset stem from a preferential selection of certain B cell clones into the marginal zone compartment. Marginal zone B cells, together with B1 B cells, have been proposed to constitute a population of innate-like B cells, recognizing a limited number of conserved antigenic structures (289). However, whereas in the B1 population some specificities, e.g. to phosphorylcholine or phosphatidylcholine, have been shown to be dominant (290, 291), a similar clonal enrichment in the marginal zone B cell compartment has proven more difficult to demonstrate. In addition, while a restricted Ig variable region gene usage has been reported for the B1 B cell population (292, 293), the heavy chain repertoire of murine marginal zone B cells has been shown to be heterogeneous and no evidence of oligoclonality could be observed in antibody sequences derived from marginal zone B cells of light chain transgenic mice (219). However, the reduction of N nucleotide additions in CDR3 sequences isolated from marginal zone B cells of Balb/c mice as well as the shorter CDR3 regions observed in rat antibody sequences derived from marginal zone B cells, compared to antibodies from
follicular B cells, have provided evidence that the marginal zone B cell repertoire might be restricted (141, 219).

The analysis of Ig transgenic mice has underscored a clonal enrichment of B cells in distinct B cell compartments depending on the composition of their BCR. Transgenic B cells specific for various types of antigens have been reported to segregate into the marginal zone: clones with specificities to phosphorylcholine, HEL, VSV as well as self-reactive B cell clones have been found to be enriched in this compartment in the respective transgenic lines (47, 50, 286, 294). Based on this finding, the idea has arisen that B cells are selected into the marginal zone or the other B cell compartments depending on the specificity of their BCR (48). However, the kinetics of expression of a functional BCR as well as its expression levels may be altered in Ig transgenic mice and might influence the selection process (295, 296). In fact, BCR surface density has been shown to play a role in determining B1 versus B2 cell development (217, 297). Thus, the major force driving B cells into the marginal zone compartment remains ill-defined. A combination of multiple parameters, including BCR specificity, BCR and coreceptor signaling and, ultimately, responsiveness to homing signals is likely to influence the recruitment of B cells into the distinct subsets.

In our studies of the marginal zone B cell response to two different VLPs, we compared antibody sequences derived from antigen-specific follicular and marginal zone B cells, as an attempt to detect a potential bias in the repertoire of the latter population. We found that the response of marginal zone B cells to bacteriophage-derived viral particles was highly polyclonal and diverse, as assessed by variable region gene segment usage as well as CDR3 length and composition (283). This indicated that marginal zone B cells were capable of generating a highly variable antibody response to immunization with VLPs and argues against a significant restriction in the diversity of the marginal zone repertoire. The results obtained with our system are in accordance with the clonotypically heterogeneous response of adoptively transferred marginal zone B cells reported in response to immunization with the hapten NP (211). Thus, although the marginal zone B cell compartment might be enriched for B cells reactive to conserved microbial antigens, it appears that this subset is still endowed with the ability to mount diverse immune responses. This might be required for an efficient early antibody production to the big variety of potentially blood-borne pathogens.
3.3 Long-term responses to VLPs: a critical role for complement in the regulation of bone marrow plasma cells generation

Plasma cells are the end-stage effectors of the B cell lineage. These terminally differentiated non-dividing cells are entirely devoted to the synthesis and secretion of antibody and therefore, as the final mediators of humoral immune responses, they play a critical role in protection against infectious agents controlled by antibodies. In differentiating from naïve B cells, plasma cells must undergo dramatic architectural remodeling, in order to accommodate high rate synthesis of Ig molecules. This process entails (i) building the secretory apparatus by increasing the ER membrane system; (ii) equipping the cell with larger quantities of ribosomal subunits, mitochondria to provide energy, and components of amino acid synthesis and metabolic processes; (iii) managing the quality of the product by increasing chaperones, folding enzymes and components of the quality control system; and (iv) upregulating transcription of Ig genes and inducing the processing of Ig heavy chain mRNA to the secreted form (298, 299). Changes in surface phenotype accompany the morphological transformations occurring during plasma cell differentiation. Numerous cell surface proteins are downregulated, including B220, CD45, CD19, CD21, CD22, MHCII and, most notably, the BCR (253). Further phenotypic changes observed during terminal B cell maturation into antibody-secreting cells include the induction of the proteoglycan Syndecan-1, which recognizes extracellular matrix and growth factors, as well as expression of VLA-4, the predominant integrin on plasma cells, mediating cell-cell contacts with bone marrow stromal cells (300, 301). In addition, chemokine receptors CXCR5 and CCR7 are downregulated on antibody secreting cells, whereas expression of CXCR4 remains high; these changes mediate homing of plasma cells from the follicles to the red pulp and the bone marrow (151). The transformation of a B lymphocyte into an antibody-secreting plasma cell is accomplished by an interplay between B lineage-specific transcriptional programs controlling B cell differentiation and an unfolded protein response. The transcription factor Blimp-1 serves as a master regulator of plasma cell development; it promotes expression of genes required for Ig secretion and extinguishes gene expression programs involved in proliferation and germinal center function. This is achieved by repressing the transcription factors Bcl-6 and Pax-5, which inhibit terminal B cell differentiation in germinal centers and are responsible for maintaining B cell identity (302). In addition, Blimp-1 plays an important role in the upregulation of XBP-1, a second transcription factor which is essential for plasma cell differentiation and antibody secretion (303). The massive increase in Ig synthesis and
secretion triggers the unfolded protein response, a response to ER stress caused by the accumulation of misfolded protein, which further augments the biosynthetic capacity of differentiating plasma cells (304).

Although, the regulatory cascades that initiate and maintain the plasma cell phenotype have recently begun to be identified, the signals involved in commitment of activated and germinal center B cells to a plasma cell fate are still largely unidentified. In particular, the question of what regulates the decision between plasma cell and memory B cell differentiation remains unresolved. The study of B cell differentiation *in vitro* has shown that signals through CD40, IL-4 or ligation of CD27 favor a memory phenotype, whereas presence of IL-2 and IL-10 and absence of CD40 engagement promotes differentiation to plasma cells (235, 236, 238, 305, 306). In germinal centers these signals are likely to be delivered by T helper cells; however it is not clear how they can determine the fate of differentiating B cells as the germinal center reaction proceeds. It has been reported that developmentally distinct T helper cells, expressing Ly6C, control plasma cell production *in vivo* (307). Nevertheless, development of both long-lived plasma cells and memory B cells, but not early production of antibody secreting cells, has been shown to be driven by T cells expressing the SAP gene (308). This is indicative of a common nature of T cells required for development of bone marrow plasma cells and memory B cells but underscores differences in late and early B cell help. Two recent studies have shed more light on the requirements for generation and maintenance of the bone marrow plasma cell compartment. The expression of the B cell maturation antigen (BCMA) on plasma cells and its interactions with the ligands BLyS and April was shown to be critical for survival of bone marrow plasma cells in the first report (309). The second investigation identified the transcription factor Aiolos as being essential in the differentiation program leading to high affinity bone marrow plasma cells, but dispensable for development of memory B cells and short-lived antibody-secreting cells (310). In contrast to Blimp-1 and XBP-1, whose activities affect all plasma cells, Aiolos is the first transcription factor described to be specifically required for differentiation and/or survival of the long lived high affinity plasma cell population residing in the bone marrow.

An alternative model has been put forward to explain the regulation of the plasma cell versus memory B cell decision. This model proposes that germinal center B cells commit to a plasma cell fate on the basis of their high affinity for antigen (311). The evidence that high affinity BCR favors plasma cell differentiation over memory cell fate derived from the analysis of single antigen-specific B cells from these two compartments indicating that high affinity variants selectively differentiated into plasma cells (234). In addition, Bcl-2 transgene
expression resulted in the accumulation of low affinity memory B cells, but the number of high affinity B cells in the bone marrow was not altered (233). This finding has provided further indirect evidence that bone marrow plasma cell development depends on high antigen affinity of the BCR, whereas selection into the memory B cell compartment is less stringent. The results we have obtained in Cr2" mice immunized with VLPs (summarized in Figure 3.1) fit with this model for the differential production of plasma cells and memory B cells from the germinal center reaction. In response to VLPs, generation of antigen-specific memory B cells was normal in Cr2" mice, but differentiation of germinal center B cells into long-lived plasma cells residing in the bone marrow was substantially reduced, and, as a consequence, Cr2" mice were unable to maintain high titers of specific antibodies (265). These results suggest that coligation of the CD21/CD19/CD81 coreceptor and the BCR by complement-coated antigen increases the avidity of the interaction of B cells with antigen, as required for promoting differentiation of germinal center B cells into bone marrow plasma cells. In the absence of CD21, the threshold of signal strength required for differentiation into long-lived plasma cells may not be reached by most germinal center B cells, preventing the induction of transcriptional programs leading to their terminal differentiation into plasma cells. However, signaling may still be sufficient for the generation of a memory B cell population. The block in plasma cell differentiation we observed in Cr2" mice occurred at the level of post-germinal center plasma cell precursors (Figure 3.1). In the absence of differentiation or survival signals mediated by the interaction of B cell complement receptors with complement-coated antigen, VLP-binding B cells exiting germinal centers, characterized by a PNA low phenotype, failed to induce the regulators of plasma cell differentiation Blimp-1 and XBP-1 as well as the anti-apoptotic protein Bcl-2 (265). Furthermore, the same population of PNA low post-germinal center B cells was unable to induce the expression of surface molecules characteristic of plasma cells, such as syndecan-1, CD44, and the integrins VLA-4 and LFA-1 (265). Hence, the generation of precursors of bone marrow plasma cells in germinal centers relies on binding of activated B cells to complement-decorated antigen. Whether this interaction is required to increase the avidity of differentiating germinal center B cells for antigen, as proposed by the model discussed above, or whether a distinct signal which directly leads to upregulation of Blimp-1 is transduced through the CD19 component of the B cell coreceptor upon binding of complement-coated antigen by CD21 remains to be determined. In any case, at some point of the pathway, one of the components of the regulatory cascade controlling plasmacytic differentiation must be intersected and Blimp-1 transcription must be induced. The unraveling of the upstream signals that regulate Blimp-1 might help in dissecting
between the two possibilities. It has been reported that in response to BCR signals, the transcription factor Bcl-6 is degraded (312). Since Bcl-6 represses the expression of Blimp-1 (313), the increased BCR signaling through coligation of the CD21/CD19/CD81 coreceptor and the BCR by complement-coated antigen might enhance degradation of Bcl-6 protein and aid in relieving its repression of Blimp-1. However, the relevance of this mechanism of inducing Blimp-1 expression remains to be defined.

As noted in other studies, short-term antibody responses were not affected in Cr2−/− mice (265). This is a further indication that the early production of short-lived non-germinal center-derived plasma cells is differently and, possibly, less tightly regulated than the generation of bone marrow plasma cells responsible for long-term antibody production. It appears reasonable to postulate that the immune system has evolved mechanisms to keep the induction of cells capable of secreting antibody for extended periods of time under tight control. Consistent with this hypothesis, germinal center B cells seem to sense the activation of the innate immune system, revealed by activated complement components bound to microbial surfaces, as a license to differentiate into the population of cells that plays a critical role in maintaining humoral immunity against pathogens.

![Figure 3.1: Distinct pathways of B cell differentiation and their requirements for Cr2. Phenotypic properties distinguishing the precursor population of bone marrow plasma cells, identified in the spleen, from germinal center B cells as well as the respective expression levels of Blimp-1, XBP-1 and Bcl-2 are indicated.](image-url)
3.4 Long-term responses to VLPs: the relative importance of antigen retention on FDCs versus generation of long-lived bone marrow plasma cells

As repeatedly noted, complement receptors also fulfill a function in the trapping and retention of antigen. After immunization, complement-tagged antigen can bind to FDCs through complement receptors type 1 (CD35) and 2 (CD21) (115, 256). FDCs can also bind IgG-containing complexes of antigen and specific antibody as a result of high levels of expression of FceRII, the low-affinity receptor for IgG (314, 315). By presenting antigen on their surface, FDCs are thought to supply antigen during the later stages of the immune response, as free antigen has been cleared from the circulation and its levels are declining. In fact, antigens have been shown to be stored as FDC-associated immune complexes for extended periods of time (99). Persistence of immune complexes on FDCs has been associated with the maintenance of humoral memory. It has been proposed that the periodical stimulation of memory B cells by persisting antigen causes their differentiation into short-lived antibody-secreting cells, and is required for maintaining antigen-specific serum antibody at high levels for long time periods (147). Several pieces of evidence have been provided to support this notion. Firstly, B cells specific for VSV have been observed to proliferate in association with persisting antigen and FDCs in long-lived germinal centers (146). Secondly, VSV neutralizing antibody titers, as well as antibody-forming cells in the bone marrow, have been demonstrated to decline more rapidly in mice devoid of FDC networks (249), further corroborating the importance of FDC-associated antigen in the maintenance of humoral memory. Similarly, in chimeric mice lacking complement receptors on FDCs, which display drastically reduced antigen deposition in germinal centers and B cell follicles, impaired B cell memory has been reported (119, 256). Finally, the increase of specific antibody levels upon addition of immune complexes of the relevant antigen has suggested that memory antibody titers are regulated by the amount of persisting immune-complexed antigen (316).

However, the concept that persisting antigen maintains immunological B cell memory has been put into question by several studies. In transgenic mice in which all B cells express only the membrane-bound form of IgM, which lack detectable immune complexes on FDCs as a consequence of their inability to secrete antibody, memory B cell development and maintenance has been reported to be unaffected (143, 261). In agreement with this finding, antigen experienced B cells have been shown to persist even when their BCR was switched to
an irrelevant specificity, arguing against a role for the immunizing antigen in survival of memory B cells (144). Nevertheless, from these experiments only an assertion on the importance of retained antigen for the survival of memory B cells can be made, since the used systems did not permit to dissect the requirement of antigen for maintenance of specific antibodies, the ultimate effectors of humoral memory. Hence, the most important challenge to the notion that antigen persisting on FDCs maintains humoral memory derives from the observation that plasma cells residing in the bone marrow can survive and secrete antibody for long periods of time in an apparently antigen-independent fashion (152, 153). Persistent antibody titers could therefore be maintained by long-lived plasma cells in the absence of continual differentiation of memory B cells into antibody-secreting cells.

Our analysis of the effect of depletion of FDCs and the associated antigen at late time points after immunization, i.e. when the bulk of the germinal center reaction had terminated, is consistent with long-lived bone marrow plasma cells playing a major role in maintaining persistent antibody titers in responses to VLPs. In fact, removal of Qβ antigen late after immunization had no impact on serum antibody titers, frequencies of specific plasma cells or the maintenance of memory B cells (272). In sharp contrast, elimination of FDC-associated antigen shortly before the peak of the germinal center reaction, and thus before the memory B cell response could fully be established, had a dramatic effect on the generation of humoral memory (272). Specific antibody titers, as well as plasma cell and memory B cell frequencies were drastically reduced after early treatment with LTβR-Ig fusion protein and the consequent disappearance of FDCs and persisting antigen (272). However, since the treatment eliminated existing germinal centers and abolished the formation of new germinal centers, the effect of an absence of antigen persisting on FDCs cannot be discerned from the impact of perturbed germinal center reactions. The defective humoral response after injection of LTβR-Ig might therefore be secondary to impaired germinal center formation caused by loss of FDCs, and not be due to reduced deposits of antigen in developing germinal centers. In fact, the requirement for immune complexes in the initiation of germinal center reactions has recently been questioned and an antigen-unspecific role of FDCs in germinal center development has been postulated (261, 262).

Still, our FDC-depletion studies demonstrate an important output of germinal center reactions with respect to memory formation early after immunization, whereas the contribution of germinal centers in the maintenance of humoral memory at later time points after immunization appears to be negligible. These results are in agreement with previous experiments showing that disruption of germinal centers by injection of anti-CD40L antibody
early after immunization with haptenated proteins had a more profound effect on the formation of high affinity bone marrow antibody-forming cells than a late inhibition of germinal center reactions (267). Similarly, serum antibody levels were not affected if treatment with anti-CD40L occurred after well formed germinal centers were present in the spleen; however only the effect on short-term antibody responses was addressed in these studies (268).

The notion that early events after immunization determine the outcome of the antibody response and the establishment of B cell memory is consistent with our findings in Cr2−/− mice. An early defect in the generation of bone marrow plasma cell precursors had a major impact on the ability of these mice to maintain elevated levels of specific antibody (265). Furthermore, despite the presence of normal numbers of functional memory B cells, Cr2−/− mice exhibited a drastic reduction in long-term anti-Qβ antibody titers (265), emphasizing the importance of bone marrow plasma cells in maintaining antibody production in responses induced by VLPs. It could be argued that reduced long-term retention of Qβ antigen in these mice prevented activation of memory B cells and their differentiation into plasma cells, thereby affecting long-term persistence of antibodies. However, our study of the effect of depletion of FDC-associated antigen late after immunization indicated that, despite low-level turnover of Qβ-specific memory B cells in long-lived germinal centers, the contribution of continually differentiating memory B cells to the maintenance of anti-Qβ antibody titers was minimal (272). This analysis therefore corroborated the major role of long-lived bone marrow plasma cells for maintaining antibody levels in the B cell response studied. The longevity of the Qβ-specific antibody-secreting plasma cell population inhabiting the bone marrow was confirmed in BrdU-labeling experiments. This approach indicated that bone marrow plasma cells induced by immunization with Qβ had a life span of at least 80 days (unpublished data), consistent with previous observations (317).

Why, then, would the immune system endeavor to keep antigen deposits available long time, if the effectors of B cell memory can survive in the absence of antigen and antigen-independent antibody production can be sustained? The capacity of the bone marrow to accommodate plasma cells is limited to 400,000 plasma cells in the entire bone marrow, or 0.5% of total mononucleated cells in this tissue (318). Thus, not all the produced plasma cells can become resident in the bone marrow and profit from this privileged niche and, eventually, antibody-secreting cells specific for a previously encountered antigen will be replaced by newly produced, incoming plasma cells. In a situation when the host frequently encounters new antigens, and especially in the case of acute infections when massive numbers of plasma
cells are produced and home to the bone marrow, the differentiation of memory B cells driven by persisting FDC-associated antigen might represent a fail-safe mechanism to compensate the loss of “old” bone marrow plasma cells and ensure that antibody production against relevant, previously faced pathogens are maintained. However, an unlimited capacity of FDCs to trap antigen and a life span exceeding that of plasma cells would have to be postulated. If only a limited load of antigens are encountered by the immune system, as is the case in “clean” mouse facilities, the contribution to humoral memory of plasma cells arising from memory B cells stimulated by persisting antigen might not become evident. Or, possibly, such analyses should be extended to the lifetime of a mouse for an obvious contribution to be detected. Hence, to date the crucial player in B cell memory has not reached consensus. Might the contrasting outcome of the many different models that have been put forward to explain how humoral memory is maintained be an indication that several distinct mechanisms coexist in order to afford efficient protection against reinfection?

3.5 Conclusions

Early and long-term B cells responses to many of the antigens encountered by the immune system not only are mediated by distinct B cell populations but are also differentially regulated. In humoral responses induced by intravenous injection of VLPs, the rapid activation and isotype-switching of B cells of the marginal zone compartment allows for early local production of IgG antibodies of substantial diversity. This early IgG secretion in the marginal zone possibly facilitates opsonization of pathogens gaining access to the bloodstream. As VLPs rapidly reach B cell follicles and are efficiently trapped on FDCs, follicular B cells are activated with only a short delay but require a longer period of time for exhaustively switching to IgG. However, it is this subset of B cells which is almost exclusively responsible for establishing humoral memory. In contrast to the rapid but transient response of marginal zone B cells, differentiation of follicular B cells into precursors of bone marrow plasma cells is tightly regulated: an interaction with complement-coated antigen retained on FDCs in the early stages of the response is essential and is crucial for the generation of long-lasting B cell responses to VLPs. Indeed, serum antibody titers to VLPs are maintained by a long-lived, slowly declining population of bone marrow plasma cells. However, these antibody-secreting cells are reinforced by an existing memory B cell population, whose induction by VLPs appears to be less stringent.
Thus, the study of early and long-term B cell responses to VLPs has confirmed and extended the role of marginal zone B cells early after antigen exposure and has provided new insight into the mechanisms of regulation of humoral memory. Although infections by live bacteria or viruses might not faithfully be mimicked by the use of VLPs as a model antigen, the study of the characteristics of B cell responses to VLPs provides insight into the mechanisms underlying induction and regulation of antibody responses to inert vaccines, in particular those relying on VLPs as a carrier for vaccinating antigens.
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4 APPENDIX

4.1 References


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4.2 Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AFC</td>
<td>antibody-forming cell</td>
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<tr>
<td>AID</td>
<td>activation-induced cytidine deaminase</td>
</tr>
<tr>
<td>ASC</td>
<td>antibody-secreting cell</td>
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<tr>
<td>BCR</td>
<td>B cell antigen receptor</td>
</tr>
<tr>
<td>BrdU</td>
<td>bromodeoxyuridine</td>
</tr>
<tr>
<td>C region</td>
<td>constant region of Ig</td>
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<tr>
<td>CD40L</td>
<td>CD40 ligand</td>
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<tr>
<td>CDR</td>
<td>complementarity-determining region</td>
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<tr>
<td>d</td>
<td>day</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorter</td>
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<td>follicular dendritic cell</td>
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<td>GC</td>
<td>germinal center</td>
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<tr>
<td>h</td>
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<td>hen egg lysozyme</td>
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<td>i.p.</td>
<td>intraperitoneal(ly)</td>
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<tr>
<td>i.v.</td>
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<td>immunoglobulin</td>
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<td>IMDM</td>
<td>Iscove’s modified Dulbecco’s medium</td>
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<td>LT</td>
<td>lymphotoxin</td>
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<td>MEM</td>
<td>minimal essential medium</td>
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<td>Abbreviation</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
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<td>membrane immunoglobulin</td>
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<td>MZ</td>
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<td>NP</td>
<td>(4-hydroxy-3-nitrophenyl)acetyl</td>
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<td>phosphate-buffered saline</td>
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<td>polymerase chain reaction</td>
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<td>receptor</td>
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<td>recombination-activating gene</td>
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<td>s.c.</td>
<td>subcutaneous(ly)</td>
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<td>standard deviation</td>
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<td>terminal deoxynucleotidyl transferase</td>
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<td>Th</td>
<td>T helper</td>
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<td>TNF</td>
<td>tumor necrosis factor</td>
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<tr>
<td>TNP</td>
<td>2,4,6 trinitrophenyl</td>
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<tr>
<td>VLP</td>
<td>virus-like particle</td>
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<td>VSV</td>
<td>vesicular stomatitis virus</td>
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<td>wk</td>
<td>week</td>
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<td>WT</td>
<td>wild-type</td>
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* Equal contribution


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PATENT

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