Primary and memory B cell responses to Qβ-VLP in mice

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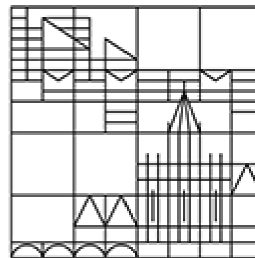
Presented by
Franziska Zabel

at the



Faculty of Mathematics and Natural Sciences

Department of Biology

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Head of commission: Prof. Dr. Thomas Brunner

First supervisor: Prof. Dr. Marcus Groettrup

Second supervisor: Prof. Dr. Martin F. Bachmann

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Abbreviation

Ab	antibody
ACK	ammonium chloride potassium (German: Kalzium) buffer
Ag	antigen
AID	activation induced deaminase
APRIL	a proliferation-inducing ligand
APC	antigen presenting cell
BAFF	B cell activating factor of the TNF family
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BCR	B cell receptor
Blimp	B lymphocyte inducing maturation protein
BLys	B lymphocyte stimulator
BD	Becton Dickinson
BM	bone marrow
BSA	bovine serum albumin
c	constant
C	complement
CD	cluster of differentiation
CD40L	CD40 ligand
CR	complement receptor
CS	class switched
CSR	class switched recombination
d	day
DC	dendritic cell
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
Ds	double stranded
ER	endoplasmatic reticulum
FACS	fluorescence activated cell sorting
Fc	F constant (regards the Fc part of an Ab)
FCM	flow cytometry
FcR	Fc receptor
FCS	fetal calf serum
FDC	follicular dendritic cell
GC	germinal center

H_2O	water
H_2O_2	hydrogen peroxid
Ham	hamster
Hepes	2-(4-(2-Hydroxyethyl)- 1-piperazinyl)-ethansulfonic acid
HRPO	horseradish peroxidase
IF	Immuno fluorescence
IFN	interferon
Ig	immunoglobulin
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin
ITIM	immunoreceptor-tyrosine-based inhibitory motif
i.v.	intravenous
kDa	kilo Dalton
k.o.	knock-out
LPS	Lipopolysaccharide
LT	Lymphotoxin
mAB	monoclonal antibody
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MHC II	major histocompatibility complex class II
Ms	mouse
MZ	marginal zone
NMS	normal mouse serum
OD50	optical density 50% of maximum
OPD	1,2-Phenylenediamine dihydrochloride
PALS	periarteriolar lymphoid sheath
PBS	phosphate buffered saline
PBST	phosphate buffered saline supplemented with Tween-20
PFA	Paraformaldehyde
p.i.	post immunization
PRR	pattern recognition receptor
R	receptor
RBC	red blood cell
PNA	peanut agglutinin

RNA	ribonucleic acid
Rt	rat
SLO	secondary lymphoid organ
SRBC	sheep red blood cells
ss	single stranded
Sw	switched
TACI	transmembrane activator and calcium modulator cyclophilin ligand interactor
TAPA	Target of the anti-proliferative antibody
TCR	T cell receptor
TD	thymus dependent (T cell help dependent)
TFH	T follicular helper cells
TI	thymus independent (T cell help independent)
TNF	tumor necrosis factor
TLR	Toll like receptor
VLP	virus like particle

Summary

Extensive studies have been undertaken to describe naïve B cells differentiating into memory B cells at a cellular and molecular level. However, relatively little is known about the fate of memory B cells upon antigen re-encounter. We have previously established a system based on virus-like particles (VLPs) which allows to track VLP-specific B cells by flow cytometry as well as histology. Using allotype markers, it is possible to adoptively transfer memory B cells into a naïve mouse and track responses of naïve and memory B cells as well as antibody responses in the same mouse under physiological conditions.

In contrast to previous reports using proteins, we have observed that VLP-specific memory B cells did not efficiently proliferate but quickly differentiated into plasma cells upon cognate antigen challenge. This was paralleled by an early onset of a strong humoral IgG response. Also upon tracking of distinct memory B cell populations, neither IgM^+ nor IgG^+ memory B cells proliferated extensively or entered germinal centers. Remarkably, plasma cells derived from memory B cells preferentially homed to the bone marrow early and produced superior amounts of antibody compared to plasma cells generated during the primary B cell response. Indeed, secondary plasma cells produced about 5 times more antibody than the corresponding primary plasma cells residing in the bone marrow. Hence, viral like particles provided a sufficient strong stimulus to drive terminal differentiation of memory B cells into highly effective secondary plasma cells preferentially homing the bone marrow. From a physiological point of view this may be explained by the immediate need for protective IgG antibodies in the presence of systemic viral particles.

Interestingly, memory B cells failed to respond after multiple rounds of stimulation by cognate antigen, as the majority of Abs was only produced by memory derived secondary plasma cells after the first boost. In contrast, after second boost the humoral response was dominated by plasma cells derived from first-round memory B cells of the host. Under these conditions, the memory B cell pool

was replaced by a new wave of memory B cells derived from primary B cells. Thus, memory B cells generated during a secondary response were largely derived from naïve B cells and may therefore harbor slightly different specificities than the concomitantly produced antibodies. As a consequence, the B cell response may remain dynamic and antigenic sub-specificities encountered during the primary response are not endlessly carried forward preventing adaptation of the B cell responses to newly emerging variants.

We further investigated the role of $CD4^+$ T helper cells during memory B cell responses. Here, we observed a graded T helper cell dependence. Proliferation of class-switched memory B cell followed by rapid generation of plasma cells and early IgG responses were generally highly T cell dependent. In contrast, late plasma cell generation as well as late IgG responses were mostly T helper cell independent. From a physiological point of view, this may reflect the goal of the immune system to rid pathogens that are present for extended time periods even at the risk of raising T cell independent memory IgG responses.

All observations and conclusions made in this study may be representative for many VLPs and viral particles, as we assessed the majority of experiments with two different VLPs.

These insights are important for our general understanding of B cell responses and may be of value for improving vaccination regimens to optimize generation of long-lived plasma cells.

Zusammenfassung

Es existieren unzählige Studien, welche die Differenzierung von naiven B-Zellen in B-Gedächtniszellen auf zellulärer wie molekularer Ebene beschreiben. Hierbei ist jedoch nur wenig über die Reaktionen der B-Gedächtniszellen bei Zweitkontakt mit ihrem Antigen bekannt. Wir haben ein System entwickelt, welches auf der Verwendung von Virus-ähnlichen Partikeln basiert und es uns ermöglicht, den Verlauf spezifischer B-Zell-Antworten mittels der Durchflusszytometrie und histologisch darzustellen und zu verfolgen. Unter Verwendung von Allotypen-Markern ist es möglich, spezifische B-Gedächtniszellen in eine naive Maus zu transferieren und nach Immunisierung die zellulären sowie humoralen Antworten von naiven B-Zellen und B-Gedächtniszellen in ein und derselben Maus unter physiologischen Bedingungen separat zu verfolgen.

Im Vergleich zu anderen Studien, welche klassische Protein-Antigene verwendeten, konnten wir beobachten, dass B-Gedächtniszellen nach erneutem Antigenkontakt nur wenig proliferierten und bevorzugt in Plasmazellen differenzierten. Dies zeigte sich in einer sehr frühen und starken IgG^+ Antikörperantwort. Wir haben verschiedene B-Gedächtniszell-Populationen untersucht. Weder IgM^+ noch IgG^+ B-Gedächtniszellen teilten sich im hohen Masse, noch sind sie eine Keimzentrumsreaktion eingegangen. Erstaunlicherweise konnte zu einem frühen Zeitpunkt die Migration von Plasmazellen, welche sich aus B-Gedächtniszellen differenziert hatten, in das Knochenmark beobachtet werden. Diese sekundären Plasmazellen waren sehr potent, da sie fünfmal mehr Antikörper produzieren konnten als Plasmazellen, die während einer Primärantwort gebildet wurden. Die Virus-ähnlichen Partikel waren demzufolge fähig eine Ausdifferenzierung der B-Gedächtniszellen in hoch effektive sekundäre Plasmazellen zu induzieren, welche im Anschluss bevorzugt ins Knochenmark migrierten. Aus physiologischer Sicht ist bei systemischer VLP-Präsenz im Organismus eine schnell schützende humorale IgG^+ -Antwort die Folge.

Interessanterweise konnten wir beobachten, dass transferierte B-Gedächtniszellen nicht im Stande waren, mehrere Male auf ihr korrespondierendes Antigen zu reagieren, da die Mehrheit der Antikörper nur von sekundären Plasmazellen nach erstem Antigenkontakt gebildet wurden. Nach zweitem Antigenkontakt stellten Plasmazellen, die von neu-generierten B-Gedächtniszellen des Empfängers während des ersten Antigenkontakts stammten, den grössten Anteil der Antikörperantwort. Unter diesen Bedingungen wurde der Pool an B-Gedächtniszellen nach jedem Antigenkontakt von neu aktivierten primären B-Zellen ersetzt. So entstanden während einer sekundären Reaktion die B-Gedächtniszellen mehrheitlich aus naiven B-Zellen, das zu geringen Unterschieden in den Spezifitäten neu produzierter im Vergleich zu bereits existenter Antikörper führte. Die Konsequenz ist, dass die B-Zellantwort nach Immunisierung offenbar dynamisch bleibt, da Antigen-Spezifitäten, die während der Primärantwort gebildet wurden, nicht ewig weitergeführt werden, was eine Anpassung der B-Zell-Antwort auf neu entstehende Varianten verhindern würde.

Des Weiteren haben wir die Rolle von $CD4^+$ T-Helferzellen in B-Gedächtniszellantworten untersucht und konnten eine graduelle Abhängigkeit beobachten. In hohem Masse war die Zellteilung von Isotypen-gewechselten B-Gedächtniszellen verbunden mit einer frühzeitigen Differenzierung zu Plasmazellen und einer frühen IgG^+ Antikörperproduktion von T Helferzellen abhängig. Hingegen waren die Generation von Plasmazellen zu einem späteren Zeitpunkt und die damit einhergehende späte IgG^+ Antikörperantwort nur teilweise von T Helferzellen abhängig. Aus physiologischer Sicht könnten diese Beobachtungen das Ziel des Immunsystems wiederspiegeln, Pathogene, die über lange Zeit präsent sind, zu eliminieren, selbst auf die Gefahr hin eine T -Zell unabhängige IgG^+ Gedächtnisantwort einzugehen.

Alle Beobachtungen und Schlussfolgerungen dieser Studie scheinen allgemein relevant für VLPs und viralen Partikeln, da die Mehrheit der Experimente mit zwei unterschiedlichen VLPs durchgeführt wurde.

Die gewonnenen Erkenntnisse sind für das allgemeine Verständnis der B-Zell-Antworten sehr wichtig und können für die Bildung von langlebigen Plasmazellen zur Optimierung von Impfungen und Impfregimes genutzt werden.

1 Introduction

There was a time when life expectancy was less than half of what it is today. Only a few centuries ago the main cause of death was infectious diseases. People usually only suffered from chronic diseases, if they were caused by infections as people usually did not grow old. The main approach to prolong life in the last century was the improvement of health care by enhanced hygiene and development of prophylactic and therapeutic medications. Indeed, it started with fighting infectious diseases by the development of vaccination procedures.

A pioneer in this field was Edward Jenner, an English physician, who greatly contributed to the discovery of mediating immunity against human small pox virus in the 18th century (Jenner, 1798; Jenner, 1809). He observed that milkmaids where resistant to infection by smallpox virus and he hypothesized that they had acquired immunity through infection with the related cowpox virus. He was able to prove this point by inoculation a boy with cowpox virus and subsequent challenge of the boy with life smallpox virus. Indeed the boy was protected. Edward Jenner established subsequently a new procedure to immunize against smallpox virus based on cowpox virus, which was widely accepted and used throughout Europe. A long the way he also coined the term vaccination as he used a cow (Latin: “vacca”) virus for immunization. Jenner’s studies were extended by the German microbiologist Robert Koch who identified that microorganisms caused infections (Koch, 1878; Koch, 1880). At the same time in Paris, Louis Pasteur also worked on the concept of infectious diseases such as cholera and rabies and provided insights into the mechanisms induced by vaccination (Pasteur, 1882; Pasteur et al., 2002; Pasteur and Illo, 1996). By studying immune responses against tetanus and diphtheria toxin, Emil von Behring and Shibasaburo Kitasato discovered proteins, which we now call antibodies, providing anti-toxic activity which allowed to introduce serum therapies to the medical world (von Behring and Kitasato, 1991). Interestingly, at the turn of the 20th century other scientists revealed a very different way to combat infectious diseases. The Scottish bacteriologist Alexander Fleming as well as Paul Ehrlich from Germany discovered antibiotics such as

Penicillin (Alexander, 1929) or Arsphenamin (Ehrlich and Bertheim, 1912), which opened a new world to tackle bacterial diseases such as anthrax or syphilis.

Since von Behring and Kitasato found protective antibodies in body fluids formerly known as humors, immunity mediated by antibodies was called humoral immunity. Humoral responses are mediated by a certain B cell subtype, the plasma cell. Plasma cells (PC), also called antibody secreting cells, are B cells which terminally differentiated upon activation by their B cell receptors (BCR) and were identified by Astrid Fagraeus in the 1940s (Fagraeus, 1947; Fagraeus, 1948). B lymphocytes belong to the arm of adaptive immunity and are capable to respond to a huge variety of antigens by providing a remarkable diversity by their B cell receptors. B cells can mediate host immunity early by rapidly secreting antibodies upon activation and late by the generation of memory B cells as well as PCs which in turn can maintain the antibody level over time. Hence, many prophylactic vaccines focus on the induction of potent and long-lived B cell responses (Zinkernagel, 2003).

Vaccination still plays an important role in fighting infectious diseases and to lower mortality and morbidity rates. The main objective of a vaccine is to develop long-lived immunity to ideally protect the host from reinfection or to reduce the severity of the disease. Not only individual immunity but also herd immunity can be achieved by vaccination of the majority of people in an area, which may completely eradicate certain pathogens. As an example, Edward Jenner developed a “vaccine” against small pox virus which was used for world-wide vaccinations. Although it certainly took almost 200 years, the World Health Organization (WHO) declared in 1979 that small pox virus was eradicated from the world. In times of globalization, only world-wide herd immunity can completely eradicate pathogens as the risk of local followed by global outbreak is eminent. As an example of polio – the US government announced in 1994 that polio was eradicated in the US (CDC, 1994). Europe was declared polio free in 2002 (WHO, 2002). However, in 2013 several cases of polio viral infections occurred in the Dominican Republic and polio remains endemic in a number of African countries. The current situation in the Middle East is even more worrying. In general, Syria has poor vaccination coverage for polio virus. Based on the social political changes and long-lasting war-like

situation in the country, the endemic spread of the virus is predicted to happen soon and medical doctors from several European countries warn of a new spread of the polio virus back into Europe.

Thus, there is still an urgent need of further education about vaccination world-wide, the development of new vaccines as well as vaccination strategies and the realization of vaccination in cooperation with the World Health Organization, UNICEF and the new global leader, The Bill & Melinda Gates Foundation. If all these associations work together we are hopefully able to declare the world polio-free within the next decade.

1.1 B cell development

B cells have been initially discovered in a specialized lymphoid organ in birds – the bursa of Fabricius (Glick, 1956). Some claim that the B cell was called according to the first discovery in the bursa. However, in mammals, general hematopoiesis as well as B cell development takes place in the bone marrow (BM) and therefore others claim that B lymphocytes are named after the site of development – B for BM. Noteworthy: fetuses develop B lymphocytes in the liver.

The development of B lymphocytes from hematopoietic stem cells in the BM is controlled by multiple factors (Figure 1.1.1). Two cytokine receptors (Flk2/Flt3 and IL-7R) and six transcription factors (PU.1, Ikaros, E2A, Bcl11a, EBF, and Pax-5) are indispensable for the development of B cell precursors (Busslinger, 2004; Singh et al., 2005). Ikaros and PU.1 play an essential role early in development. Ikaros controls the development of the lymphoid progenitors (Georgopoulos et al., 1994), whereas PU.1 directs the progenitor cell either to the myeloid or lymphoid lineage (Scott et al., 1994) and regulates the transcription of the cytokine receptors, which are required at early time points for proper B cell development (Mackarehtschian et al., 1995; Peschon et al., 1994). For immune globulin (Ig) rearrangements EA2 and EBF are essential and function at the pro-B cell stage (Bain et al., 1994; Hagman and Lukin, 2005; Lin and Grosschedl, 1995; Zhuang et al., 1994). Expression of PAX5 is finally seminal to commit B cell progenitors to the lymphoid lineage as activation of B-cell-specific genes

and V(D)J recombination by E2A and EBF is insufficient in this process (Busslinger, 2004; Nutt et al., 1997).

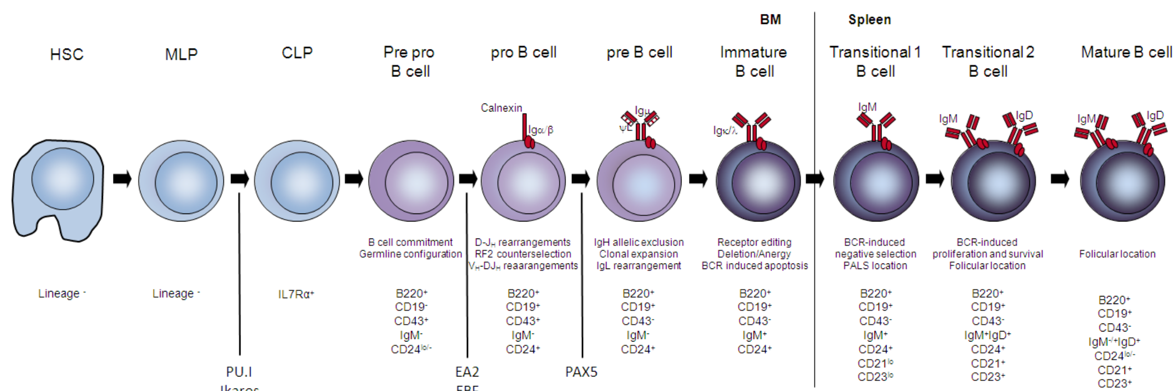


Figure 1.1.1│B2 cell development. Stages of B-cell lymphopoiesis from hematopoietic stem cell to mature B cell are shown. Relevant transcription factors such as PU.I, Ikaros, and PAX5 are indicated. Surface marker expression at different developmental stages is shown. (Hagman and Lukin, 2005; Strasser, 2005). HSC hematopoietic stem cell, MLP myeloid progenitor, CLP common lymphoid progenitor, Pre precursor, Pro progenitor.

B cells do not develop in distinct areas of the bone marrow. They mature in close relation with stromal reticular cells, which supply the IL-7 cytokine driving commitment to the B cell lineage. The earliest committed B cell precursors are pre-pro B cells (Hardy et al., 1991). They express CD45 – the common leucocyte antigen also called B220 (the B cell specific version of CD45) during their lifespan (Hardy et al., 2000). “B cells” at the pre-pro stage have their immunoglobulin (Ig) loci in germline configuration. Beside encoding the constant regions of the immunoglobulin (Ig) heavy chains (IgH) and κ or λ light chains (IgL), the inherited germline genes also encode for clusters of gene segments of the variable regions for IgH as well as IgL - the variable (V), diversity (D, in heavy chain only) and joining (J) exons (Tonegawa, 1983). Numerous V(D)J gene segments for IgH and IgL exist which are randomly reassembled during V(D)J recombination and are therefore primarily responsible for the variety of BCRs to bind to abundant antigens. In the further course of the development of mature B cells, different surface molecules are expressed which characterize certain stages such as CD43

expression on pro-B cells (Hardy and Hayakawa, 2001; Hardy et al., 2000). The transition from the pro-B cell stage to pre-B cell stage is accompanied by down-regulation of CD43 and up-regulation of CD24, which is also called heat stable antigen (HSA) and serves as adhesion molecule. The specific B cell lineage marker CD19 is expressed from the pro B cell stages onwards.

Once B cells escape from negative selection in the bone marrow they migrate to the periphery as transitional B cells. Transitional B cells are characterized by distinct expression levels of molecules that characterize B cells in general, e.g. complement receptors (C_R) such CD21^{low} or the low affine Fc receptor for IgE ($Fc_{\varepsilon}R$ II)^{low}.

Once transitional B cells escape peripheral negative selections and find a survival niche, they become part of the functional B cell repertoire.

According to their origin, phenotype and function, two major B cell subtypes – B1 and B2 can be distinguished. B2 cells are generated during postnatal life in the BM and recirculate throughout the lymphatic and blood system as follicular B cells or reside in the marginal zone (MZ) in the spleen as MZ B cells. (Hardy and Hayakawa, 2001; Nagasawa, 2006). On the other hand, B1 cells develop primarily during the fetal (B1a) and perinatal life (B1b) (Herzenberg, 2000) and are located in peritoneal and pleural cavities. They therefore develop slightly differently from B2 cells as it is shown in Figure 1 (Roy et al., 2009).

The most mature B cells reside in B cell follicles in secondary lymphoid organs (SLO) and represent the follicular B cells. They develop throughout life from transitional B cells and participate in T cell dependent (TD) and independent (TI) immune responses. Upon binding to cognate antigen, follicular B cells can undergo somatic hypermutation (SHM) as well as class switching (sw) of their BCRs and are further selected during affinity maturation. They essentially contribute to the adaptive immune response by generating memory B cells and long-lived (LL) PCs which maintain the humoral immune response. In contrast, MZ B cells are located at the border between the white and red pulp in spleen and can be self-replenished. Due to their location, MZ B cells are exposed to blood borne antigens and rapidly respond by secreting low affinity antibodies. MZ B cells are the primary producers of T

cell independent IgM antibodies. Another major mature B cell population represents B1 cells, which are found in the body-cavity sierosa and represent the main source of natural antibodies. MZ and B1 cells belong to the "innate-like" lymphocytes and secure host survival providing an early and fast humoral response (Lopes-Carvalho and Kearney, 2004).

All these different B cell populations in the periphery can be distinguished based on their location, function and surface marker profiles (Fig. 2) (Kantor and Herzenberg, 1993). Interestingly there have been recently two different B1 subtypes described, which may be even developed from different progenitor stem cells (Ghosn et al., 2012) and provide different effector functions. Figure 2 summarizes the different B cell populations by their surface marker expression.

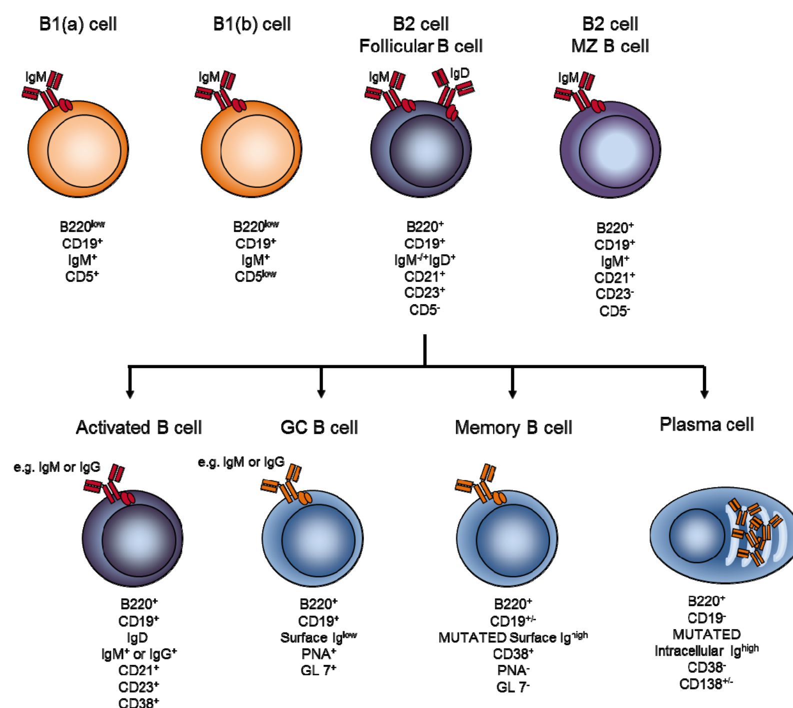


Figure 1.1.2│ B cell subtypes can be distinguished by their differential surface marker expression. (Herzenberg, 2000; Lopes-Carvalho and Kearney, 2004; Shlomchik and Weisel, 2012)

1.2 B cell activation

B cell activation by T cell independent (TI) antigen

When mature B cells migrate from the BM to the periphery and do not encounter their cognate antigen, they die within a few weeks to months. The BM produces approximately $5x10^7$ B cells every day, of which only 10% survive. This leads to a very dynamic process and ensures a high variety of B cells which are capable to mediate host protection at any time. B cells usually only respond to their cognate antigen in the presence of T help. Antigens which are able to induce antibody responses in the absence of T cell help are classified as TI antigen. There are two groups of TI antigens type I and type II (TI-1 and TI-2). The first group includes the so-called mitogens, which are capable to induce a polyclonal response of mature and immature B cells (Mond et al., 1995b) which do not involve the BCR. Bacterial cell wall components such as lipopolysaccharide (LPS) are antigens that trigger activation by engagement of e.g. Toll like receptors (TLR).

The TI-2 antigens comprise bacterial capsular and cell wall polysaccharides, viral glycoproteins, synthetic polymers and their haptenated counterparts. They are of high molecular weight and supply repeating antigenic epitopes (Dintzis et al., 1989). One main feature of TI antigens is therefore their ability to crosslink the BCRs on B cells to initiate signaling which in turn induces a rapid and strong humoral response (Mond et al., 1995a; Mond et al., 1995b; Vos et al., 2000). Approximately 10 to 20 receptors are needed to be cross-linked by TI multivalent antigen molecule to mediate BCR signaling (Dintzis et al., 1976; Sulzer and Perelson, 1997).

Many TI-2 antigens are not proteins and therefore cannot be degraded into peptides, which generally precludes cognate T cell help. However, their repetitive structure clusters and crosslinks membrane Ig (mIg) leading to prolonged and persisting BCR signaling (Fehr et al., 1996). In addition, those bacterial antigens often carry a number of further B cell activators which engage TLRs or CR (Cooper et al., 1988) and supply a so called second signal (Mond et al., 1995a) which synergizes with cross-linking mIg. Although no direct interaction with T helper cells occur in TI B cell responses, T_H cells as well as dendritic cells (DC) and macrophages nevertheless contribute to B cell activation

indirectly, by cytokine secretion of the tumor necrosis factor (TNF) superfamily such as BLyS (also called BAFF) or APRIL (Litinskiy et al., 2002; Macpherson and Lamarre, 2002). There are similarities in the activation of MZ B cells and B1 cells as these B cell responses often take place in the absence of cognate T cell help (Martin et al., 2001). Normally T cell independent immune responses mediate only a short lived humoral response because generation of memory B cells is not established due to the absence of CD40 activation (Foy et al., 1994a; Kawabe et al., 1994) and no long-lived plasma cells are generated. Nevertheless, there has been a report of T cell-dependent isotype switch of MZ B cells, indicating that these cells may still contribute to B cell memory to some degree (Gatto et al., 2004). In summary, the advantage of TI B cell responses is the rapid antibody response providing host protection.

B cell activation by T dependent (TD) antigen

Early B cell activation has been studied with common model antigens called hapten. Haptens are small non-natural chemical molecules e.g. nitrophenol (NP), which can only elicit a B cell mediated humoral immune response when coupled to a protein carrier such as ovalbumin. The so-called carrier effect induces the activation of T cells which in turn help B cells responding to the hapten.

Soluble proteins, bacterial toxins and other model antigens such as the above mentioned haptenated proteins as well as sheep RBC are examples of TD antigens. Professional antigen presenting cells (APC) such as DC or macrophages take up the antigen, process it and present determinants on MHC II molecules to the cognate TCR on T helper cells (Parker, 1993). B cells are being activated by binding the native antigen via their BCR and in addition gain further signals by cytokines from T cells as well as DCs and cell bound signals from cognate T_H. Actually, B cells can interact with already activated cognate T cells or act as APCs and activate T cells by presenting antigenic peptides on their MHC II molecules (Chesnut and Grey, 1981). Therefore, B cells are able to bind and present Ag especially at low Ag concentrations, as they can concentrate antigens through interaction with specific BCRs,

whereas professional APCs are more efficient at high Ag concentrations but are generally much faster in Ag presentation than B cells (Lanzavecchia, 1990). The interaction of T cells with APCs by peptide-MHC II complexes up-regulate further co-stimulatory molecules on the surface of both cell types (Tseng and Dustin, 2002). DCs express high levels of B7-1 and B7-2 as well as CD40 molecules which interact with CD28 and CD40L on T cells. Upon activation by cognate interaction with DCs, T cells move from the T cell zone towards the B cell area to interact with cognate B cells. B cells that have been stimulated through their BCR gain further stimulation upon recognition of peptides presented on their MHC II molecules by specific TCRs, causing CD40-CD40L interaction as well as stimulation by T cell derived cytokines which drives further activation and proliferation of B cells and delivers survival signals (Noelle et al., 1992a; Noelle et al., 1992b; Noelle et al., 1992c; Noelle et al., 1992d; Van den Eertwegh et al., 1993). At the border of T and B cell zone, in response to this strong co-stimulation by T helper cells, follicular B cells become extrafollicular and differentiate into plasmablasts and secrete either IgM or class-switched Abs (Liu et al., 1991; MacLennan et al., 2003). When $CD4^+$ T cells are being activated, they can be categorized by their distinct cytokine secretion pattern as T helper cell type 1 and type 2. T_H1 cells secrete IL-2, IFN-γ and lymphotoxin (LT), which mediate B cells to class switch to IgG2a and IgG2b. In contrast, T_H2 cells promote B cells expressing Ig of the isotype IgG1, IgG3 and IgE by secretion of IL-4, IL-5 and IL-10 (Coffman et al., 1988; Parker, 1993). This scheme has recently been refined and a number of additional T_H cell subsets has been added; most notable in this context are follicular T_H cells, which are specialized in driving B cell responses (Breitfeld et al., 2000; Havenith et al., 2013; Haynes, 2008). Furthermore, TLR-stimulation in B cells has been found to be the major factor driving IgG1 versus IgG2a responses (Coutelier et al., 1987; Jegerlehner et al., 2007).

These early foci of plasmablasts secreting specific Abs are of short-lived nature as the response progresses and some activated B cells migrate into the B cell follicles and initiate the germinal center (GC) reaction, during which SHM, CSR and affinity maturation of the BCR occur in SLOs (Gatto and

Brink, 2010). The CD40-CD40L interaction is critical for the outcome of T cell-dependent antibody responses (Klaus et al., 1994a; Klaus et al., 1994b) and formation of the GC reaction (Banchereau et al., 1994). Defects of CD40L in humans cause the X-linked hyperimmunoglobulin syndrome, which is characterized by high levels of IgM accompanied with low levels of swIg in sera (Foy et al., 1994b).

Germinal centers contain oligoclonal B cells as one to three B cells have been reported to colonize each GC (Jacob and Kelsoe, 1992). With time, a high number of centroblasts form the dark zone and proliferate (MacLennan, 1994). Their BCRs undergo SHM through point mutations within the variable regions at a high rate of 10^3 per base pair per generation (10^6 fold the normal rate of somatic mutation) (Berek and Milstein, 1987; McKean et al., 1984; Rajewsky et al., 1987), a process which requires the enzyme activation induced deaminase (AID) (Muramatsu et al., 2000). Due to chemotaxis the CXCR5 expressing centroblasts respond to CXCL13 derived from follicular dendritic cells (FDC) and move throughout the germinal center – from the dark to the FDC-containing light zone (Allen et al., 2004). In the light zone, centrocytes are selected through iterative cycles of somatic antigen receptor diversification (in dark zone) and the selection of B cells with receptors of best fit for the Ag (in the light zone) (Rajewsky, 1996). FDCs which retain unprocessed Ag bound to CR and FcR on their surface support affinity maturation of those B lymphocytes with increased affinity for the Ag in a process whereby GC B cells compete for the FDC-bound antigen (Mandel et al., 1980). T follicular helper cells (T_{FH}) also play an essential role during the affinity maturation (Vinuesa et al., 2010) and provide survival signals by e.g. IL-21 to B cells with increased Ag affinity (Bessa et al., 2010; Linterman et al.; Zotos et al.). LT α and β, members of the TNF cytokine family produced by B cells have been shown to play a central role in generation and maintenance of FDC networks within GCs (Fu et al., 1998; Futterer et al., 1998). If selection of B cells with increased Ag affinity was not successful, centrocytes may move back to the dark zone upon CXCR4 expression responding to stromal cells derived CXCL12 (Allen et al., 2004) to acquire further point mutations and to escape from apoptosis. Besides SHM, class switching of the constant region of Igs occurs as a second AID

dependent maturation process during the GC reaction. The IgH constant region of the μ exon (Cμ) is exchanged by an alternative set of downstream IgH C regions to express Ab of the same specificity but different isotype such as IgG1, IgG2a, IgG2b, IgG3, IgA or IgE. Various Ab classes mediate different effector functions (Harriman et al., 1993). Notably, the CS of the BCR, which can also occur extrafollicular (mentioned above) (MacLennan et al., 2003), changes the Ig half-life, the ability to bind to FcR and the activation of the complement system. Centroblasts that fail to compete for antigens and also do not migrate back to the dark zone undergo apoptosis and are cleared by macrophages (Ahmed and Gray, 1996; McHeyzer-Williams and Ahmed, 1999). Cell death in GCs is usually mediated by programmed death receptors and CD95 (Good-Jacobson and Shlomchik, 2010; Good-Jacobson et al., 2010).

Follicular B cells are dependent on the expression of the TF Bcl6 to enter the GC reaction (Dent et al., 1997). Neither naïve B cells nor PCs express Bcl6 (Reljic et al., 2000; Shaffer et al., 2000), which seems to counterbalance Blimp-1 expression to prevent terminal differentiation to plasma cells. Blimp-1 is a TF that drives PC differentiation (Angelin-Duclos et al., 2000; Shaffer et al., 2002) and its up-regulation can release a B cell from the GC reaction to become a plasmablast. In contrast, Bcl6 expression has been shown to play an important role in the formation and maintenance of memory B cells (Fearon et al., 2001; Schittek and Rajewsky, 1990), which persist after an infection to mediate host protection during Ag re-encounter. The process of leaving a GC or that a GC reaction comes to an end is a highly complicating process and has not been described in detail yet. Limiting Ag can be one reason that the GC reaction is stopped. There are many evidences that T_{FH} and their expression of IL-21 play an important role. However, in IL-21 and IL-21 R deficient mice a memory B cell compartment is still established, which indicates further factors that can drive a GC reaction (Zotos and Tarlinton, 2012).

Complement receptors

The complement receptors 1 (CD35) and 2 (CD21) are predominantly expressed on B cells as well as FDCs in mice (Batista and Harwood, 2009; Kurtz et al., 1990) and have shown to play an essential role in the innate and adaptive immunity.

CD21 and CD35 bind to opsonized Ag by C3 and C4 complement components. CD21 forms a co-receptor for the BCR and activates the surface molecule CD19 on B cells which lowers the threshold of B cell activation by the BCR (Carter and Fearon, 1992; Matsumoto et al., 1993).

Therefore, B1 cells as well as MZ B cells are able to respond in the absence of cognate T cell help, as these cells express high levels of CD21 and complement opsonized Ags can co-engage the complex of CD21/CD19/CD81 with the BCR on these cells. On follicular B2 cells, which usually can acquire T cell help, CD21 can nevertheless enhance the immune response by lowering the number of BCRs that need to be engaged (Dempsey et al., 1996; Jegerlehner et al., 2002). The expression of CRs is also important to FDCs in GC reactions. It has been shown that intact Ag reaches the B cell follicles in an Ig and complement dependent manner which ensures the persistence of Ag on FDCs during affinity maturation of activated B cells (Bessa et al., 2012; Fischer et al., 1998; Haberman and Shlomchik, 2003; Link et al., 2012; Rossbacher et al., 2006). In addition, the maintenance of memory B cells seems to be dependent on CR for Ag retention on FDCs in SLOs and/or on stromal cells in the BM (Barrington et al., 2002).

TLR expression on B cells and their role in B cell activation

Toll like receptors were first identified in Drosophila (Hashimoto et al., 1988). They cannot only be found in insect but in virtually all vertebrates which indicate them as an evolutionary conserved pattern recognition receptor (PRR) family (Lemaitre et al., 1996; Medzhitov et al., 1997; Nomura et al., 1994a; Nomura et al., 1994b; Poltorak et al., 1998). Therefore, TLRs bind to certain microbial molecules exhibiting an invariant and foreign structure (a so called pattern) that are conserved

among pathogens, and which do not occur in hosts. Several TLRs and their ligands have been identified and described in detail (Table 1).

Table 1 │TLRs and their ligands in humans and mice (O'Neill et al., 2013)

TLR	Ligand	Occurrence	On/In B cells?
TLR 1 **act as heterodimer with TLR2**	Lipopeptides	surface	yes
TLR 2 **act as heterodimer with TLR1 or 6**	Lipopeptides	surface	no
TLR 3	dsRNA	intracellular	yes
TLR 4	LPS	surface	yes
TLR 5	Flagelin	surface	no
TLR 6 **act as heterodimer with TLR2**	Lipopeptides	surface	yes
TLR 7	ssRNA	intracellular	yes
TLR 8	ssRNA	intracellular	no
TLR 9	CpG - DNA	intracellular	yes
TLR 10	Unknown		
TLR 11	Profilin	surface	no
TLR 12	Profilin		no
TLR 13	ribosomal RNA	intracellular	no

The engagement of TLRs on B cells can also lower the threshold of the BCR signaling and can serve as the second signal during B cell activation (Cooper et al., 1988; Mond et al., 1995a). In addition, engagement of TLR7/8 or TLR9 is the primary driver of IgG2a responses (Jegerlehner et al., 2007).

As one of the main features of viruses, they carry genetic information such as DNA and RNA. Viral and eukaryotic nucleic acids are quite different from each other, including the absence of a 5`triphosphate mRNA, as well as the presence of double stranded RNA and double stranded DNA in the cytoplasm (Ronald and Beutler, 2010; Yan and Chen, 2012). As mentioned above, there are different TLRs which can sense nucleic acids in APCs (mainly DCs) as well as B cells (table 1). TLR3 is activated by double stranded RNA, TLR7/8 by single stranded RNA and TLR9 by DNA. TLR9 only binds DNA which is rich in non-methylated CG motifs (CpGs). Those structures are mainly found in bacteria but also in viral DNA (Barton et al., 2006; Krieg et al., 1995).

1.3 Memory B cell compartment

The hallmark of the adaptive immune response is the generation of an immunological memory that mediates long-term protection during pathogenic re-encounter. In humoral responses, circulating memory B cells that are ready for rapid response to antigenic challenge and long-lived plasma cells constantly produce antibodies to ensure the host's protection. A memory B cell is characterized as a clone that has responded to antigen and returned to a resting state. Memory B cells are generally generated in GCs, where the BCR acquires mutations in the variable region and switches to another Ab class resulting in enhanced Ag affinity. Those activated and matured B cells can leave the GC as memory B cells or can differentiate into a PCs. Memory B cells remain mostly in a resting state and at expanded frequency in niches such as the BM or circulate throughout the system of SLOs searching for their cognate Ag. However, only PCs can actually provide direct effector function by secreting Abs. They can be found in SLOs or in the BM (MacLennan, 1994) and are often referred as LL-PCs.

Survival of memory B cells and PCs is independent of T cell help and persisting antigen (Crotty et al., 2003; Gatto et al., 2007b; Manz et al., 1998; Maruyama et al., 2000; Vieira and Rajewsky, 1990). The GC reaction can continue for months which is due to long-term retention of antigen on FDCs (Bachmann et al., 1996). Thus, there might be no general Ag independence of the maintenance of GC B cells, as during the latent phase of viral infections Ag is also permanently supplied to a certain extent (Zinkernagel et al., 1996). Nevertheless, both cell types are the main players during Ag re-exposures: the present Abs produced by LL-PCs can immediately neutralize the pathogen and activate further cells to clear the infection. In addition, memory B cells respond quickly and differentiate into PCs and also contribute to the humoral response.

Memory B cells can be defined operationally by their surface marker expression - CD27 in humans (Tangye et al., 1998) and CD38 in mice (Gordon et al., 2001; Ridderstad and Tarlinton, 1998). However, CD38 could not be established in humans. A combination of markers such as CD80, CD73, PD-L2, and CD21/23 characterize memory B cells and their subsets in more detail (Anderson et al., 2007; Dogan et al., 2009). Nevertheless, the most exclusive marker is still the BCR itself. Memory B cells usually derive from GCs. However, there also exist extra-GC memory B cells (Defrance et al., 2011; Taylor et al., 2012; Tomayko et al., 2010). In both cases, memory B cells can either carry BCRs of the µ or sw isotype. Only GC derived memory B cells exhibit increased Ag affinity as this maturation process does not occur elsewhere. As mentioned earlier, Bcl6 expression is required in B cells to enter GCs. Further analysis revealed that Bcl6 expression is indispensable for B cells to undergo SHM and to become LL-PCs, as Bcl6-deficient B cells could not acquire mutations in their BCRs (Toyama et al., 2002). Since Dogan and colleagues discovered multiple layers of memory and their different effector functions (Dogan et al., 2009), a few other groups contributed with additional insights about memory B cell populations (Pape et al., 2011; Racine et al., 2011; Yates et al., 2013).

Many LL-PCs migrate to the BM, which serves as one of their exclusive survival niche (Sze et al., 2000). LL-PCs are best described as positive for intracellular Ig and negative for surface Ig. In electron microscopic pictures, PCs show a distinct appearance of an increased cell size with a massively

enlarged ER allowing massive production of Abs. The massive protein production is counterbalanced with the unfolded protein responds which requires the TF XPB-1 and Blimp-1 (Taubenheim et al., 2012). In addition, Blimp-1 and IRF-4 are up-regulated, while bcl-6 is down-regulated in PCs (Calame, 2001; Nakayama et al., 2006; Radbruch et al., 2006). CD138 was described as exclusive PC marker, but only half of the PCs express CD138 formerly known as Syndecan-1. While plasmablasts still express low levels of B220 or CD19 and are therefore probably not fully differentiated, both surface markers are not expressed on PCs. To migrate to the BM, PCs must express the chemokine receptor CXCR4 which responds to CXCL12 produced by stromal cells in the BM. CXCR4 is also expressed on GC B cells which ensures the migration of centrocytes from the light zone towards the dark zone. It is still under investigation, whether and how the expression profile of CXCR4 changes during stages of a GC B cell to a PC. This might not be the case when GC B cells with higher affinity for the Ag compared to other clones preferentially leave the GC reaction earlier and differentiate into PCs (Phan et al., 2006) migrating to the BM by CXCR4 to ensure high affine clones for long term survival providing a protective humoral response. Although CD69 was once described as T cell activation marker (Shiow et al., 2006), it rather plays an essential role in the retention of different cell types in the lymphoid organs (Shinoda et al., 2012). Beside CXCL 12 and IL 7 production by stromal cells in the BM that make PCs “feel home”, eosinophiles, macrophages as well as megakaryocytes further secrete IL 6 and APRIL (Cassese et al., 2003; Chu and Berek, 2013; Tarlinton et al., 2008; Winter et al., 2010) In this setting it seems to be unlikely, that LL-PCs in the BM only persist in the presence of Ag (Manz et al., 2005). First, Ag must reach the BM and be retained for certain extent to be able to interact with cells. Second, PCs do not carry the BCRs on their surface nor MHC II molecules, which are involved in the Ag presentation machinery.

1.4 Antigens

The size, structure and type of antigen play an essential role in the activation of the immune system. Interestingly, the word antigen was coined by a Hungarian physician and microbiologist named Detre. He characterized this word already in 1899. Initially he thought those substances were precursors of antibodies. However, in 1903 he realized that those molecules induce the production of antibodies and called them anti-somato-gen, which was later referred as antigen (Lindenmann, 1984).

Antigens can be generally divided into self and non-self. Non-self antigens such as pathogens usually do not occur behind the mechanical barriers of healthy organisms and are recognized by pattern associated recognition receptor (PARR) or lymphocyte receptor (Lo et al., 1995; Moller, 1977) once they breach the barriers. A misguiding of the immune response can occur to foreign antigens which are harmless but perceived as dangerous as they deliver some sort of danger signal (Kohl, 2006). An example of those antigens are allergens which promote allergic reaction. Allergy is a dysfunction of the immune system responding to harmless antigens and leads to hypersensitivities. In contrast, self-antigens are present in the host and can induce an immune response if dislocated and are presented in the context of “danger” or an infection (Kato et al., 2011). ATP release out of a cell which mediates e.g. a danger signal to the immune system (Matzinger, 1994). Self-antigens are usually the target in auto immune diseases (Karges et al., 1995) but can also serve as targets in tumor immunology (Speiser et al., 1997).

The majority of antigens chemically belong to the families of proteins, polysaccharides nucleic acids and lipids, which virtually include all compounds of bacteria, viruses and other microorganisms. Receptors of the innate immune system mostly recognize lipids, nucleic acids and carbohydrates. However, stimulation of these receptors alone is usually not sufficient to induce an immune response.

Antigens can also be categorized according to their size. The size orchestrates whether an antigen can freely drain or must be transported to SLOs. Small proteins (< 70 kDa, <5 nm) are rapidly

channeled into B cell follicles via a conduit system, which virtually represents a “highway” for small antigen trafficking (Gonzalez et al., 2009; Pape et al., 2007; Phan et al., 2009; Roozendaal et al., 2009). Proteins larger than 70 kDa may also be brought to SLOs by migrating DCs (Qi et al., 2006). In contrast, particulate antigens such as VLPs can freely drain throughout the lymphatic and blood system towards SLOs, when their size does not exceed 200 nm (Manolova et al., 2008). Once they reach the subcapsular sinus (SCS) of the LN, they are further transported by SCS macrophages. However, whole viruses or particles (> 200 nm) acquire cell mediated transport by macrophages already from the side of entry (Carrasco and Batista, 2007; Junt et al., 2007) and by resident DC within LNs (Gonzalez et al., 2010). Dependent on their location, immune complexes, which can be highly diverse in size, can be transported by different cell types, such as macrophages, follicular dendritic cells (restricted to B cell follicles) or MZ B cells (Link et al., 2012; Pape et al., 2007; Roozendaal and Carroll, 2007; Suzuki et al., 2009).

Virus-like particles as model antigen

The mechanisms and rules underlying the induction of humoral immune responses have been intensively studied by common experimental antigens such as haptens, lysozyme and sheep red blood cells. Some of those model antigens are poor at inducing potent B cell responses, even when coupled to protein carrier as in the case of haptens. To be able to study B cell responses comparable to pathogenic infections, an antigen similar to viruses should be the best model antigen.

Virus-like particles (VLPs) are such antigens exhibiting many structural and geometrical features which they share with viruses.

Virus-like particles (VLPs) can be generated by the expression and assembly of the capsid proteins of viruses or bacteriophages (Kozlovska et al., 1993; Pumpens et al., 1995; Qiu et al., 1994). The coat protein of the *Escherichia coli* derived RNA bacteriophage Qβ as well as AP205 were cloned into the pQβ10 vector to be recombinantly expressed in *E. coli* (Cielens et al., 2000; Klovins et al., 2002). Their icosahedral capsids are composed of 180 subunits which reveal a highly ordered and repetitive

structure (Figure 1.4.1). Particles of an average size of 30 nm diameter are formed. Since those VLPs do not carry relevant genetic information, they cannot replicate in the host and are therefore non-infectious.

Their size as well as their highly organized structure make them potent immunogens even in the absence of adjuvant (Bessa et al., 2010; Bessa et al., 2008; Gatto et al., 2007a; Gatto et al., 2004; Jegerlehner et al., 2002; Lechner et al., 2002). The particular appearance of viral particle surfaces is very distinct and similar structures are virtually absent in the host. As a consequence, the immune system has developed receptors which target highly repetitive structures considered as geometric pathogen-associated molecular pattern (PAMP) (Bachmann and Zinkernagel, 1997). However, this highly organized structure also leads to efficient crosslinking of B cell receptors which is the key for potent B cell activation. During the production process, *E. coli* derived RNA is enclosed which serves as ligand for TLRs 7/8 which contributes to the immunogenicity of VLPs. Their size is optimal to be able to drain freely throughout the lymphatic system and they easily reach SLOs. Thus, VLPs are efficiently trapped on FDCs to prolong GC reactions and maintenance of the humoral response (Link et al., 2012).

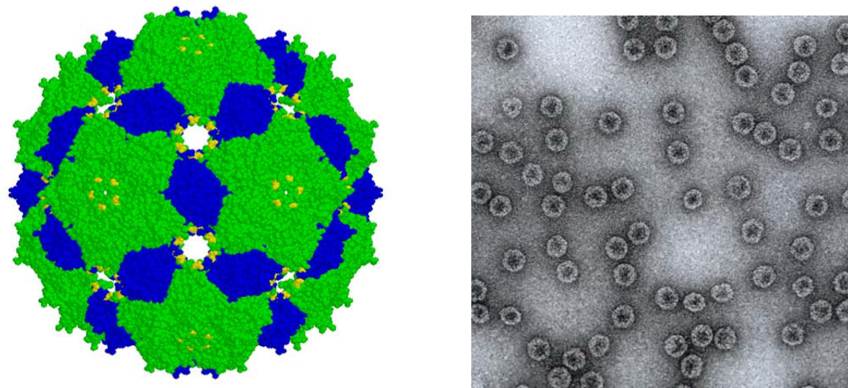


Figure 1.4.1| The bacteriophage derived VLP Qβ. The icosahedral schematic structure and electron microscopy image of several particles is shown.

1.5 Introduction related to experimental projects

1.5.1 Re-stimulation of memory B cells

For induction of long-lived humoral memory, interaction of B cells with their cognate antigen alone is not sufficient, since this process is T cell dependent (Parker, 1993). $CD4^+$ T cells help B cell activation through cell bound molecules, such as e.g. CD40L (Banchereau et al., 1994) and secreted molecules such as IL-4, IFNγ or IL-21 (Parrish-Novak et al., 2000; Rudge et al., 2002) which results in isotype-switching, B-cell proliferation and differentiation into memory B as well as plasma cells (PCs) (McHeyzer-Williams and McHeyzer-Williams, 2004; Rajewsky, 1996).

The long-lived humoral immune response is mainly driven by the germinal center (GC) response. Upon activation by cognate antigen, follicular B cells move to the edge of B cell follicles for interaction with specific $CD4^+$ T helper cells. Subsequently, some of the activated B cells move back into the B cell follicles and initiate the GC response, which results in mutated and affinity matured memory B cells and antibody forming cells (AFCs) (Allen et al., 2007; Blink et al., 2005; Jacob and Kelsoe, 1992; Reif et al., 2002). Typically, memory B cells express isotype-switched IgG or IgA immunoglobulins on their surface. However, immunization with protein or polysaccharide antigens can also result in a pool of IgM^+ memory B cells (Foote et al., 2012; Klein et al., 1997; Pape et al., 2011; Schittek and Rajewsky, 1992; Tangye and Good, 2007). Memory B cells appear to efficiently recirculate throughout the lymphatic system and are found at comparable frequencies in all lymphoid organs as well as in the blood (Bachmann et al., 1994). The majority of memory B cells resides in the marginal zone (MZ) and at a lower frequency in the splenic follicles close to GCs (Aiba et al., 2010; Anderson et al., 2007). In contrast, PCs are mostly found in lymphoid organs where they have been induced as well as in the bone marrow which serves as the primary survival niche up to several years (Bachmann et al., 1994; Slifka et al., 1995; Sze et al., 2000). They can usually only be detected in the blood for a short duration early after immunization (Blink et al., 2005; Manz et al., 1998). In general, GC-derived memory B cells as well as PCs persist independently of antigen

presence (Bachmann et al., 1994; Manz et al., 1998; Maruyama et al., 2000; Schittek and Rajewsky, 1990).

Upon secondary immunizations the humoral response is usually faster, stronger and of the IgG isotype. The cellular basis of this memory response is only starting to emerge. Recently, functional differences between IgM^+ and IgG^+ memory B cells have been described upon Ag recall (Dogan et al., 2009). Dogan et al. used a transgenic mouse model which irreversibly marked B cells GFP positive when activation induced deaminase (AID) has been expressed. AID is involved in somatic hypermutations and class switching of the B cell receptor (BCR), which normally occurs in GCs. Upon immunization with sheep red blood cells, they observed GFP^+ memory B cells which expressed either IgM or IgG on their surface. After antigenic re-exposure, the majority of the GFP^+ IgM^+ memory B cells initiated GC reactions and gave rise to a $IgG1^+$ memory pool (Dogan et al., 2009). In contrast, GFP^+ IgG^+ memory B cells failed to efficiently proliferate or enter GCs but rapidly differentiated into PCs. Different observations were made with a soluble protein antigen (phycoerythrin, PE) after secondary immunizations. The generated $IgG1^+$ memory B cells expanded and a proportion differentiated into PCs. However, unlike to the previous study, IgM^+ memory B cells were not observed to enter GCs which was probably due to the presence of IgG antibodies (Benson et al., 2009; Pape et al., 2011). Additional studies after secondary tetanus toxoid immunizations in humans showed a peak of PCs at around day 6 in the blood (Frolich et al., 2010; Odendahl et al., 2005) followed by a small increase of memory B cells at day 14 (Frolich et al., 2010). Analysis of the BCRs showed increased affinities due to further increased mutations after secondary immunizations without distinct dissection of the isotypes. This observation suggests that memory B cells went into a second GC reaction upon Ag re-challenge.

In order to be able to follow naïve and memory B cell responses in the same animal, we established an adoptive transfer system using memory B cells specific for virus-like particles (VLPs). Studies of memory B cell responses using prime-boost regimens in the same host have the disadvantage that

specific IgG antibodies are present, which generally suppress B cell responses. Therefore, we used an adoptive transfer model to study the cellular response preventing the bias of the cellular response if specific IgG would be present. We used VLPs derived from the bacteriophage Qβ as model antigen, which induce strong and long-lasting GC derived memory B cell, plasma cell as well as antibody responses. Both, the repetitive surface of the VLPs as well as the RNA that is spontaneously packaged during production contribute to the magnitude of the response (Bessa et al., 2009; Gatto et al., 2007b; Hou et al., 2011; Jegerlehner et al., 2007). Using this strategy, we were able to follow naïve and memory B cell responses under physiological conditions without the need of additional tools and treatments. We found that these VLPs induced IgM^+ as well as IgG^+ memory B cells. Adoptive transfer experiments demonstrated that in contrast to previous reports, both subsets failed to efficiently proliferate or enter GCs. Unexpectedly, both populations rapidly differentiated into unique secondary plasma cells, which homed to the BM and produced superior amounts of antibodies compared to their primary counterparts derived from naïve B cells.

1.5.2 T cell help dependency in memory B cell responses

B cells are activated when antigens such as viral particles bind to and crosslink their BCRs. After binding, the pathogen is taken up and processed for antigen presentation on MHC II molecules to enable interaction with T_H cells (Noelle and Snow, 1991; Parker, 1993). Activated B cells subsequently migrate to the edge of the T cell zone in SLOs and find and interact with their cognate T cell counterparts causing further reciprocal stimulation by cell bound factors such as CD40L stimulating B cells or CD28 stimulating T_H cells (Klaus et al., 1994a; Klaus et al., 1994b) as well as secreted cytokines such as IL4, IFNγ or IL21 (Parrish-Novak et al., 2000; Rudge et al., 2002). With the help of T cell derived co-stimulatory factors, B cells efficiently expand and initiate GC formation. Within GCs, follicular T_H cells further aid the B cell response, eventually leading to the formation of memory B cells and plasma cells, some of which are long-lived. These events represent the hallmark of the

humoral immune response to avoid re-infections for years or even lifelong. In the absence of T cell help, B cells may also sufficiently activated by antigens exhibiting a repetitive structure and efficient crosslinking of the BCRs (Bachmann et al., 1993; Dintzis et al., 1983; Perelson and Wiegel, 1981) and/or binding to Toll like receptors (TLRs) (Krieg et al., 1995; Mond et al., 1995a; Vos et al., 2000) which can serve as the 2nd signal during T cell independent (TI) B cell activation. However, the antibody responses induced in the absence of T_H cells are usually more short-lived and not very efficient as they only mediate host protection for a short time.

In contrast to T cell independent (TI) B cell responses, B cells form GCs within the B cell follicles during T cell dependent (TD) B cell responses. Their BCRs undergo isotype switching, hypermutation with subsequent selection for best fit for the antigen during a process called affinity maturation. Hypermutation and affinity maturation is largely restricted to B cells, as evidence for a similar process happening in the TCR is very limited (Dutton et al., 1998). BCR affinity has been reported to affect plasma cell versus memory B cell differentiation, with the plasma cell precursors generally exhibiting the higher affinities (Paus et al., 2006; Phan et al., 2006) which in turn leave the GC for differentiation, whereas low affine B cells stay and acquire more mutations in their BCR.

Memory responses usually occur much faster, are stronger and require lower doses compared to primary responses (Croft et al., 1994; Sallusto et al., 2004; Vieira and Rajewsky, 1990). Furthermore, it has been reported that memory immune cells need less co-stimulatory factors than naïve cells (Dutton et al., 1998). While T_H cell dependence for memory T cell responses has been investigated in great detail (Kaech et al., 2002; Shedlock and Shen, 2003; Sun and Bevan, 2003; Suvas et al., 2003), much less is known about the T_H cell dependence of memory B cell responses. We used the virus like particle Qβ as model antigen, which induces strong B cell responses due to the repetitive structure as well as the packaging of RNA as a natural TLR7/8 ligand. It is known that such particulate antigen can drive T cell independent IgM responses and are able to cause at least partially T cell independent secondary antibody responses (Jegerlehner et al., 2007; Jegerlehner et al., 2002). Therefore, we were

assessing whether VLP specific memory B cells are able to respond independently of specific and/or non-specific (memory) T cell help.

2 Objectives

Primary B cell responses have been characterized in detail. The activation of B cells including different subpopulations such as B1 cells, MZ B cells as well as conventional B2 cells is understood in great detail. Only B2 cells undergo a GC reaction leading to maturation of their BCRs by SHM, CSR and affinity maturation to become highly specific and effective B cells which mediate the long-term host protection. B cells leave the GC reaction either as memory B or plasma cell. Although there have been some recent studies investigating different memory B cell populations and functions, not much is known about the fate of memory B cell responses after cognate Ag encounter. There mainly exist two memory B cell populations – IgM^+ as well as IgG^+. Studies by Dogan et al. as well as Pape and colleagues could reveal different effector functions for IgM^+ and IgG^+ memory B cell sub-populations using protein Ag (Dogan et al., 2009; Pape et al., 2011).

To study B cell memory using viral particles, we performed adoptive transfer experiments of memory B cell into a naïve host to track memory B cell versus naïve B cell responses in one animal under physiological conditions using Qβ-VLP as model antigen.

By conducting adoptive transfer experiments we wanted to address several questions:

- Which memory B cell population responds best to antigenic challenge: IgM^+ or IgG^+ memory B cells?
- Do IgM^+ and/or IgG^+ memory B cells proliferate extensively if even at all upon Ag re-challenge?
- Do IgM^+ and/or IgG^+ memory B cells differentiate quickly into plasma cells?
- Do naïve host cells similarly respond to the antigen in the presence of memory B cells as observed in primary B cell responses?

- How efficient is the GC response mounted? Do memory B cells undergo a second GC reaction? Do only host naïve B cells enter a GC reaction?
- Can memory B cells respond to multiple rounds of stimulation?
- How are $CD4^+$ T cells in memory B cell responses involved?

Our goal was to extend our knowledge about memory B cell responses by answering these questions and to implement the findings to optimize vaccination regimens for the generation of protective memory B cells. Regarding the results and conclusions, we might be able to establish optimal conditions for memory B cells to either re-enter GCs for better affinity maturation or to differentiate into plasma cells for rapid antibody production upon antigen re-encounter.

3 Material and methods

3.1 Buffers

Puffer & solutions	Components
ACK buffer (RBC lysis)	0.15 M ammonium chloride, 0.01 M potassium hydrogen carbonate, pH 7.2-7.4
FACS buffer	PBS pH 7.4, 2%FCS, 5 mM EDTA
PBST	PBS pH 7.4, 0.05% Tween-20
ELISA buffers	
Blocking buffer	PBST, 2% BSA
Coating buffer	0.1 M $NaHCO_3$, pH 9.6.
Serum dilution buffer	Blocking buffer
OPD buffer (citric acid buffer for substrate)	0.066 M Na_2HPO_4, 0.035 M citric acid, pH 5.0
Substrate solution buffer	1 tablet (10 mg) OPD, 9 µl of 30% H_2O_2 to 25 ml citric acid buffer
Stop solution	5% H_2SO_4 in H_2O
Immunofluorescence buffers	
Fixation	100% acetone or 4% PFA
Blocking buffer	1% BSA, 1% NMS in PBS
Dilution buffer	0.1% BSA, 1% NMS in PBS

3.2 Reagents and Antibodies

All common chemical compounds that are not further specified were purchased from Sigma Aldrich, Switzerland or Fluka, Switzerland. PBS (1x) was obtained from the Cantonal drugstore at the University Hospital Zurich.

Material & Reagents	**Producer**	**Order number**	**Specificities & Comments**
A/A antibiotic/antimycotic	Invitrogen	15240120	100x
Alexa Fluor 488 Protein Labeling Kit	Invitrogen	A10235	
Alexa Fluor 467 Protein Labeling Kit	Invitrogen	A20173	
DMEM w/o Glu	Invitrogen	12491-015	
ELISA plates – Maxi Immunosorb	Nunc	442404	
Fluorescence Mounting medium	Dako	S3023	
FACS lysing solution	Becton Dickinson (BD)	349202	8 ml FACS lysis solution, 2 ml H_2O, 4 µl Tween-20
HEPES	Invitrogen	15630080	
OPD	Fluka	95302	
Antibodies / Proteins			***Dilutions/Clone***
biotin Rat a Ms CD4 (L3T4)	BD Pharmingen	553728	500
biotin rat a ms CD8a (Ly-2)	BD Pharmingen	553029	500
rat a Ms CD16/32 (FcγIII/II rcpt)	BD Pharmingen	553142	200
HRPO Streptavidin	Jackson Immuno	016-030-084	1000
PerCP rat a ms CD4	BD Pharmingen	553052	400 / L3T4
PE Ham a Ms CD11c	BD Pharmingen	553802	500
PE rat a ms CD11b	BD Pharmingen	553311	500
FITC Rat a Ms CD8a (Ly-2)	BD Pharmingen	553031	300
FITC Rat a Ms CD4	BD Pharmingen	553729	300 / L3T4
biotin Rat a Ms CD11b	BD Pharmingen	553309	400
biotin ham a ms CD11c	BD Pharmingen	553800	500
Alk.Phos. Don a Gt IgG (H+L)	Jackson Immuno	705-055-147	1000
PE rat a ms Ly-6G and Ly-6C (Gr-1)	BD Pharmingen	553128	500

Antibodies & Proteins	**Producer**	**Order number**	**Dilution / Clone**
PE rat a ms CD4	BD Pharmingen	553653	500 / L3T4
PE rat a ms IgD	eBioscience	12-5993	500
PNA-biotin	Vector	B-1075	1000
PerCP rat a ms CD8a (Ly-2)	BD Pharmingen	553036	400
HRPO gt a ms IgG (Fcγ spec)	Jackson Immuno	115-035-071	1000
APC ms a ms CD45.1	eBioscience	17-0453-82	400
PE F(ab')2 gt a ms IgM (mu chain spec)	Jackson Immuno	115-116-075	500
PE F(ab´)2 gt a ms IgG (Fcγ spec)	Jackson Immuno	115-116-071	1000
Alexa Fluor 647 rat a ms CD45R/B220	BD Pharmingen	557683	400
PE-Cy7 Streptavidin	BD Pharmingen	557598	500
APC-Cy7 Streptavidin	BD Pharmingen	554063	400
APC rat a ms IgM	BD Pharmingen	550676	400
PE-Cy7 rat a ms CD45R/B220	BD Pharmingen	552772	400
PE rat a ms CD8a (Ly-2)	BD Pharmingen	553032	500
PE-Cy7 rat a ms CD4	eBioscience	25-0041	400 / L3T4
Alexa Fluor 546 gt a rat IgG (H+L)	Invitrogen	A11081	500
Biotin rat a ms IgM	BD Pharmingen	553406	
APC-Cy7 ms a ms CD45.1	BioLegend	110716	300
Streptavidin-Alexa488	Invitrogen	S32354	1000-5000
Streptavidin-Alexa546	Invitrogen	S11225	1000-5000
Streptavidin Alexa647	Invitrogen	S21374	1000-5000
Biotin ms anti-ms IgG1(a]	BD Pharmingen	553500	250 / 10.9
Biotin ms anti-ms IgG1(b]	BD Pharmingen	553533	250 / B68-2
Biotin ms anti-ms IgG2a(a]	BD Pharmingen	553502	250 / 8.3
Biotin ms anti-ms IgG2a(b]	BD Pharmingen	553504	250 / 5.7
a ms CD45.2 APC	eBioscience	17-0454	400
APC-Cy7 a ms CD45R/B220	BioLegend	103224	400
PE-Cy™7 Rat Anti-Mouse CD19	BD Pharmingen	552854	300
PE-Cy7 ham a ms CD3e	BD Pharmingen	552774	300
Qβ-Alexa488	Cytos		1-5 ug/ ml
Qβ-Alexa647	Cytos		1-5 ug/ ml
AP205-Alexa488	Cytos	E046P01	1-5 ug/ml

Antibodies & Proteins	Producer	Order number	Dilution / Clone
Qb	Cytos	batch Qx2.2	1-5 ug/ml
AP205	Cytos	E046P01	1-5 ug/ml
rabbit anti-Qβ polyclonal serum	Cytos		1500
gt-anti ms IgG	EY Laboratories	AT-2306-02	1000
gt anti ms IgM	Sigma	M8644	1000
Sheep anti-rb biotin	The Binding Site	AB311	
Rb anti-rt biotin	DakoCytomation	E 0468	200
Gt-anti-ms biotin	DakoCytomation	E0433	
APC-Cy7 mouse anti mouse CD45.1	BD Pharmingen	560579	400
APC Rat anti mouse IgG1	BD Pharmingen	550874	400
PerCP rat anti mouse IgG 2 a+b	BD Pharmingen	340271	15 ul /50 ul staining

3.3 Mice

Wild-type C57BL/6 mice were purchased from Harlan (Horst, The Netherlands). A breeding pair of Ly5.1 (B6.SJL-Ptprc<a> Pepc<b>/BoyJ(#002014)) mice were obtained from Jackson Laboratory (USA) and further bred at the germ-free BZL mouse facility at the University Hospital (Zurich, Switzerland). IgHa mice were bred at Biosupport (Schlieren, Switzerland). All mice experiments were conducted in accordance to ethical principles and guidelines of the Cantonal Veterinary Office.

3.4 Antigen

Qβ-virus like particles (VLPs) are derived from the bacteriophage Qβ. The VLP self-assembles during the production process in *E. coli*. Along the way, *E. coli* derived mRNA (ssRNA) is packaged within the particle. The Qβ-VLP consists of 180 subunits and forms a particulate and repetitive structure. The purification process is described elsewhere (Cielens et al., 2000).

3.5 Immunization

Mice were immunized with 50 µg of Qβ-VLP intravenously (i.v.) to generate memory B cells, which were isolated from the spleen and adoptively transferred. To challenge mice, 20 µg Qβ-VLP were administered intravenously. For immunization, the VLPs were diluted with sterile PBS and injected in a volume of 150 µl into the tail vein.

3.6 Adoptive transfer of memory B cells

Spleens were collected from congenic Qβ-VLP-immunized Ly5.1 or IgH^a mice in DMEM media supplemented with 2% FCS, antibiotics and 10 mM HEPES. A single cell suspension of the splenocytes was prepared. Red blood cells (RBC) were lysed by ACK buffer (0.15 M ammonium chloride, 0.01 M potassium hydrogen carbonate, pH 7.2-7.4). Splenocytes were then either directly transferred into recipient mice or as B cells were further purified by MACS and/or cell sorting. In general, Ly5.1 memory B cells were transferred into Ly5.2 positive C57BL/6 recipient mice, which allowed us to track the memory and the endogenous B cell response. Memory B cells generated in IgH^a mice were transferred into C57BL/6, which expresses the IgH^b-allotype. Hence, we were able to distinguish antibodies resulting from the transferred memory B cells (IgH^a positive) or from newly activated naive B cells of the host (IgH^b positive). Recipient mice (Ly5.2 or IgH^b) received 1/10 of a donor-mouse (Ly5.1 or IgH^a) derived memory spleen. A number of ~ $1x10^7$ cells of total splenocytes containing ~ 0.03-0.08% ($3\text{-}8x10^3$) VLP specific memory B cells were transferred. The corresponding purified populations were transferred with a frequency of total splenocytes: 50% (~ $0.5x10^7$ cell) for $B220^+$ MACS purified memory B cells, 1% (~ $1x10^5$ cells) for IgG^+ memory B cells and 2% (~ $2x10^5$ cells) for the IgM^+ memory B cells. Control mice received 1/10 of whole spleen of naïve Ly5.1 or naïve IgH^a mice, respectively.

3.7 ELISA

Blood was taken at indicated time points. For determination of Qβ-VLP-specific antibody titers, ELISA plates (Nunc Immuno MaxiSorp, Rochester, NY) were coated overnight with 100 µl of Qβ-VLPs (1 µg/ml) and binding of serum antibodies was detected by horse-radish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch). Pairs of allotype-specific markers were used to determine antibodies produced either by B cells of the IgH^a strain (biotin ms anti-ms IgG1[a] (10.9), biotin ms anti-ms IgG2a[a] (8.3)) or IgH^b strain (biotin ms anti-ms IgG1[b] (B68-2), biotin ms anti-ms IgG2a[b] (5.7)). Antibody binding was detected by horse radish peroxidase labeled streptavidin (BD). Absorbance readings at 450 nm of the 1,2-Phenylenediamine dihydrochloride color reaction were analyzed as OD50 which represents the reciprocal dilution reaching half maximum of the OD.

3.8 ELISPOT

Qβ-VLP specific antibody-forming cell (AFC) frequencies were determined as described (Bessa et al., 2008). Briefly, 24-well plates were coated with 10 µg/ml Qβ-VLPs. Single cell suspensions of bone marrow cells were added in DMEM containing 2% FCS and incubated for 5 h at 37°C. Cells were washed off and plates were incubated with goat anti-mouse IgG (EY Labs), followed by alkaline phosphatase-conjugated donkey anti-goat IgG antibody (Jackson ImmunoResearch) before development of alkaline phosphatase color reactions.

3.9 Flow Cytometry (FCM)

Single cell suspensions of splenocytes and bone marrow cells were prepared. Prior staining, RBC lysis of samples was performed. If not specifically indicated all antibodies for FCM and IF were purchased from Becton Dickenson (BD) Biosciences and eBioscience (eBio). Fc receptors were generally blocked with an anti-CD16/32 antibody. To detect specific memory B cells, splenocytes were stained for

differential surface expression and were characterized as PE-Cy7 - B220^{+}, negative for PE – IgD, CD4, CD8, CD11b, CD11c and Gr-1 and positive for binding to fluorescence labeled Qβ-VLP (Alexa 488). GC B cells were analyzed for binding to biotinylated peanut agglutinin (PNA, Vector laboratories) and subsequent detection with streptavidin labeled APC-Cy7. To be able to distinguish subtypes of specific memory B cells, B lymphocytes were stained for binding to PE - anti-IgM or PE - anti-IgG. Specific plasma cell numbers were obtained by staining splenocytes and bone marrow cells which were characterized as PE-Cy 7 - B220^{low} and negative for PE – IgD, CD4, CD8, CD11b, CD11c and Gr-1. Cells were permeabilized using FACS lysing solution (BD) and stained for intracellular binding to labeled Qβ-VLP (Alexa 488), PE - anti-IgM or PE- anti-IgG. Surface binding was blocked by unlabeled Qβ-VLP, anti-IgM or anti-IgG. Transferred B cells were analyzed for allotype marker (Ly5.1) expression and stained with anti -Ly5.1-APC antibody. Qβ-VLPs were labeled by fluochrome Alexa 488 according to the producer's instructions (Invitrogen). The cell acquisition was measured by the FACS Canto II (BD) and data was analyzed by Flow Jo version 7.6.4 (Tree Star software).

3.10 Cell purification and sorting

In order to transfer distinct memory B cell populations, splenocytes of memory mice were either purified by B220^{+} MACS beads (Miltenyi) or sorted as IgM^{+} or IgG^{+} memory B cells. B220^{+} MACS purified B cells were further sorted positive for B220-PE-Cy7 (as control for MACS purification) and negative for PE – IgM, IgD, Gr-1, CD11b, CD11c, CD4 and CD8 expression. This population represented the class switched (CS) specific and unspecific B cells. A second population which was also B220^{+} MACS purified was further sorted positive for B220-PE-Cy7 (as control for MACS purification) and negative for PE – IgG, IgD, Gr-1, CD11b, CD11c, CD4 and CD8 expression. This population represented the specific and non-specific IgM^{+} memory B cells. Samples were sorted with FACS Aria. Purities of 95-98% were yielded.

3.11 Immunofluorescence (IF)

Fresh spleen tissue was embedded in optimum cutting temperature (OCT) compound (Sakura) and was snap frozen in liquid nitrogen. 6-8 µm cryostat sections on Superfrost Plus glass slides (Fisher Scientific) were air-dried and could be stored up to a couple of weeks in a cassette at room temperature. After rehydration in ice-cold acetone for 10 min, sections were blocked with 1% (w/v) BSA (Sigma) and 1% (v/v) normal mouse serum (Jackson laboratories). All stainings were performed in PBS containing 0.1% (w/v) BSA and 1% (v/v) normal mouse serum. B cell follicles were identified with Alexa 647 labeled anti-B220. GC B cells were visualized by binding to biotinylated PNA (Vector laboratories). Specific B cells were detected for binding to Alexa 488 labeled Qβ. Transfer derived B cells were stained with biotinylated CD45.1. Biotinylated antibodies were detected with Alexa 546-labeled streptavidin (Molecular Probes). Images were acquired on an Axioplan microscope with an AxioCam MRm (Zeiss) camera using Open laboratory software (Improvision) and edited with Adobe Photoshop software.

3.12 Statistics

Statistical analysis was obtained by software Graph Pad Prism 6. With regards to data sets, the significance of two groups were calculated by different t-tests such as double student's T-test with Welsh's correction, unpaired t-test, Mann-Whitney test. For the comparison of three groups, the statistical analysis was performed with an F-test (ANOVA). Statistical significance was defined as $p < 0.05$.

4 Results

4.1 Qβ-VLP drive rapid differentiation of memory B cells into secondary plasma cells producing increased levels of antibodies

4.1.1 Accelerated antibody response in the presence of memory B cells

In order to be able to selectively trace the response of memory B cells to VLP challenge, we established an adoptive transfer model (Figure 4.1.1). To this end, Ly5.1 congenic mice were immunized with Qβ-VLP and 4-6 weeks later either total splenocytes or B220^{+} MACS purified B cells were isolated and transferred into Ly5.2^{+} recipient mice. After 24 h the mice were challenged with Qβ-VLP.

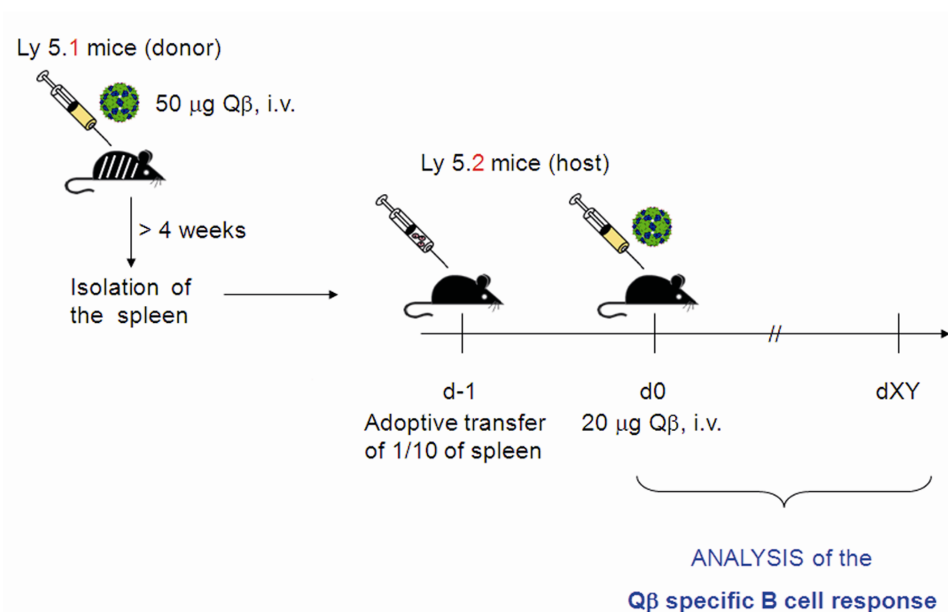


Figure 4.1.1 | Experimental set up. Congenic mice were immunized with 50 µg of Qβ-VLP in PBS intravenously. After 4-6 weeks the spleen was isolated. Only 1/10 of whole splenocytes of immunized Ly5.1^{+} containing memory B cells were either unpurified or purified into Ly5.2^{+} recipient mice transferred. Subsequently, the recipient mice were challenged with 20 µg of Qβ-VLP in PBS intravenously after 24h after the adoptive transfer.

In order to minimally perturb the memory B cells, we tried to limit the experimental handling of the cells as much as possible. Thus, recipient mice received either non-purified splenocytes or B220^{+}

MACS purified B cells. As control, splenocytes from naïve Ly5.1 mice were transferred into Ly5.2 recipients. 24 hours after the transfer, mice were challenged with 20 µg of Qβ-VLP. The hallmark of memory B cell responses is an accelerated antibody response. Indeed, we observed that the humoral response at early time points was higher in memory B cell (memory transfer & B220$^+$ purified transfer) recipient mice compared to control mice which only received naïve splenocytes (Fig. 2A). However, the antibody titers at later time points (t > d12) were comparable between mice receiving primed or naïve B cells. Memory B cells therefore accelerated the early antibody response while they made a minor contribution at later time points (Figure 4.1.1 A). By using congenic mice expressing the allotype a (IgH^a) instead of b (IgH^b), we were able to distinguish the antibody production resulting from transferred memory B cells or from *de-novo* activated naïve B cells within the recipients. Hence, mice of the IgH^a strain were immunized and after 4-6 weeks splenocytes containing memory B cells were isolated and adoptively transferred into naïve mice expressing IgH^b. As expected, the antibody response was dominated by the transferred memory B cells (Figure 4.1.2 B).

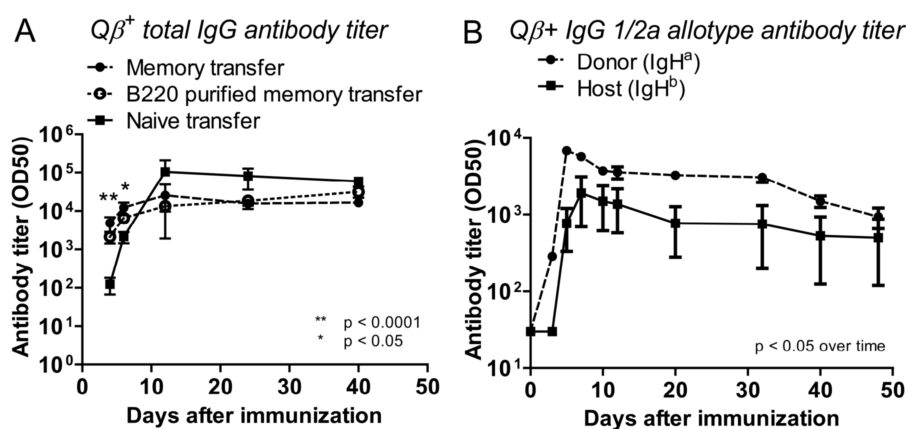


Figure 4.1.2│ Transferred memory B cells dominate the early humoral response. Qβ positive memory B cells derived from immunized congenic mice (Ly5.1 or IgH^a) were transferred into recipient mice (Ly5.2 or IgH^b) and challenged with Qβ-VLP. Mice which received an adoptive transfer of naïve splenocytes served as a control and presented a primary response. (A) Qβ-VLP specific total IgG antibody titer of mice which received undepleted memory, B220$^+$ MACS purified or naïve splenocytes. Mean with SEM. *P* values represent the significance of Memory and B220 purified memory transfer versus naïve transfer at indicated time points. (B) Qβ-VLP specific

IgG1 and IgG2a antibody titer originated of allotype a or b of mice which had received splenocytes from immunized mice containing memory B cells. Mean with SEM. Mice per group n=3. Data are representative of at least three independent experiments.

4.1.2 Only small numbers of specific memory B cells are found in recipient mice after antigenic challenge

The magnitude of the memory B cells response at the cellular level was assessed next and compared to responses of naïve mice. Congenic Ly5.1$^+$ mice were immunized. After 4-6 weeks splenocytes were isolated and either directly or by B220$^+$ MACS beads purified and transferred into recipient mice (Ly5.2$^+$). 24 hours later the mice were challenged with Qβ-VLP. After indicated time points we checked for the population of activated specific B cells (% VLP specific B cells within B220$^+$, IgM^{low}, IgD^{low}) within the spleen. As expected, after immunization of naïve mice (Gatto et al., 2007b; Jegerlehner et al., 2007), up to 25% of B cells with an activated phenotype (B220$^+$, IgM^{low}, IgD^{low}) were specific for Qβ at the peak of the response (Figure 4.1.3 A, B). Surprisingly, the overall Qβ-specific B cell response was strongly reduced (~ 10x) in the presence of transferred splenocytes containing memory B cells as well as B220$^+$ purified B cells (Figure 4.1.3 B). Reduced expansion of specific host B cells (Figure 4.1.3A) correlated with the observation that host B cells mounted a relatively poor antibody response when memory B cells were present (Figure 4.1.3 B).

In order to dissect the memory versus naive B cell response we analyzed the expression of the allotype marker Ly5.1 within the specific activated B cell population (Qβ-VLP$^+$ B220$^+$, IgM^{low}, IgD^{low}). Unexpectedly, between 70 - 90% of Qβ-specific B cells were host (Ly5.2$^+$) and not donor (Ly 5.1$^+$) derived in mice receiving splenocytes from immunized mice (Figure 4.1.3 C). This indicates that memory B cells do not proliferate nearly as well as naïve B cells and even inhibit the host primary B cell response. This suppression is in agreement with a study by Jenkins and colleagues (Pape et al.

Science 2011) who reported some suppression of the host response in the presence of switched immunoglobulin (swIg) memory B cells.

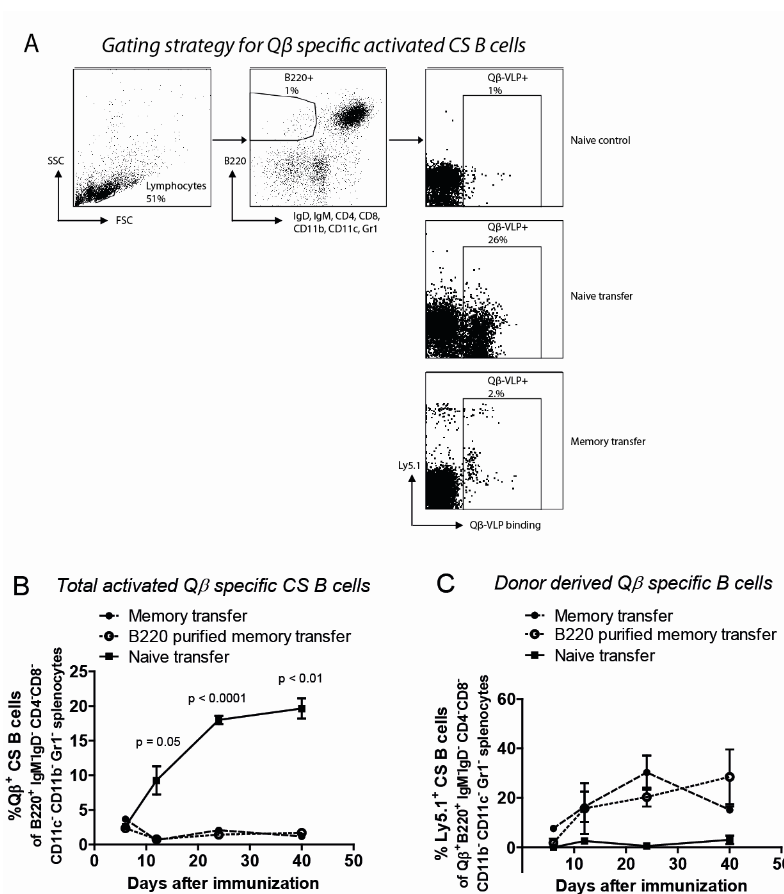


Figure 4.1.3│ Memory B cells do not efficiently proliferate after antigen re-encounter. Memory B cells derived from congenic immunized mice (Ly5.1$^+$) were transferred into recipient mice (Ly5.2$^+$) either undepleted or B220$^+$ MACS purified and challenged with Qβ-VLP. Mice which received an adoptive transfer of naïve splenocytes served as a control and presented a primary response. (A) Gating strategy of FCM analysis on day 13 after immunization upon adoptive transfer. (B) Frequencies of Qβ specific B cells within the activated B cell population (B220$^+$, IgM$^-$, IgD$^-$, CD4$^-$, CD8$^-$, CD11c$^-$, CD11b$^-$, Gr-1$^-$). (C) Distribution of responding transferred memory B cells (donor, Ly5.1) within the activated specific B cell population (Qβ$^+$B220$^+$, IgM$^-$, IgD$^-$, CD4$^-$, CD8$^-$, CD11c$^-$, CD11b$^-$, Gr-1$^-$). Mean with SEM. P value represents the significance of Memory and B220 purified memory transfer to Naïve transfer. Mice per group n=3. Data are representative of at least three independent experiments.

The question whether transferred memory B cells homed to other organs than the spleen, which could also contribute to secondary B cell responses, was addressed next. To this end, we quantified the number of transferred lymphocytes and specific B cells (Figure 4.1.4 A) in different lymphoid and non-lymphoid organs. No transferred lymphocytes were found in the kidney and liver (data not shown) and only a small number in blood, BM and lung. However, the majority of transferred non-specific lymphocytes were detected in lymph nodes and the spleen (Figure 4.1.4). A small number was also found in the lung. In contrast, the majority of specific B cells was only detected in spleen (Figure 4.1.4 C). Hence, secondary B cell responses did not occur besides the BM or secondary lymphoid organs and in particular the spleen, confirming that memory B cells only inefficiently expanded upon antigenic challenge.

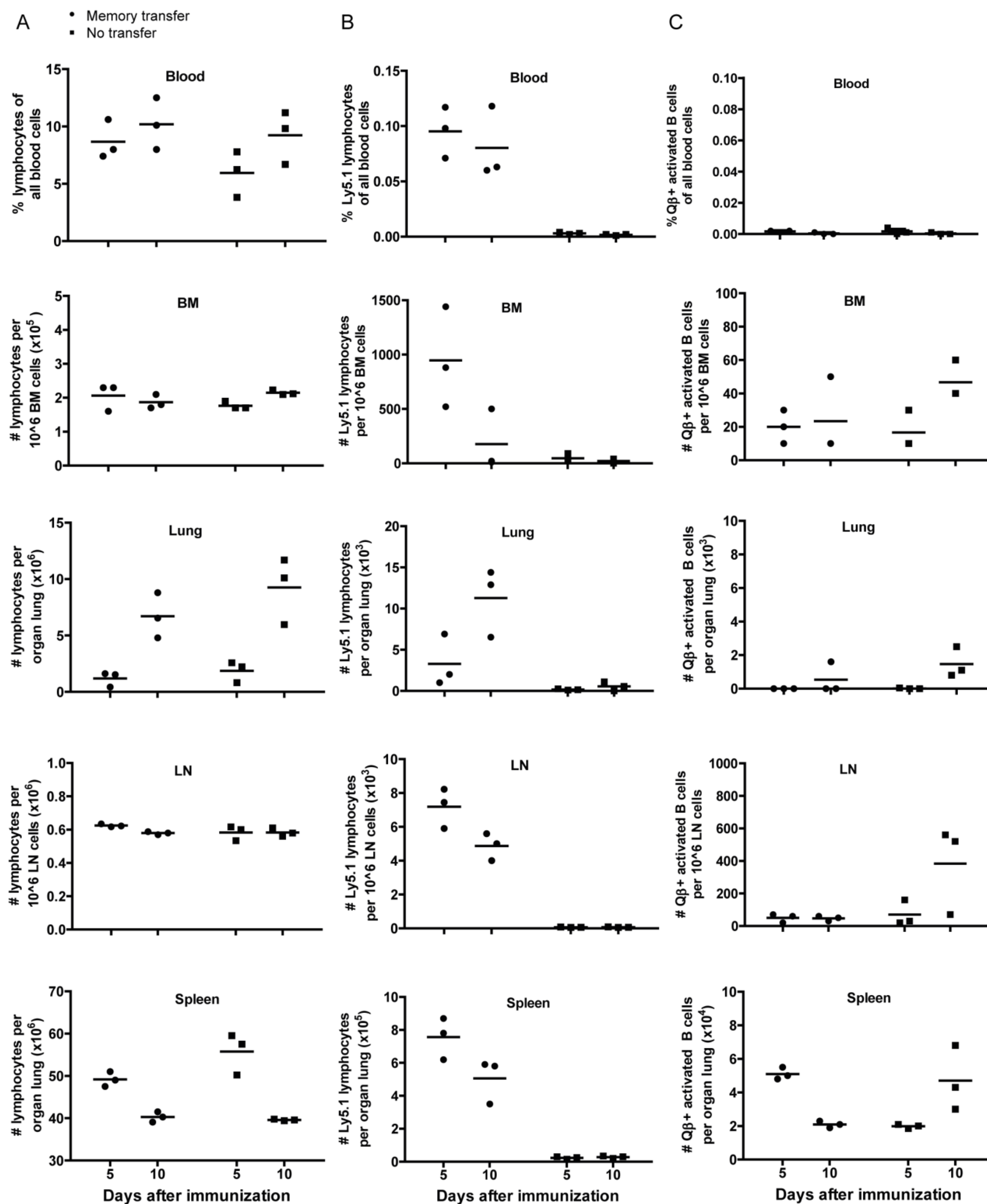


Figure 4.1.4│ Recovery of transferred splenocytes after adoptive transfer. Memory B cells derived from immunized congenic mice (Ly5.1$^+$) were transferred into recipient mice (Ly5.2$^+$) and 24 h later challenged with Qβ-VLP. Control mice did not receive an adoptive transfer and were only immunized. BM, blood, lymph nodes (LN), lung and spleen were analyzed to detect frequencies and numbers of transfer derived (Ly5.1$^+$) or Qβ-VLP specific splenocytes. (A) Analysis of proportions (blood only) or numbers of lymphocytes. (B) Recovery of Ly5.1$^+$

lymphocytes in recipient mice after day 5 or day 10. (C) Detection of Qβ-VLP specific lymphocytes throughout different organs. Mice per group n=3 of three independent experiments.

4.1.3 Memory B cells do not re-enter GCs upon Ag re-challenge

Induction of GC-formation was assessed next by flow-cytometry and histology (Figure 4.1.5). One half of the spleen was used for flow cytometry and the other half for histological analysis. Congenic ($Ly5.1^{+}$) mice were immunized, spleens 4-6 weeks later isolated and either unpurified or $B220^{+}$ MACS beads purified transferred into recipient mice ($Ly5.2^{+}$). After 24 hours the mice were challenged with Qβ-VLP. The PNA expression was assessed within the specific activated B cell population (% PNA expression within $Q\beta^{+}$, $B220^{+}$, IgM^{low}, IgD^{low}) in the spleen. Although a significant population of PNA^{+} B cells could be observed in the presence of transferred splenocytes containing memory B cells, the majority of these cells was host-derived (Figure 4.1.5 A-C). Differences were even more pronounced if GC-formation was assessed by histology. We identified B cell follicles by B220 expression and stained for PNA and Qβ-VLP binding. Congenic B cells were visualized by Ly5.1 staining. Beside reduced numbers of established GCs in the presence of splenocytes from immunized mice, virtually all GCs were host ($Ly5.1^{-}$) and not donor ($Ly5.1^{+}$) derived (Figure 4.1.5 D-H). Figure 4.1.5 G shows a rare exception of a memory B cell-derived GC which was positive for the congenic Ly5.1 marker. Similar findings were made by Pape et al. (Pape et al., 2011). They could observe suppression in GC formation when swIg memory B cells were present.

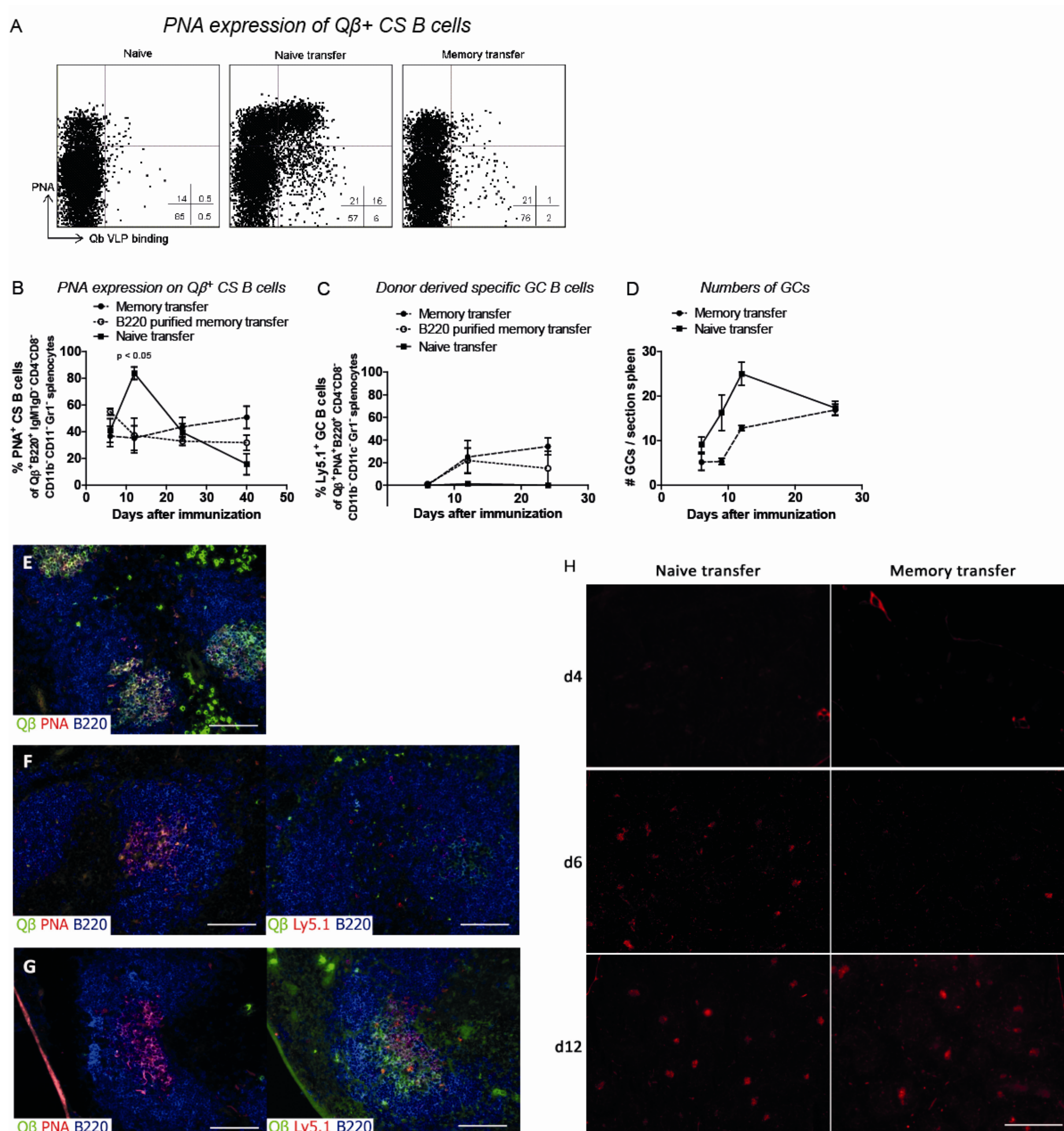


Figure 4.1.5│ Memory B cells inefficiently form germinal centers. An adoptive transfer experiment was performed to assess the ability of memory B cells entering germinal centers. PNA expression was assessed by FCM (A-C) and histology (D-G). (A) Gating strategy of FCM analysis of Qβ specific CS B cells expressing PNA on day 12 after challenge. (B) Distribution of PNA expression gated on $Q\beta^{+}B220^{+}$, IgM^{-}, IgD^{-}, $CD4^{-}$, $CD8^{-}$, $CD11c^{-}$, $CD11b^{-}$, $Gr\text{-}1^{-}$ B cells. (C) Distribution of Ly5.1 expression gated on $PNA^{+}Q\beta^{+}B220^{+}$, IgM^{-}, IgD^{-}, $CD4^{-}$, $CD8^{-}$, $CD11c^{-}$, $CD11b^{-}$, $Gr\text{-}1^{-}$ B cells. (D) Quantitative analysis of numbers of GCs by their PNA expression per section of spleen. (E-H) Green: Staining for specific B cells by Qβ-VLP binding (stained with Qβ-VLP labeled Alexa 488); Blue: Staining with anti-B220 antibody; Red: indicated at either PNA or anti-Ly5.1. (E) GC formation during a primary

response on day 26 after immunization. Scale bar 100 µm (F, G) Simultaneous immune fluorescence staining of spleen sections for detection of donor derived GCs. (F) Example of a host derived GC (no signal for Ly5.1) on day 9 after antigen re-challenge. Scale bar 100 µm (G) Example of a rare donor derived GC (positive signal for Ly5.1) on day 26 after antigen re-challenge. Scale bar 100 µm (H) Spleen sections have been stained for GC formation by PNA expression. Scale bar 1000 µm. Mice per group n=2 (only for D) or 3 of at least three independent experiments.

4.1.4 Rapid differentiation of memory B cells into secondary plasma cells

Plasma cell numbers in the spleen were enumerated by FCM in a next set of experiments (Figure 4.1.6 A). Plasma cells were defined as positive for intracellular antibody, negative for B220, CD4, CD8, CD11b, CD11c and Gr1. Congenic ($Ly5.1^+$) mice were immunized, spleens 4-6 weeks later isolated and unpurified transferred into recipient mice ($Ly5.2^+$). After 24 hours the mice were challenged with Qβ-VLP. Despite the smaller number of Qβ-specific activated CS B cells in the presence of splenocytes from immunized mice, a sizeable population of specific PCs could be detected in mice which received splenocytes from immunized mice when compared to control mice (naïve splenocyte transfer) (Figure 4.1.6 B). A large fraction of these PCs was donor ($Ly5.1^+$) and therefore memory B cell-derived (Figure 4.1.6 C). This indicated that transferred memory B cells could quickly differentiate into PCs. To exclude that the plasma cell population was co-transferred during the adoptive transfer of unpurified splenocytes, a group of mice was left unchallenged. Neither a significant specific plasma cell population nor a humoral response could be observed in these mice, indicating that PCs were induced in the host after immunization (data not shown).

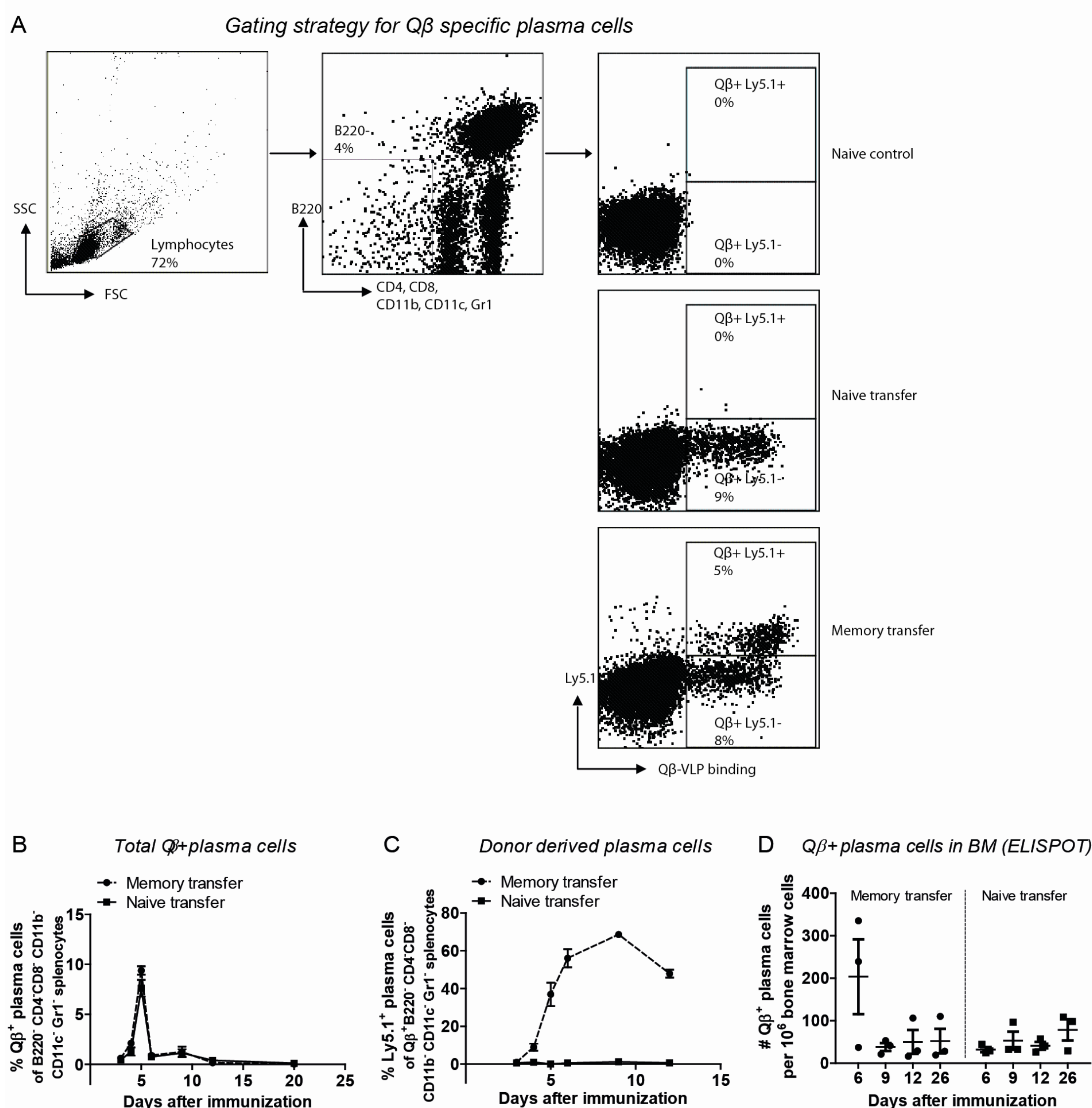


Figure 4.1.6│ Memory B cells preferentially differentiate into secondary PCs. Memory B cells derived from congenic immunized mice (Ly5.1$^+$) were transferred into recipient mice (Ly5.2$^+$) and challenged with Qβ-VLP. Mice which received an adoptive transfer of naïve splenocytes served as a control and presented a primary response. (A) Gating strategy of FCM analysis of Qβ$^+$ PCs on day 5 after immunization upon adoptive transfer. (B) Frequencies of Qβ specific PCs within the activated B cell population (B220$^-$, CD4$^-$, CD8$^-$, CD11c$^-$, CD11b$^-$, Gr-1$^-$). (C) Distribution of responding transfer derived PCs (donor, Ly5.1) within the activated B cell population (Qβ$^+$B220$^-$, CD4$^-$, CD8$^-$, CD11c$^-$, CD11b$^-$, Gr-1$^-$). (D) Analysis of specific PC numbers in BM by ELISPOT assay. Mean with SEM. Mice per group n=3. Data are representative of at least three independent experiments.

4.1.5 Memory B cell-derived secondary plasma cells rapidly migrate to the bone marrow

The BM is known to serve as an important survival niche for long-lived PCs. We therefore analyzed the BM for specific AFC numbers by ELISPOT. To this end, $Ly5.1^+$ mice were immunized and spleens isolated after 4-6 weeks. 24 hours after the transfer the mice were challenged with Qβ-VLP. There were strongly increased numbers of specific PCs (Figure 4.1.6 D) in the presence of transferred splenocytes containing memory B cells early after challenge, which correlated with the increased antibody titer on early time points (Figure 4.1.2 A, B). Frequencies of BM PCs early after challenge were 5-20 fold increased in the presence of memory B cells, indicating that 80-95% of PCs are memory B cell derived under these conditions. Thus, memory B cells rapidly differentiated into PCs and migrated to the bone marrow.

4.1.6 IgM^+ as well as IgG^+ memory B cells fail to efficiently proliferate

It has been reported that IgM^+ memory B cells preferentially proliferate upon antigenic challenge while IgG^+ memory B cells differentiate into PCs (Dogan et al., 2009). Would that also be the case for VLP specific memory B cells? To address this question, we negatively purified IgM^+ and IgG^+ memory B cell populations from spleens of congenic immunized mice and transferred them into Ly5-disparate recipient mice. Mice were challenged after 24 hours post transfer with Qβ-VLP. During primary responses we found that VLPs induced IgM^+ (5-10% of total $Q\beta^+$ memory B cells) as well as IgG^+ (90-95% of total $Q\beta^+$ memory B cells) memory B cells (data not shown). Neither IgM^+ nor IgG^+ memory B cells efficiently proliferated and differentiated into a sizeable population of specific B cells with an activated phenotype (Figure 4.1.7 A) when compared to primary responses (transfer of naïve splenocytes). In contrast both populations differentiated into PCs (Figure 4.1.7 B). This indicates that both memory B cell populations differentiate into PCs upon challenge with viral particles.

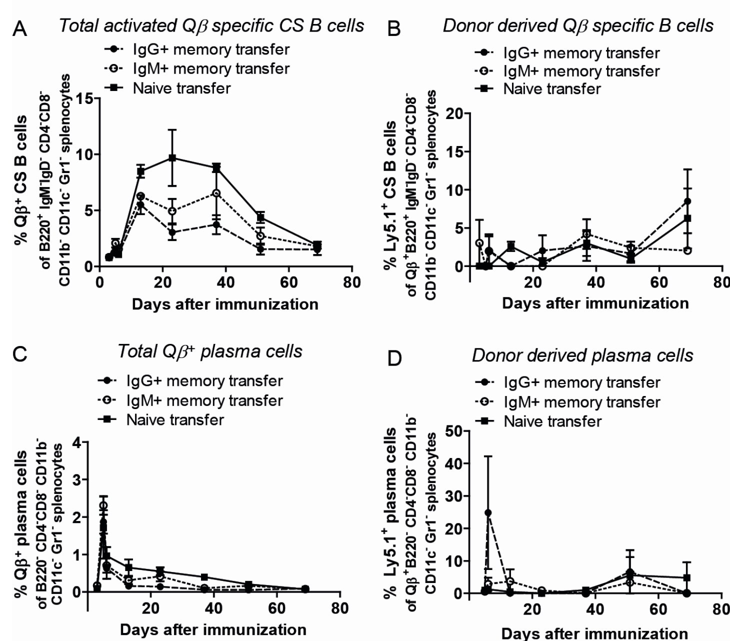


Figure 4.1.7| Transfer of purified memory subtypes show a similar proliferation and differentiation profile upon antigen challenge as unpurified memory splenocytes. Memory B cells derived from congenic immunized mice (Ly5.1$^+$) were negatively purified and transferred as IgM$^+$ or IgG$^+$ memory B cells into recipient mice (Ly5.2$^+$) and challenged with Qβ-VLP. Mice which received an adoptive transfer of naïve splenocytes served as a control and presented a primary response. (A) Frequencies of Qβ specific B cells within the activated B cell population (B220$^+$ IgM$^-$, IgD$^-$, CD4$^-$, CD8$^-$, CD11c$^-$, CD11b$^-$, Gr-1$^-$). (B) Distribution of responding transferred memory B cells (donor, Ly5.1) within the activated specific B cell population (Qβ$^+$B220$^+$, IgM$^-$, IgD$^-$, CD4$^-$, CD8$^-$, CD11c$^-$, CD11b$^-$, Gr-1$^-$). (C) Frequencies of Qβ specific PCs within the activated B cell population (B220$^-$, CD4$^-$, CD8$^-$, CD11c$^-$, CD11b$^-$, Gr-1$^-$). (D) Distribution of responding transfer derived PCs (donor, Ly5.1) within the activated specific B cell population (Qβ$^+$B220$^-$, CD4$^-$, CD8$^-$, CD11c$^-$, CD11b$^-$, Gr-1$^-$). Mean with SEM. Mice per group n=3. Data are representative of at least three independent experiments.

4.1.7 The secondary memory B cell derived plasma cells secrete more antibodies

As described above, memory B cells rapidly differentiated into PCs which could be detected in the spleen by FCM and BM by ELISPOT. Interestingly, spot-sizes of ELISPOT experiments were strongly increased in the presence of splenocytes from immunized mice containing memory B cell derived PCs

(Figure 4.1.8 A-C). This suggested that memory B cell-derived PCs produce increased amounts of antibody. To address this point quantitatively, we performed FCM experiments to detect intracellular binding of labeled Qβ-VLP in PCs. Consistent with the hypothesis of higher intracellular antibody levels, we observed increased binding of Qβ-VLP in memory B cell-derived PCs compared to PCs derived from naïve B cells (Figure 4.1.8 D, E) both in spleen and bone marrow. To directly measure levels of intracellular antibodies, we performed intracellular IgG staining (Figure 4.1.8 F). These experiments confirmed increased amounts of antibody within secondary PCs derived from memory B cells. Hence, secondary memory B cell derived PCs produced increased levels of specific antibodies compared to primary PCs derived from naive B cells.

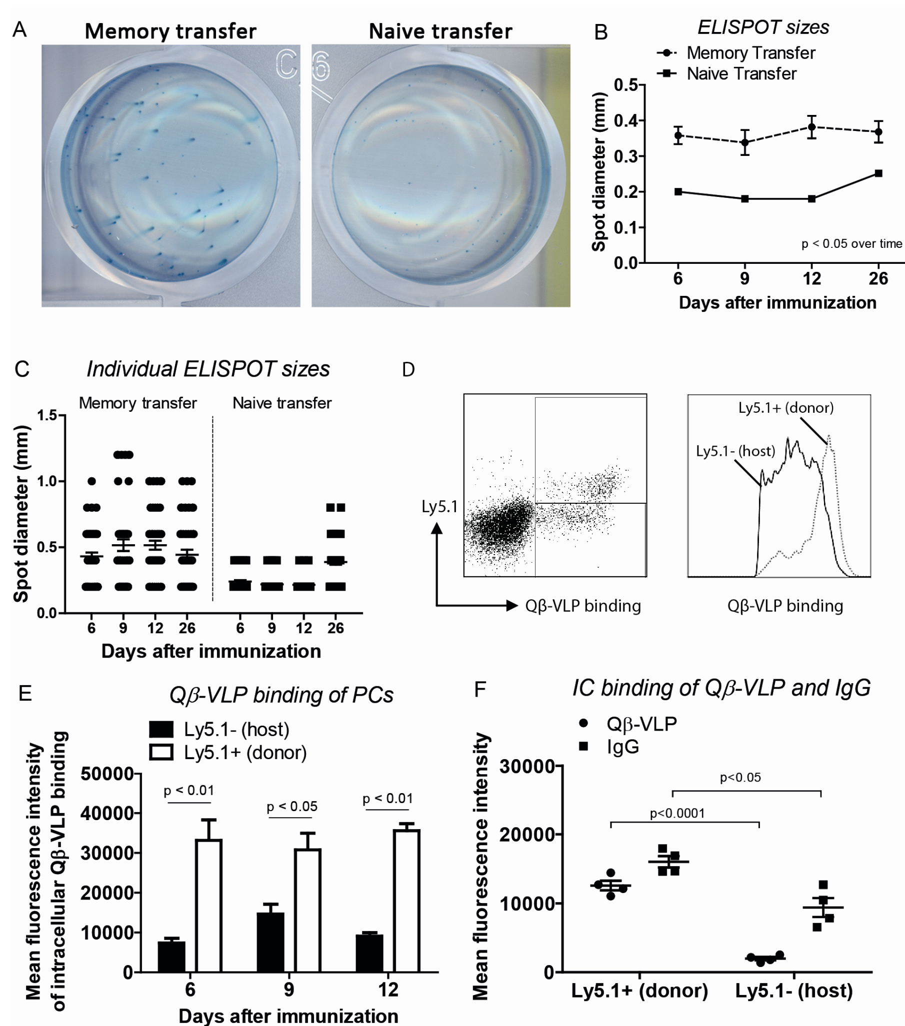


Figure 4.1.8 | Plasma cells derived from memory B cells are highly potent for Qβ. Congenic mice ($Ly5.1^+$) were immunized and unpurified whole splenocytes were transferred into recipient mice ($Ly5.2^+$). Transfer of naïve splenocytes served as a control. BM was analyzed for specific PCs. (A) Example of ELISPOTS on day 6 after immunization. (B) Overall and (C) individual analysis of ELISPOT sizes. (D) Dot plot and histogram of intracellular binding of Qβ-VLP of PCs of mice which received memory splenocytes. (E) MFI of intracellular Qβ-VLP binding of PCs in recipient mice which are either donor ($Ly5.1^+$) or host derived ($Ly5.2^+$). (F) MFI of simultaneous intracellular staining of PCs with increased binding for Qβ-VLP as well as anti-IgG. Mean with SEM. Mice per group n=3 or 4 (only F), respectively. Data are representative of three independent experiments.

4.2 Multiple stimulations of memory B cells by cognate Ag

4.2.1 Memory B cells fail to produce antibodies after multiple rounds of stimulation

Use of allotypic mice expressing IgH^a on a C57BL/6 background allows to specifically follow antibody responses of adoptively transferred B cells (IgH^a) vs. endogenous B cells (IgH^b). In a first set of experiments we observed that the majority of antibodies in mice which had received splenocytes from immunized mice containing memory B cells was derived from transferred memory B cells (IgH^a). As a next step we were exploring whether the antibody response would also be dominated by the donor derived memory B cells after further round(s) of stimulation. To address this question, we adoptively transferred primed splenocytes containing memory B cells and challenged the recipient mice twice with Qβ-VLP (Figure 4.2.1).

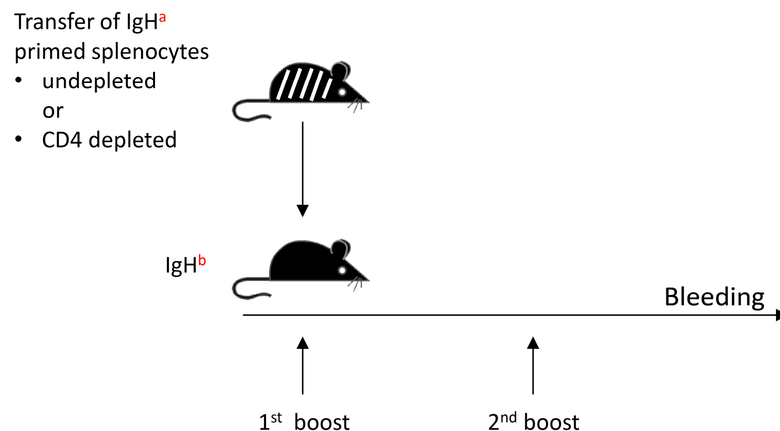


Figure 4.2.1| Experimental set up to assess multiple rounds of memory B cell stimulation.

To this end, splenocytes of immunized IgH^a congenic mice were transferred into recipient mice (IgH^b) and challenged with Qβ-VLP on day 0 and 75. As expected, at early time points the humoral response was dominated by donor derived memory B cells (IgH^a) (Figure 4.2.2). The antibody titer declined over time with a half-life dictated by the life span of plasma cells (Gatto et al., 2007b).

However, boosting those mice a second time with the cognate antigen induced an antibody response which was dominated by host derived B cells (IgH^b). Interestingly, the antibody titer of donor derived memory B cells declined constantly unperturbed by the second challenge with Qβ-VLP.

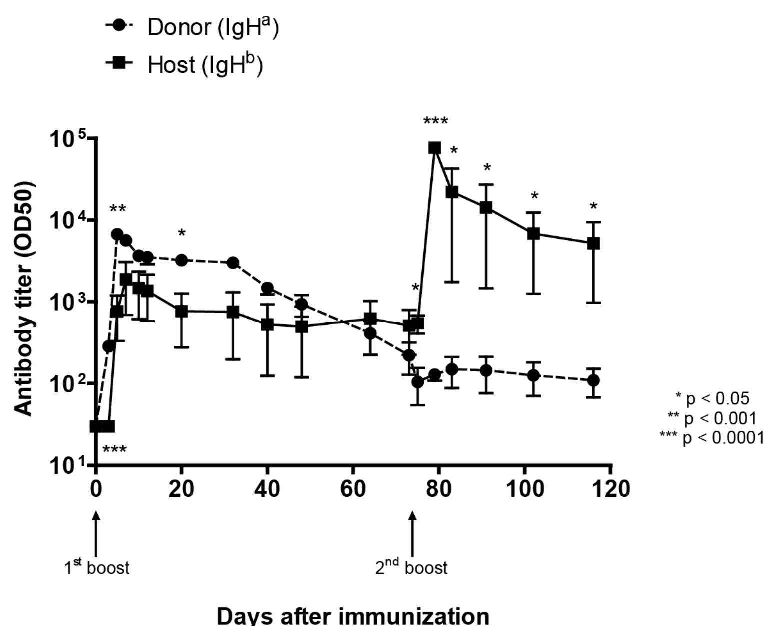


Figure 4.2.2│ Donor derived memory B cells fail to produce high amounts of antibodies after second challenge. Congenic IgH^a mice were immunized with Qβ-VLP. After 4-6 weeks splenocytes containing memory B cells were transferred into recipient mice (IgH^b) and challenged with Qβ-VLP on day 0 and 75 after the adoptive transfer. Specific IgG1 and IgG2a titers were obtained by ELISA. Mean with SEM. *P* values were calculated by an unpaired t-test. Mice per group n=3. Data are representative of three independent experiments.

4.2.2 CD4-depleted memory B cells fail to produce antibodies after multiple rounds of stimulation

We were wondering whether the depletion of specific memory $CD4^+$ T cells before adoptive transfer of splenocytes (IgH^a) of immunized congenic mice containing memory B cells would have an influence on the Ab production after multiple stimulation of B cells in recipient mice (IgH^b). Therefore, an adoptive transfer experiment was set up as described before (Figure 4.2.1), but memory $CD4^+$ T cells were depleted by $CD4^+$ MACS beads purification.

As observed previously, the early Ab response after the first boost was mediated by donor derived memory B cells (Figure 4.2.3). However, at later time points (> d35) the host response was stronger. The donor and host derived antibody titers declined similarly over time. In contrast to the first boost, after the second boost, the humoral response was dominated by the host B cells. However, donor derived memory B cells contributed only marginally to the humoral response.

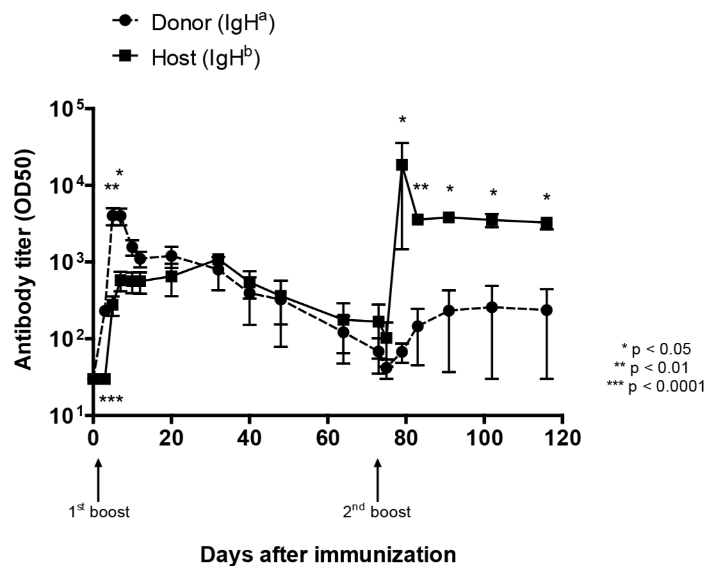


Figure 4.2.3│ CD4-depleted memory B cells fail to produce antibodies after multiple rounds of stimulation. Congenic IgH^a mice were immunized with Qβ-VLP. After 4-6 weeks splenocytes containing memory B cells were negatively purified by $CD4^+$ MACS beads, transferred into recipient mice (IgH^b) and challenged with Qβ-VLP on day 0 and 75 after adoptive transfer. Specific IgG1 and IgG2a titers were obtained by ELISA. Mean with SEM. *P* values were calculated by an unpaired t-test. Mice per group n=3. Data are representative of three independent experiments.

4.2.3 Host-derived memory B cells fail to respond after multiple rounds of stimulation

Whether it was possible to re-stimulate host derived B cells a second time was assessed next. To this end, we transferred splenocytes of the recipient mice (IgH^b) back into initial donor mice (IgH^a) (Figure

4.2.4). Therefore, we isolated spleens of recipient mice (IgH^b) which had received an adoptive transfer from donor mice (IgH^a) and had been challenged twice with Qβ-VLP, and adoptively transferred them back into IgH^a positive mice. After 24 hours, mice were challenged and found that the humoral response was dominated by B cells of the recipient mice (IgH^a).

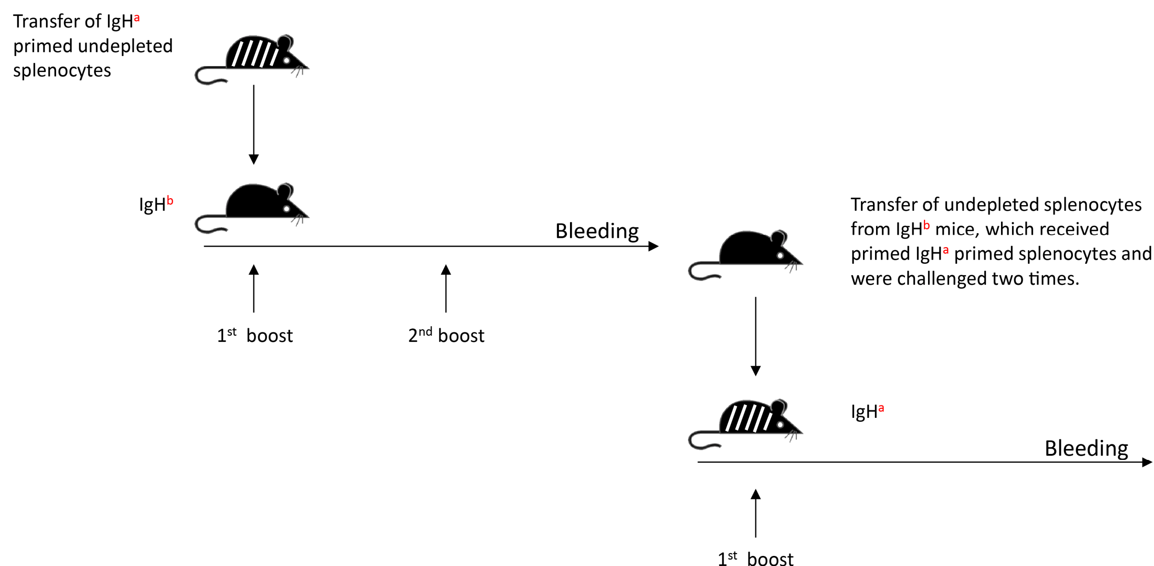


Figure 4.2.4│ Advanced experimental set up to assess multiple rounds of memory B cell stimulation.

The transferred memory B cells of the hosts of the first adoptive transfer (IgH^b) therefore also failed to respond to third stimulation by the cognate antigen (Figure 4.2.5). Hence, also under these conditions, memory B cells failed to respond multiple times to antigenic challenge.

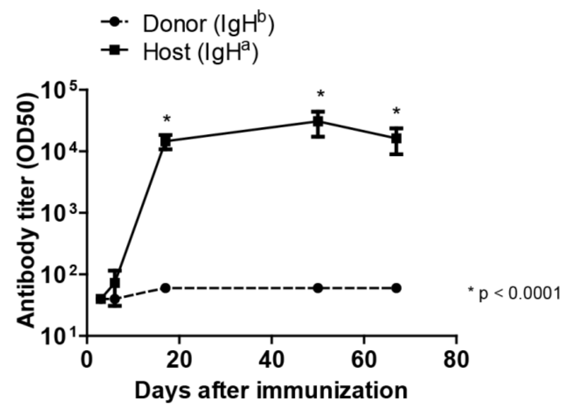


Figure 4.2.5│ Host derived memory B cells also fail to respond after multiple rounds of antigen stimulation. Splenocytes of mice (IgH^b), which had received an adoptive transfer and were stimulated twice, were transferred back into IgH^a mice and challenged with Qβ-VLP. Allotype specific IgG1 and IgG2a antibodies were assessed by ELISA. Mean with SEM. *P* values were calculated by an unpaired t-test. Mice per group n=5. Data are representative of two independent experiments.

4.3 Do memory B cells require T cell help?

4.3.1 Memory B cells accelerate humoral immune response in the absence of $CD4^+$ memory T cells

In a first set of experiments we could observe that after transfer of undepleted memory splenocytes containing memory B cells the humoral response occurred earlier and was much stronger compared to a primary response. To assess if memory T helper cells are required to induce this increased antibody production we negatively purified splenocytes of immunized congenic ($Ly5.1^+$) mice by using $CD4^+$ MACS beads before transfer into recipient mice ($Ly5.2^+$). Recipient mice were challenged 24 hours later with Qβ-VLPs. Antibody responses were similarly accelerated in the presence and absence of memory $CD4^+$ T cells (Figure 4.3.1 A). This result is consistent with the general observation that antiviral antibody responses are usually not enhanced by memory $CD4^+$ T cells (Charan et al., 1987).

In a next adoptive transfer experiment, we immunized IgH^a mice to be able to trace the memory B cell derived antibodies. Four weeks later we isolated splenocytes, which were CD4-depleted by MACS purification and transferred into C57BL/6, which expressed the IgH^b-allotype. Hence, we were able to distinguish antibodies resulting from the transferred memory B cells (IgH^a positive) or from newly activated naive B cells of the host (IgH^b positive). We could observe that the majority of the humoral response was produced by memory B cell (donor) derived (IgH^a positive) plasma cells (Figure 4.3.1 B). Surprisingly, the antibody response of host derived plasma cells was stronger at later time points. Even in the absence of memory $CD4^+$ T cell help, transferred memory B cells within the undepleted splenocytes pool from immunized mice differentiated quickly into plasma cells which were responsible for the increased titer at early time points.

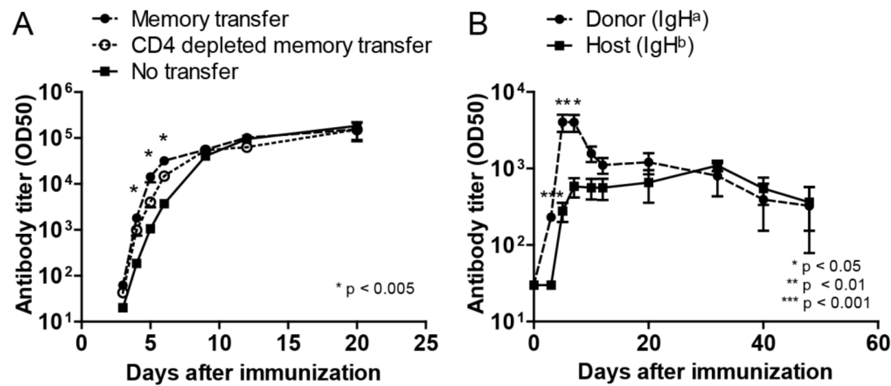


Figure 4.3.1│ Accelerated humoral response after antigen re-encounter in the presence of memory B cells and absence of specific CD4$^+$ memory T cells. Qβ positive memory B cells derived from immunized congenic mice (Ly5.1 resp. IgH^a) were transferred into recipient mice (Ly5.2 resp. IgH^b) and challenged with Qβ-VLP. Mice which received no transfer served as a control and presented a primary response. (A) Qβ-VLP specific total IgG antibody titer of mice which received undepleted or CD4 MACS negatively purified memory splenocytes. Mean with SEM. *P* values were obtained by an unpaired t-test and represents significance of memory transfer and CD4 depleted memory transfer vs. no transfer. (B) Qβ-VLP specific IgG1 and IgG2a antibody titer originated of allotype H^a or H^b of mice which had received CD4 negatively purified (MACS beads) memory splenocytes. Mean with SEM. *P* values were calculated by an unpaired t-test with Welsh's correction. Mice per group n= 3 (only day 20 in Figure A) or 6. Data are representative of at least three independent experiments.

4.3.2 Memory B cells inefficiently expand in the presence or absence of CD4 memory T cells

The magnitude of the cellular response was assessed to reveal whether specific memory T_H cells enhance proliferation of memory B cells. To this end, congenic Ly5.1$^+$ mice were immunized and after 4-6 weeks, memory splenocytes were isolated and memory T_H cells were either depleted or not depleted before transfer. Thus, splenocytes were negatively purified by CD4$^+$ MACS beads prior to transfer. Recipient mice as well as naïve mice were immunized and populations of activated B cells (B220$^+$, IgM$^-$, IgD$^-$) were assessed for antigen specificity in the spleen. As we already have observed in previous experiments, the specific cellular B cell response was reduced after transfer of undepleted memory splenocytes containing memory B cells. This was also the case, when CD4$^+$ T cell help was

depleted (albeit to a slightly lesser extent) (Figure 4.3.2 A). This demonstrates that suppression of specific host B cell responses was not due to possibly antigen-specific suppressive donor-derived $CD4^+$ T cells. In addition, the majority of this reduced antigen-specific B cell population, when CD4 memory T cells were depleted, was mostly host cell-derived ($Ly5.1^-$) (Figure 4.3.2 B). Hence, presence of antigen-specific memory $CD4^+$ T cells had a limited influence on specific humoral and cellular memory B cell responses.

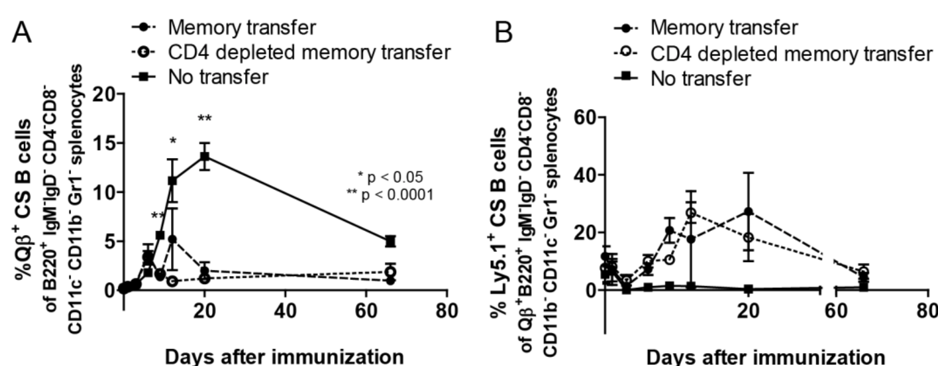


Figure 4.3.2 | Memory B cells do not efficiently proliferate after antigen re-encounter independent of specific T cell help. Memory B cells derived from congenic ($Ly5.1^+$) immunized mice were either transferred as undepleted or CD4 negatively MACS purified splenocytes into recipient mice and challenged with Qβ-VLP. Mice which received no transfer served as a control and presented a primary response. (A) Frequencies of Qβ specific B cells within the activated B cell population ($B220^+$, IgM^-, IgD^-, $CD4^-$, $CD8^-$, $CD11c^-$, $CD11b^-$, $Gr\text{-}1^-$). Mean with SEM. *P* values represent significances of mice which received any memory B cells vs. “no transfer”, which were obtained by an unpaired t-test with Welsh’s correction. (B) Distribution of responding transferred memory B cells (donor, Ly5.1) within the specific activated B cell population ($Q\beta^+$ $B220^+$, IgM^-, IgD^-, $CD4^-$, $CD8^-$, $CD11c^-$, $CD11b^-$, $Gr\text{-}1^-$). Mean with SEM. Mice per group n=3. Data are representative of three independent experiments.

4.3.3 Memory B cells differentiate quickly into plasma cells independent of memory T cell help

We further analyzed the influence of memory $CD4^+$ T cells on the formation of specific PCs in the spleen. Spleens of congenic immunized mice ($Ly5.1^+$) were isolated and transferred either undepleted or CD4 depleted into recipient mice ($Ly5.2^+$). After 24 hours the mice were challenged with Qβ-VLP. Despite a suppressed cellular B cell response (Figure 4.3.2 A), similar plasma cell numbers were found after antigenic challenge (Figure 4.3.3 A) during primary and secondary B cell responses. The differentiation of memory B cells into plasma cells was independent on specific T cell help as no differences of PC formation were observed between transfers of undepleted or CD4 depleted splenocytes from immunized mice. At the peak of the response, the majorities of plasma cell populations were donor ($Ly5.1^+$) derived independent of the presence of memory T_H cells (Figure 4.3.3 B).

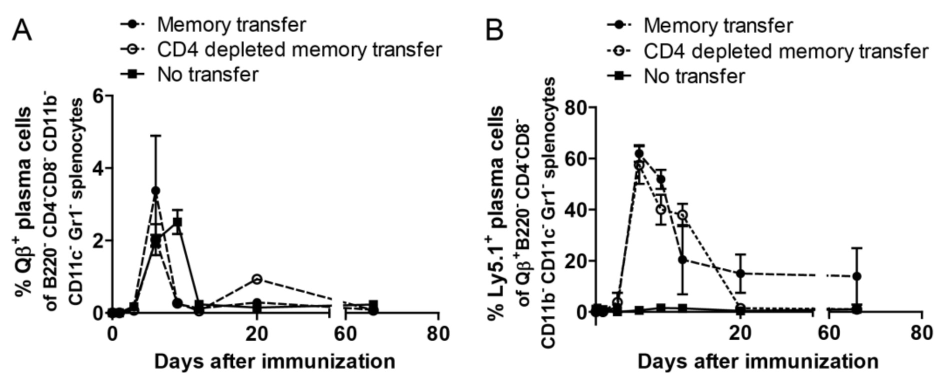


Figure 4.3.3| Upon antigen re-challenge memory B cells differentiate into splenic secondary plasma cells independent of memory T cell help. Memory B cells derived from congenic immunized mice ($Ly5.1^+$) were either undepleted or CD4+ depleted transferred into recipient mice ($Ly5.2^+$) and challenged with Qβ-VLP. Mice which received no transfer of splenocytes served as a control and presented a primary response. (A) Frequencies of Qβ specific plasma cells within the activated B cell population ($B220^-$, IgM^-, IgD^-, $CD4^-$, $CD8^-$, $CD11c^-$, $CD11b^-$, $Gr\text{-}1^-$). (B) Distribution of responding transferred plasma cells (donor, $Ly5.1^+$) within the plasma cell population ($Q\beta^+$ B220, $CD4^-$, $CD8^-$, $CD11c^-$, $CD11b^-$, $Gr\text{-}1^-$). Mean with SEM. Mice per group n=3. Data are representative of three independent experiments.

4.3.4 Secondary derived plasma cells produce more antibodies without memory T cell help

We have shown previously that memory B cells differentiate into more efficient secondary plasma cells. Whether this process was dependent on memory $CD4^+$ T cells was assessed next. As a surrogate for Ab production, spot sizes in ELISPOT experiments were measured.

To this end, splenocytes from immunized congenic mice ($Ly5.1^+$) were isolated and either directly or CD4 T cell depleted (MACS purification) into disparate mice transferred ($Ly5.2^+$). The mice were challenged with Qβ-VLP 24 hours after the adoptive transfer. BM was isolated at indicated time points and for the ability to secrete specific Ab analyzed by ELISPOT. In addition, PCs in spleen were analyzed for their capacity to bind intracellular Qβ-VLP which correlates directly with the amount of Ab, which is present within a cell. Memory B cells differentiated into secondary plasma cells causing increased spot sizes independent of the presence of memory $CD4^+$ T cells (Figure 4.3.4 A). Increased binding of Qβ-VLP upon intracellular staining was also independent of the presence of memory $CD4^+$ T cells (Figure 4.3.4 B). The increased spot size as well as the increased capacity of intracellular Qβ-VLP binding correlated with the increased titer observed at early time points (Figure 4.3.1 A, B).

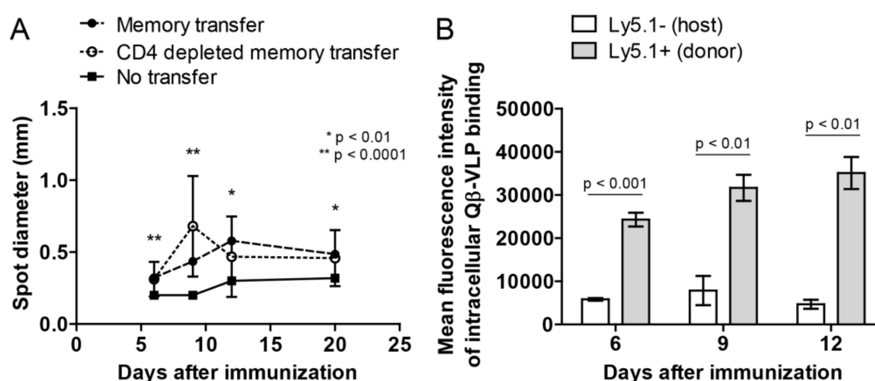


Figure 4.3.4│ Plasma cells derived from CD4 depleted memory B cell transfer produce more antibodies. An adoptive transfer experiment was set up as described before. (A) Analysis of ELISPOT sizes of plasma cells in BM on day 5 after challenge upon transfer of memory splenocytes, CD4 depleted splenocytes or no transfer. Mean with SEM. *P* values were calculated by Mann-Whitney test. (B) Comparison of intracellular binding of Qβ-VLP of

plasma cells in mice which received CD4 depleted splenocyte. Plasma cells derived from the transfer are Ly5.1$^+$ (donor) or derived from *de-novo* activated B cells are Ly5.1$^-$ (host). Mean with SEM. *P* values were calculated by an unpaired t-test. Mice per group n=3. Data are representative of three independent experiments.

4.3.5 Memory B cell responses are dependent of non-cognate T cell help

To further dissect the question wether T cell help is needed during memory B cell responses we performed an adoptive transfer experiment with CD40L$^{-/-}$ as well as MHC II$^{-/-}$ as recpient mice. MHC class II-deficient mice have virtually no CD4$^+$ T cells while CD40L-deficient mice lack the most important cell-bound ligand involved in cognate T help during B cell activation.

Congenic (Ly5.1$^+$) mice were immunized with Qβ-VLP and after 4-6 weeks spleens were isolated and purified. To deplete specific T helper cells, splenocytes were negatively purified by CD4$^+$ MACS beads and transferred into WT as well as MHCII$^{-/-}$ and CD40L$^{-/-}$ recipient mice. We compared memory B cell responses in recipient mice with primary B cell responses of naïve mice.

The antibody production in mice lacking CD40L or MHCII molecules was impaired during primary B cell responses when compared to WT mice (Figure 4.3.5 A, B). Interestingly, the primary humoral response in CD40L and MHC II knock out mice was strongly reduced at the later time point (Figure 4.3.5 B). However, transfer of splenocytes of immunized mice containing memory B cells accelerated the humoral response in all recipient mice also in the absence of MHC class II or CD40L (Figure 4.3.5 C, D). Although the presence of memory B cells could increase antibody responses in CD40L and MHC class II deficient mice, levels of specific IgG nevertheless remained reduced compared to WT mice. Interestingly, both deficient mouse strains responded similarly poor during the primary response, whereas in the presence of memory B cells, CD40L$^{-/-}$ mice were able to produce near normal IgG responses while MHC II deficient mice mounted strongly reduced responses. This difference was very pronounced at the early timepoint but evanescent at a later time point. Thus, presence of T_H cells in normal mice produced the optimal memory IgG response while CD40L-

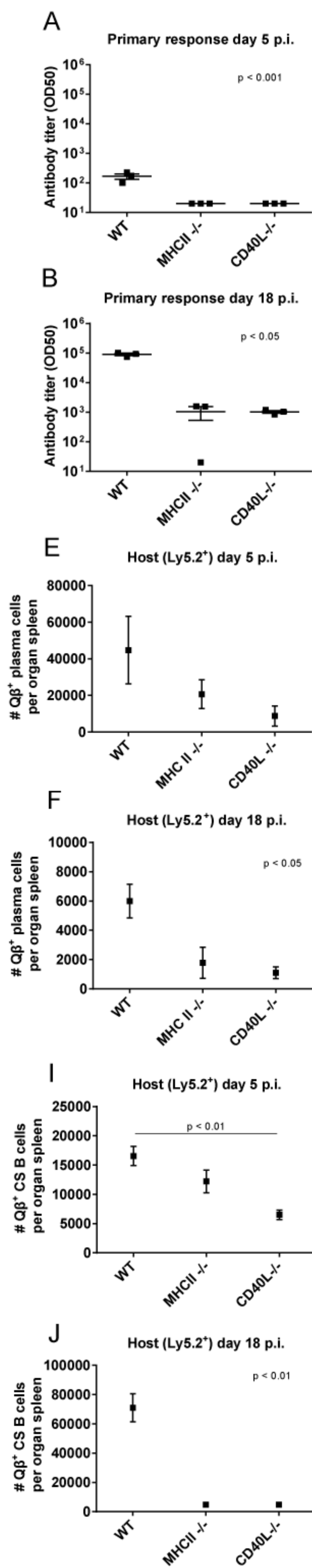
deficient mice, likely providing non-cognate bystander T_H were able to stimulate reduced but still robust memory IgG response. In contrast, in complete absence of T_H, memory IgG respose were almost absent at early time points but could reach near normal levels at later time points.

We further assessed the specific plasma cell numbers in spleen. During the primary response, both CD40L$^{-/-}$ as well as MHC II$^{-/-}$ mice showed strongly reduced PC frequencies (Figure 4.3.5 E, F) corresponding to the IgG titer (Figure 4.3.5 A, B). In contrast, during memory responses the number of specific PCs was strongly reduced in mice deficient for CD40L and MHC II when compared to WT mice at an early time point (Figure 4.3.5G). As seen for the IgG responses (Figure 4.3.5 C), the reduction in plasma cell frequencies was much more pronounced for MHC class II deficient mice compared to CD40L-deficient mice (Figure 4.3.5 G). In contrast, plasma cell frequencies were generally low but rather comparable at the later timepoint (Figure 4.3.5 H). Therefore, the minor PC population of both k.o. strains correlated with the decreased antibody titer.

The number of antigen-specific class-switched B cells was also assessed. At an early time point, reduced frequencies of specific CS B cells in k.o. mice were detected (Figure 4.3.5 I) when compared to specific CS B cells in WT mice. However, at a later time point virtually no specific CS B cells were detected in deficient mice whereas in WT mice the number of specific CS B cells increased (Figure 4.3.5 J). During recent experiments, we could observe that after transfer of splenocytes of immunized mice containing memory B cells, the specific CS B cell population after Ag challenge was reduced and mostly host derived (Ly5.2$^+$) (Figure 4.1.3 B, C). After adoptive transfer of memory B cells into CD40L$^{-/-}$ as well as MHC II$^{-/-}$ mice, the donor derived (Ly5.1$^+$) specific CS B cell population is even more decreased when compared to WT mice (Figure 4.3.5 K, L) . In contrast to reduced plasma cell numbers, which reached nearly normal levels over time in the absence of T_H cells and CD40L (Figure 4.3.5 E-H) the number of class-switched B cells remained very low at indicated time points (Figure 4.3.5 I-L) for mice deficient for CD40L as well as MHC II. This indicates that memory B cells differentiate even more preferably into plasma cells in the absence than in the presence of T cell

help. These data are consistent with earlier reports, indicating that GC B cells preferentially differentiate into plasma cells when CD40L is limiting (Arpin et al., 1995).

Primary B cell responses (no transfer)



Transfer of CD4+ depleted memory splenocytes (Ly5.1+) into recipients (Ly5.2+)

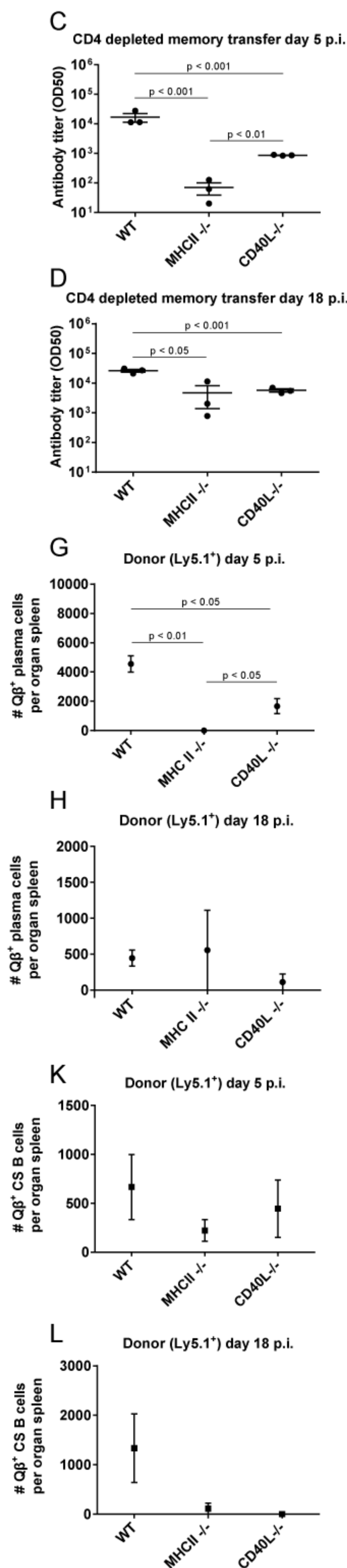


Figure 4.3.5| Memory B cell responses are dependent on non-cognate T cell help. Splenocytes of immunized congenic (Ly5.1+) mice were isolated and negatively purified by CD4+ MACS beads. Purified splenocytes containing memory B cells were transferred in WT, MHCII -/- or CD40L -/- mice and challenged with Qβ-VLP 24 hours later. (A-D) show Qβ-VLP specific total IgG antibody titer of naïve mice after immunization (A, B) and mice, which had received CD4 depleted splenocytes containing memory B cells (C, D) on day 5 (A, C) or day 18 (B, D) after immunization. Data with mean and SEM. *P* values were obtained by two-way ANOVA (A, B) and unpaired t-test (C, D). (E, F) present numbers of Qβ specific plasma cells ($Q\beta^+$, $B220^-$, $CD4^-$, $CD8^-$, $CD11c^-$, $CD11b^-$, $Gr\text{-}1^-$) per spleen (related to $1x10^8$ cells) during a primary response at indicated time points. Data with mean and SEM. *P* value was calculated by two-way ANOVA. (G, H) present numbers of Qβ specific plasma cells, which were Ly5.1 positive ($Ly5.1^+$, $Q\beta^+$, $B220^-$, $CD4^-$, $CD8^-$, $CD11c^-$, $CD11b^-$, $Gr\text{-}1^-$) in mice, which had received an adoptive transfer of CD4 depleted splenocytes containing memory B cells at indicated time points. Data with mean and SEM. *P* values were calculated by unpaired t-test. (I, J) present numbers of Qβ specific B cells ($Q\beta^+$, $B220^+$, IgM^-, IgD^-, $CD4^-$, $CD8^-$, $CD11c^-$, $CD11b^-$, $Gr\text{-}1^-$) per spleen (related to $1x10^8$ cells) during a primary response at indicated time points. Data with mean and SEM. *P* values were calculated by unpaired t-test (I) and two-way ANOVA (J). (K, L) present numbers of Qβ specific B cells which are Ly5.1 positive ($Ly5.1^+$, $Q\beta^+$, $B220^+$, IgM^-, IgD^-, $CD4^-$, $CD8^-$, $CD11c^-$, $CD11b^-$, $Gr\text{-}1^-$) in mice, which had received an adoptive transfer of CD4 depleted splenocytes containing memory B cells at indicated time points. Data with mean and SEM. Mice per group n=3. Data are representative of two independent experiments.

We also analyzed the capacity of PCs for intracellular binding for Qβ-VLP in mice, which had received an adoptive transfer. As observed before in WT mice, the transfer ($Ly5.1^+$) derived PCs showed increased antibody production levels compared to their counterparts differentiated from naïve B cells ($Ly5.1^-$) (Figure 4.3.6). Similar findings could be made for plasma cells in CD40L k.o. mice. Only host derived PCs were detected in MHC II k.o. mice at the time point measured.

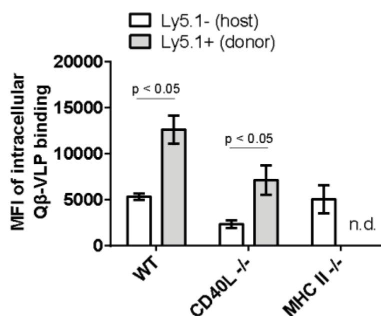


Figure 4.3.6│ Secondary plasma cells produce more antibodies. Memory B cells derived from congenic immunized mice were CD4 depleted and transferred into Ly5.2^{+} recipient mice (WT, CD40L$^{-/-}$, MHC II$^{-/-}$). After 24 h mice were challenged with Qβ-VLP. Plasma cells in spleen on day 5 after immunization of animals which had received an adoptive transfer were analyzed for their capacity to bind Qβ-VLP intracellular. Comparison of binding capacity of donor (Ly5.1^{+}) versus host (Ly5.1^{-}) derived plasma cells. Mean with SEM. *P* values were obtained by an unpaired t-test. Mice per group n=3. Data are representative of two independent experiments.

These data demonstrate that there is a graded T_H cell dependence of the memory B cell response. Most T cell dependent are the expansion of class-switched memory B cell derived of memory B cells followed by rapid generation of plasma cells and early IgG responses. Least dependent are late plasma cells as well as late IgG responses. CD40L-independent, likely non-cognate bystander T_H cells are able to partly rescue early IgG and plasma cell responses.

4.4 AP205-VLP drive rapid differentiation of memory B cells into secondary plasma cells producing increased levels of antibodies

4.4.1 Accelerated humoral response detectable in the presence of AP205-specific memory B cells

Congenic mice ($Ly5.1^{+}$) were immunized with AP205-VLP. After 4-6 weeks the spleen containing memory B cells was isolated and transferred into recipient mice ($Ly5.2^{+}$). The recipient mice were immunized with AP205-VLP 24 hours after the transfer.

Similarly to the previously observed accelerated humoral response during Qβ-VLP transfer experiments, we were also able to detect an increased antibody titer when AP205-specific memory B cells were transferred (Figure 4.4.1). When AP205-VLP specific memory B cells were present, the onset as well as the magnitude of the humoral response was elevated early after challenge when compared to a primary response. However, the primary humoral response was stronger at later time points (> d12).

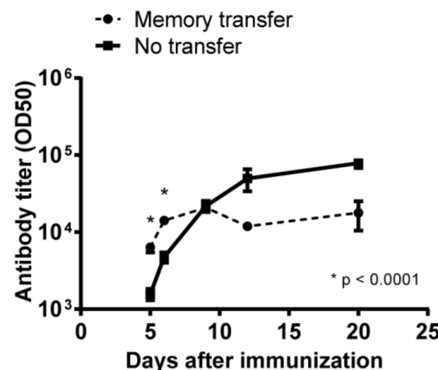


Figure 4.4.1│ Accelerated humoral response in the presence of memory B cells. Splenocytes of immunized congenic mice were isolated and adoptively transferred into disparate recipient mice. After 24 hours the mice were challenged. Mice which received no transfer served as a control and presented a primary response. Qβ-VLP specific total IgG antibody titer of mice is shown which received undepleted memory splenocytes. Mean with SEM. *P* value was calculated by an unpaired t-test. Mice per group n=3. Data are representative of two independent experiments.

4.4.2 A small population of specific B cells was detected in the presence of memory B cells after antigen re-challenge

Next, we assessed the cellular response of specific activated B cells. Thus, another adoptive transfer experiment as described above (see chapter 4.4.1) was conducted. At the indicated time points splenocytes were analyzed for specific CS B cells (% VLP specific B cells within B220$^+$, IgM^{low}, IgD^{low} CD4$^-$, CD8$^-$, CD11c$^-$, CD11b$^-$, Gr-1$^-$). After immunization of naïve mice, up to 10% of B cells with an activated phenotype (B220$^+$, IgM^{low}, IgD^{low}) were specific for AP205-VLP at the peak of the response (Figure 4.4.2 A). However, as seen before for Qβ, the overall AP205-specific B cell response was strongly reduced in the presence of transferred splenocytes containing memory B cells.

In order to dissect the memory versus naive B cell response within the activated CS B cell population (AP205-VLP$^+$ B220$^+$, IgM^{low}, IgD^{low}) we analyzed the expression of the allotype marker Ly5.1 of mice which had received an adoptive transfer (Figure 4.4.2 B). Unexpectedly, donor derived (Ly 5.1$^+$) specific B cells could only be detected at an early time point (day 6) and made up less than 20% of all AP205$^+$ CS B cells population. As a result, the majority of specific CS B cells were host (Ly5.2$^+$) derived in mice receiving splenocytes from immunized mice. Again, similar observations were made for Qβ-specific memory B cells.

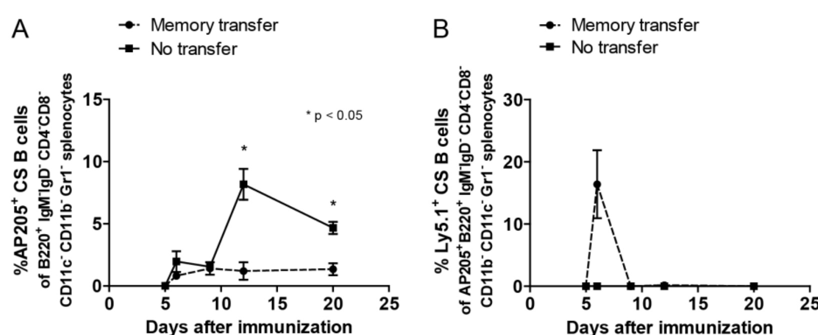


Figure 4.4.2| AP205$^+$ memory B cells do not efficiently proliferate after antigen re-encounter. Memory B cells derived from congenic with AP205 immunized mice (Ly5.1+) were transferred into recipient mice (Ly5.2+) and challenged with AP205-VLP. Mice which received no transfer served as a control and presented a primary response. (A) Frequencies of AP205 specific B cells within the activated B cell population (B220$^+$, IgM^{low}, IgD^{low}

CD4$^-$, CD8$^-$, CD11c$^-$, CD11b$^-$, Gr-1$^-$). Mean with SEM. *P* values were obtained by an unpaired t-test with Welsh's correction. (B) Distribution of responding transferred memory B cells (donor, Ly5.1) within the activated specific B cell population (AP205$^+$, B220$^+$, IgM^{low}, IgD^{low} CD4$^-$, CD8$^-$, CD11c$^-$, CD11b$^-$, Gr-1$^-$). Mean with SEM. Mice per group n=3. Data are representative of two independent experiments.

4.4.3 Differentiation of memory B cells into secondary plasma cells with enhanced ability for Ab production

Specific plasma cell numbers in spleen were analyzed, which are defined as positive for intracellular antibody, negative for B220, CD4, CD8, CD11b, CD11c and Gr1. Congenic (Ly5.1$^+$) mice were immunized with AP205-VLP, and splenocytes were transferred 4-6 weeks later into recipient mice (Ly5.2$^+$). After 24 hours the mice were challenged with the cognate antigen. In spite of a smaller population of AP205-specific activated CS B cells in the presence of splenocytes from immunized mice, a similar population of specific PCs could be detected in mice which received splenocytes from immunized mice when compared to control mice (no transfer) (Figure 4.4.3 A). Notably, a large fraction of these PCs was donor (Ly5.1$^+$) derived at early time points (Figure 4.4.3 B). Hence, transferred memory B cells could quickly differentiate into PCs and showed a minor proliferative phenotype.

As we observed in adoptive transfer experiments with the Qβ-VLP, we also assessed whether AP205-PCs derived from memory B cells would have an increased capability in producing antibodies. Indeed, those secondary PCs were able to produce more antibodies as their binding capacity of intracellular AP205-VLP binding was increased at day 5 to host derived PCs from newly activated naïve B cells (Figure 4.4.3 C).

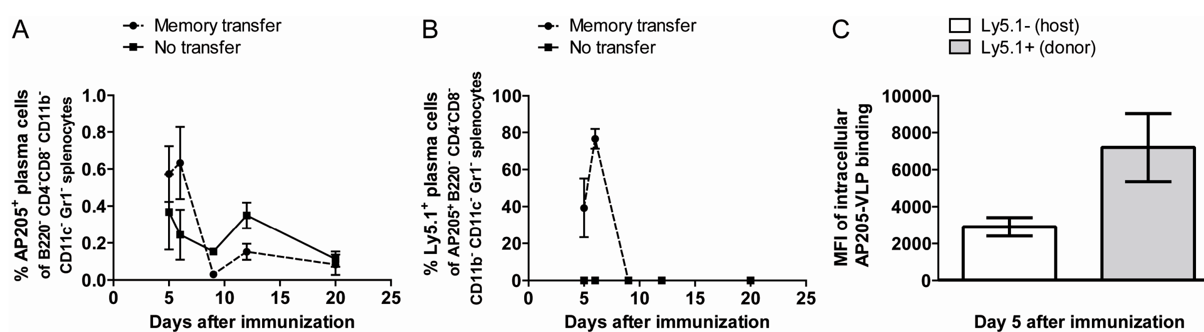


Figure 4.4.3│ Memory B cells preferentially differentiate into secondary PCs. Memory B cells derived from congenic immunized mice (Ly5.1$^+$) were transferred into recipient mice (Ly5.2$^+$) and challenged with AP205-VLP. Mice which received no transfer served as a control and presented a primary response. (A) Frequencies of AP205 specific PCs within the activated B cell population (B220$^-$, CD4$^-$, CD8$^-$, CD11c$^-$, CD11b$^-$, Gr-1$^-$). Mean with SEM. (B) Distribution of responding transfer derived PCs (donor, Ly5.1) within the activated B cell population (AP205$^+$B220$^-$, CD4$^-$, CD8$^-$, CD11c$^-$, CD11b$^-$, Gr-1$^-$). Mean with SEM (C) MFI of intracellular AP205-VLP binding of PCs in recipient mice which are either donor (Ly5.1$^+$) or host derived (Ly5.2$^+$). Mean with SEM. Mice per group n=3. Data are representative of at least three independent experiments.

Therefore, by assessing adoptive transfer experiments with a second VLP, we could observe very similar results for Qβ-VLP as well as AP205-VLP. Therefore, we can conclude that all observations and conclusions made in this study may be of general nature for VLPs and viral particles.

5 Discussion

Understanding the induction and maintenance of immune responses has been a long-standing goal of immunological research, as it may allow to optimize vaccination or to keep autoimmune responses under control. B cell responses are particularly important for the efficacy of most vaccines and a large number of model antigens have been used to study B cell responses in great detail. Haptenated proteins are one of the most intensively studied model antigens. They belong to the TD family of antigens as they only induce an immune response in the presence of T cell help. After immunization, haptens coupled to protein carriers usually induce formation of GCs, which, however, decline quickly (~d7) after the peak of the response. The output of these GCs is IgM^+ as well as IgG^+ memory B cells and mostly short-lived plasma cells. The antibody response is mainly of the IgG1 isotype and not long lasting (Lalor et al., 1992). The immune response can be prolonged with the application of adjuvants. However, haptenated proteins are rather artificial antigens and may not be ideal to study immune responses against infectious agents, as their physical appearance is quite different from pathogens.

In contrast to haptenated proteins, viruses can induce an early T cell independent IgM response followed by a T cell dependent IgG response, which mediates long-term protection (Bachmann and Zinkernagel, 1997). The GC reaction peaks after 2-3 weeks and lasts up to several months (Bachmann et al., 1996). The strong humoral response is mainly of the IgG2a subtype. The potency and longevity of the adaptive immune response induced by haptens or particulate and repetitive VLPs are quite different. Interestingly, 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 (Zinkernagel et al., 2001). It is therefore surprising that immune responses induced against haptens are usually lower rather than similar or higher compared to responses specific for viral or bacterial proteins.

Beside bacteria, fungi and parasites - viruses are an important class of pathogens which carry different features to efficiently infect the host, to prolong infection or even escape from recognition and/or elimination. The immune system has co-evolved with the pathogens and developed many defense mechanisms to fight those pathogenic invaders to finally clear the infection. In contrast to model antigens, which are usually given i.v. or s.c., viruses usually enter the host via the mucosal surfaces. At this entry-site, epithelial cells as well as underlying tissue cells have a large number of intracellular receptors that recognize viral components, in particular nucleic acids. Prominent receptors are RIG-1, Mda5, LPG2, STING and AIM2 (Barber, 2011; Gitlin et al., 2006; Hornung et al., 2009; Hornung et al., 2006; Imaizumi et al., 2002; Ishikawa et al., 2009; Satoh et al., 2010; Schmidt et al., 2009). As a result, inflammatory cytokines such as IL-1 or type I IFN are produced, which in turn activate immune cells and inhibit viral replication by a number of mechanisms, including degradation of viral nucleic acids (Bowie and Unterholzner, 2008; Yan and Chen, 2012). One of the main players during early viral infections are plasmacytoid DCs producing high amounts of IFNs (Colonna et al., 2004; McKenna et al., 2005). The innate immune system has developed multiple pathways ranging from soluble mediators to activated killer cells to combat such infections at an early time point (Zinkernagel et al., 1996).

Understanding immune responses induced by viral infections are particularly important; as such knowledge may provide the key for development of novel vaccines which are usually based on attenuated, inactivated viral particles or non-replicating VLPs. To work with VLPs provides a significant experimental advantage as they are not infectious and do not replicate but still are highly immunogenic (D'Argenio and Wilson, 2010; Pulendran et al., 2010). VLPs provide structural and additional stimulatory characteristics for efficient recognition by the immune system (Bachmann and Jennings, 2010). On one hand, their highly ordered and repetitive structure efficiently crosslinks BCRs and on the other hand the supply of ligands for TLR 7/8 and 9 can stimulate APCs as well as B cells. These features trigger potent and long lasting antibody responses comparable with those induced by

infectious viruses (Bessa et al., 2008; Gatto et al., 2007a; Kundig et al., 2006; Zinkernagel, 1996; Zinkernagel et al., 2001). Also isotypes induced by VLPs are comparable to those induced by viruses, as the dominant Ab isotype triggered by VLP immunization are IgG2a in the mouse (Coutelier et al., 1987; Jegerlehner et al., 2007; Zhang et al., 2009) and IgG1/3 in the human (Beck, 1981; Cavacini et al., 2003; Litwinska et al., 1993).

To be able to study immune responses induced by pathogens such as viruses, it is important that appropriate tools are available and to choose a suitable model Ag such as VLPs.

Different functions of antibodies in B cell responses

The main effector function of B cells is the secretion of Abs by terminally differentiated PCs. As discussed above, unlike protein model Ags, viruses as well as VLPs (carrying TLR ligands) induce IgG2a as the dominant Ab subclass, that is the most potent antibody subclass at mediating ADCC and opsonization (Coutelier et al., 1987). Abs can bind to the virus for direct neutralization as well as opsonization for Fc receptor interactions. In addition, viral surfaces trigger the alternative pathway of complement activation while viral particles bound to antibodies activate the classical pathway. In addition, infected cells can also be marked by Abs for antibody dependent cell cytotoxicity (ADCC) by phagocytes and killer cells such as NK cells. Also non-neutralizing antibodies are able to mediate protection of the host against infection (Jegaskanda et al., 2013; Jegerlehner et al., 2004). Beside their role in neutralization and opsonization of antigen, Abs also play an important role in antigen-deposition in the GC reaction.

The GC reaction in secondary lymphoid organs is the hallmark of the adaptive humoral immune response and is required for induction of long-term host protection. One important first step for the induction of GCs is the transport and retention of intact Ag on FDCs to select specific B cells during the Ag affinity maturation process with increased affinity. Very small proteins reach the B cell follicles

by conduits. They are, however, normally not deposited on FDCs as they fail to activate complement and are not recognized by natural antibodies. This conduit system is probably more important for transport of signaling substances (chemokines, cytokines, etc.) than Ags. Proteins bigger than 40 kDa are deposit on FDCs, if they form ICs, in a complement receptor dependent manner (Brown et al., 1973; Ferguson et al., 2004; Heinen et al., 1986). In line with this, it has been shown that specific Abs can have a strong influence on humoral responses (Heyman, 2000; Hjelm et al., 2006). Indeed, immune complexes formed by the protein Ag and IgM as well as IgG may promote a stronger Ab response than free protein, a phenomenon which is most likely due to cross-linking of the BCRs, complement activation and in particular efficient deposition on FDCs. Excess amounts of antibodies, however, can also suppress antibody responses, most likely by binding and neutralizing the Ag preventing Ag access to B cells. It has been reported that there is an interesting difference between IgM^+ and IgG^+ memory B cells as the latter are able to efficiently respond to antigenic stimulation in the presence of specific IgG (Pape et al., 2011). Particulate Ags are finally transported into B cell follicles in a cell associated way by cognate or non-cognate B cells or DCs (Bessa et al., 2012; Gonzalez et al., 2010; Link et al., 2012; Manolova et al., 2008). However, the role of natural and specific Abs for particulate Ags is different compared to protein Ags. Our lab recently published data on the transport of the same antigenic protein into B cell follicles, either as soluble protein consisting of 2 Qβ-monomers (28 kDa protein) or as Qβ-VLP which forms an icosahedral structure of 180 subunits (2520 kDa) (Link et al., 2012). The particulate and repetitive VLP was efficiently transported into B cell follicles and deposited on the surface of FDCs, a process which was dependent on natural IgM antibodies as well as components of the classical (C1q) complement system (C3) and complement receptors (CR1, CR2). The influence of specific antibodies was further investigated. Whereas specific IgM had little influence on the deposition of VLPs on FDCs, specific IgG Abs dramatically reduced this process. In contrast, the soluble protein form as Qβ-dimer was not found on FDCs in a primary immune response. However when mice were passively immunized with specific IgM or IgG antibodies 24 h prior challenging, efficient deposition on FDCs could be observed. These

findings demonstrate a strong influence of antigen size and repetitiveness for deposition on FDCs for the formation of GCs. They also demonstrate that VLPs effectively recruit the innate humoral immune system for deposition on VLPs, while soluble antigens only efficiently deposit on FDCs in the presence of specific IgM or IgG. This may explain why soluble proteins fail to efficiently induce GCs during primary immune responses in the absence of strong adjuvants.

TLR engagement in B cell responses

A strong influence on antibody responses provides the engagement of TLR receptors (Pasare and Medzhitov, 2005). Surprisingly, for antigens linked to the TLR-ligands, TLR expression in B cells rather than DCs is important. Specifically, TLR-signaling in B cells and not DCs promote IgG class-switching (Bessa et al., 2009; Hou et al., 2011; Jegerlehner et al., 2007). During natural infections, viruses either carry ssRNA, dsRNA or DNA. The VLPs used in our studies also supply ligands to TLR7/8, as they package bacterial RNA during production. Originally Medzhitov and colleagues claimed to have shown that TLR signaling was pivotal for the induction of Ab production (Pasare and Medzhitov, 2005). This publication was, however, seriously flawed as they analyzed an antibody response which was dependent on the presence of LPS as adjuvants and then went on to show that the antibody response was also TLR4 dependent. Further studies by others demonstrated that requirement for TLR signaling is highly system dependent and that TLR-ligands often rather fine-tunes the humoral response (Gavin et al., 2006).

Our lab has studied the role of TLR-signaling in B and T cell responses by generation of VLPs loaded with RNA (TLR7/8), CpGs (TLR9) or inert polyglutamate (no TLR ligand). Indeed, immunization with VLPs loaded with RNA or CpG induced a stronger Ab response dominated by IgG2a. However, mice immunized with VLPs devoid of nucleic acid mounted a slightly reduced humoral response which was, however, mainly consisting of the IgG1 isotype. Thus, isotype switching can be directly induced

by the stimulation of TLR receptors by immunization with VLPs carrying RNA or CpG as TLR ligands. As mentioned above, TLR-signaling in B cells rather than DCs were important. To date, such insights are only partly considered in vaccine development. Current antiviral vaccines based on VLPs do not carry TLR ligand. However, vaccination against hepatitis B with constructs of HBsAg formulated with CpG enhances humoral responses in humans (Barry and Cooper, 2007). Hence, TLR-signaling may enhance the efficacy of antiviral vaccines. An interesting point to consider is that inactivated vaccines still may carry RNA or DNA. These nucleic acids, however, are most likely inactive as their biological activity was destroyed by e.g. formaldehyde or β-propiolactone. Recombinant vaccines based on virus-like particles may be able to solve this problem. They can be loaded with natural TLR ligands to enhance a humoral response and drive isotype switching to the more protective forms and at the same time address safety issues in vaccine development by loading defined amounts of natural TLR ligands preventing severe side effects by TLR over-stimulation.

Interestingly, our studies with TLR-loaded VLPs further demonstrated that TLR-signaling in DCs as well as the induction of T_H1 responses were not crucial to drive IgG2a dominated humoral responses. It is commonly assumed, that T_H1 and IgG2a are causally related but this might not be the case in general. In contrast, our data indicate that T_H1 responses and IgG2a antibodies are simply driven by the same TLR-stimulus.

Antiviral humoral responses of the isotype IgG are generally dependent on T cell help. Most T cell subsets are probably able to deliver CD40-stimulation on B cells for TD IgG^+ responses. One T_H cell subset, is however, particularly potent, namely the follicular T_H cells as they are localized in B cell follicles and GCs. T_{FH} cells provide IL-21 to the GC B cells which is essential to promote a strong IgG humoral response in the absence of TLR-stimulation. Interestingly, in IL-21 deficient mice, immunization with RNA-loaded VLPs could compensate the absence of IL-21 and induced an almost normal IgG response, whereas immunization with “empty” VLPs induced a strongly reduced humoral response (Bessa et al., 2010). Hence, the dependence on T_{FH} of the IgG humoral responses is not very

strict in the presence of TLR-ligands. This observation is even more pronounced for IgA, since systemic IgA responses are completely T cell independent. Interestingly, mechanisms that drive IgA responses are also with other respects slightly different from IgG responses (Fagarasan et al., 2010). Upon systemic encounter of VLPs, a potent IgA response is induced which is dependent on TLR-signaling in B cells. However, T_H cells or TLR-signaling in DCs was dispensable (Bergqvist et al., 2006; Bergqvist et al., 2010; Bessa et al., 2009; Fagarasan et al., 2010). In contrast, TLR signaling in B cells but not in DCs as well as alveolar macrophages was dispensable for the induction of mucosal IgA responses. Indeed, upon uptake of the RNA loaded VLPs, DCs and alveolar macrophages secrete April and BAFF causing an up-regulation of TGF-β produced by T helper cells to promote local mucosal IgA humoral responses (Bessa et al., 2009). Hence, TLR-stimulation in B cells is indispensable for IgG2a and systemic IgA responses while mucosal IgA responses depend on TLR-signaling in DCs.

Thus, there are two major reasons why VLPs are an ideal and reliable tool to study B cell responses after primary and secondary immunizations: 1) the strong immunogenicity of VLPs which is comparable to viruses without using infectious and replicating particles and 2) there are established tools to follow specific humoral and cellular B cell responses. Fluorescently labeled VLPs may be used in immunohistochemistry and FCM to stain B cells for their specific surface as well as intracellular expression of Igs. In addition, ELISA assays have been also developed to analyze the humoral responses. In comparison, such analyses are only partly possible for haptenated proteins and other model Ags.

5.1 Qβ-VLP drive rapid differentiation of memory B cells into secondary plasma cells producing increased levels of antibodies

In contrast to primary B cell responses, which are well described at the cellular and molecular level, relatively little is known about memory B cell responses. Several factors are responsible for the problems in studying memory B cell responses. These include difficulties in distinguishing primary

from secondary B cell responses in the same host, and the presence of specific antibodies which may interfere with secondary B cell responses. The latter problem is particularly obvious for immunization with live vaccines, where specific antibodies may block replication and thus antigenic exposure. Specific antibodies may, however, also inhibit secondary responses by blocking access of B cells to specific antigen. To be able to track previously activated B cells, knock-in mice have been generated that allow specific tracking of B cells that have previously expressed AID (Dogan et al., 2009). Adoptive transfer of memory B cells into allotype disparate hosts also allows selective characterization of memory B cell responses even in the absence of specific antibodies. This latter approach is complicated by the fact that positive selection and in particular antigen-specific selection of B cells blocks responsiveness of transferred B cells and that extensive manipulation of memory B cells may in general interfere with B cell responsiveness. This issue is particularly worrisome since transferred purified memory B cells fail to extensively proliferate. In order to avoid these problems, we established an adoptive transfer system where minimally manipulated specific memory B cells were transferred. A number of control experiments demonstrated that results were qualitatively the same if non-purified splenocytes, B220$^+$ purified B cells or isotype-switched purified memory B cells were transferred. Using this system, we found that memory B cells failed to efficiently proliferate or initiate GC-formation. In contrast, we describe here for the first time the differentiation of highly effective secondary PCs derived from memory B cells.

Different memory B cell subsets have been described. Classical memory B cells may be best described as surface IgG$^+$ B cells that do not secrete antibodies. This is in contrast to PCs which secrete antibodies and are IgG$^+$ in the cytoplasm but not on the cell surface. Both subtypes are GC derived (Good-Jacobson and Shlomchik, 2010; Shlomchik and Weisel, 2012) and mediate host protection for a long period of time. In addition, surface IgM$^+$ memory B cells exist exhibiting hypermutated BCRs which also persist over extended periods of time (Schittek and Rajewsky, 1992; Tangye and Good, 2007). Recently, it has been shown that these latter memory B cells constitute a

proliferation competent pool of memory B cells which may differentiate into IgG^+ memory B cells upon antigenic challenge. In contrast, IgG^+ memory B cells rapidly differentiated into PCs upon challenge (Dogan et al., 2009). In an additional study secondary B cell responses were primarily mediated by IgG^+ memory B cells since they were better able to respond to antigenic challenge in the presence of specific antibodies (Pape et al., 2011). Only a poor IgM^+ memory B cell mediated response was detected, which was attributed to the presence of specific antibodies. After the adoptive transfer of IgM^+ memory B cell population into naïve recipient mice Pape et al. could observe that those memory B cells proliferated and re-entered GCs. More than 50% of this population class-switched from the IgM to the IgG subtype. In none of the studies a quantitative cellular analysis was performed. Using the highly immunogenic model antigen Qβ-VLP, we were able to track and quantify naïve and memory B cell responses within one animal. In contrast to recent findings, our adoptive transfer experiments did not reveal such multiple layers of B cell memory. In our experimental system, both memory B cell compartments failed to extensively expand upon challenge with VLPs but similarly differentiated into secondary plasma cells. The fact that neither un-manipulated memory B cells (as found in whole splenocyte populations) nor purified memory B cell populations efficiently proliferated renders it unlikely that manipulation of cells was responsible for the failure of memory B cells to divide and expand. As a control we chose to transfer naïve Ly5.1 splenocytes into the Ly5.2 positive recipient. No similar suppression of the host B cell response or in other words enhanced plasma cell and antibody response was observed when compared to the host response of recipient mice after memory B cell transfer.

A possible explanation for the discrepancies between our system and the model antigens used by the other groups is that we used highly repetitive VLPs of 30 nm diameter loaded with the TLR7/8 ligand RNA in this study. Such viral particles are known to drive T cell independent IgM responses and are able to cause strong and long-lasting IgG responses in the absence of adjuvant (Jegerlehner et al., 2007; Jegerlehner et al., 2002). Therefore, it is likely that viral particles are the stronger stimulus for

memory B cells than sheep red blood cells or soluble protein antigens used in the previous studies. From a physiological point of view, presence of viral particles in lymphoid organs constitutes a situation which asks for immediate antibody production rather than extensive expansion of the memory B cell pool. This may explain why memory B cells rapidly differentiate into PCs in the absence of major proliferative events. It will be interesting to directly compare responses to repetitive antigens with those to soluble proteins.

The present data indicates that during secondary B cell responses, secondary PCs are generated while naïve B cells are recruited into a parallel primary B cell response resulting in a new wave of memory B cells. Therefore, each antigenic re-exposure may trigger both secondary and primary B cell responses. In this model, the memory B cell pool induced in the primary response essentially represents the antibody repertoire produced in the secondary response (Weiss and Rajewsky, 1990). In contrast, the memory B cells generated during the secondary response are largely derived from naïve B cells and may therefore harbor slightly different specificities than the concomitantly produced antibodies. As a consequence, the B cell response remains dynamic and antigenic sub-specificities encountered during the primary response are not endlessly carried forward preventing adaptation of the B cell responses to newly emerging variants. Original antigenic sin, where the originally encountered influenza virus strain dominates subsequent responses to new viral strains, would be such an example of a constraint B cell response. However, while this phenomenon may be induced experimentally in animals, during natural infection of humans, this does not appear to be the case and, in line with our data, the B cell response is always well adapted to currently circulating viral strains (Wrammert et al., 2008).

Taken together, our data demonstrate rapid differentiation of memory B cells into powerful secondary PCs while the secondary pool of memory B cells is to a large extent derived from naïve B cells, allowing plasticity of the memory B cell repertoire upon multiple antigenic exposures.

5.2 Multiple stimulations of memory B cells by cognate Ag

Memory B cells specific for Qβ-VLP differentiate quickly into plasma cells after antigen re-encounter (see chapter 4.1). Therefore, during secondary immune responses, specific antibodies are mainly produced by secondary plasma cells derived from memory B cells, as they show enhanced production of immune globulins. Once an activated or memory B cell turns into a plasma cell, it constitutes a terminal differentiation step and the plasma cells cannot re-differentiate into memory B cells. At an early stage, generation of PCs is Ag dependent whereas at later time points antibody levels are largely maintained independently of Ag, as plasma cells are long-lived and differentiation of new antigen-specific plasma cells is limited (Gatto et al., 2007b). This is consistent with our present finding that memory derived (IgH^{a+}) humoral responses do not increase after secondary boost (Fig. 4.4.2, 3). Furthermore, these data indicate that memory B cells do not contain a “stem-like” cell that replenish the memory B cell pool subsequent to its differentiation into a plasma cell. In contrast, memory B cells essentially differentiate into PCs and the whole memory B cell pool shows little potential for self-renewal. During secondary infections, the pool of memory B cells therefore is not maintained by memory B cells but rather by recruitment of naïve B cells into the memory B cell pool. Thus, during each round of infection, memory B cells differentiate into plasma cells and a new pool of memory B cells is recruited from naïve B cells. It will be interesting to dissect whether these rules are specific for viral particles or may also apply to systems using soluble proteins for immunization.

5.3 Do memory B cells require T cell help?

Activation of naïve B cells by T cell independent (TI) or T cell dependent (TD) antigens is well described. Qβ-VLP is an antigen which requires no T cell help to generate transient IgM responses but T_H is essential for isotype switching and the generation of long lasting GC derived memory B cell

responses. As we described above the VLPs combine most feature required for the induction of potent and long lasting B cell responses.

For induction of a memory B cell response, memory B cells first require their cognate antigen for initial activation (Benson et al., 2009; Maruyama et al., 2000). Benson and colleagues published data regarding the activation of memory B cells and could observe that the proliferation and differentiation process was exclusively dependent on the presence of cognate antigen. Neither TLR agonists nor bystander T cell helper signals alone were efficient to activate memory B cells. However, there have been in-vitro studies of human memory B cells, showing that proliferation and differentiation was induced after bystander T cell help and CpG DNA (Bernasconi et al., 2002) which suggested that memory antibody titers may be maintained by constant and "random" differentiation of memory B cells into plasma cells.

Ag experienced B cells are thought to be able to respond more quickly to antigenic stimulation and require less costimulatory factors (Dutton et al., 1998) compared to a primary response. Such co-stimulating factors could be provided from cognate and non-cognate $CD4^+$ T cells by e.g. CD40-CD40L interaction or cytokine secretion, respectively. Rajewsky and colleagues were amongst the first to study this question *in vivo* (Vieira and Rajewsky, 1990). They took a hapten conjugated to a carrier as antigen and observed that the memory B cell pool was independent of the presence of CD4 T cell help and persisted up to six weeks after depletion of $CD4^+$ T cells. However, during Ag re-encounter memory B cell responses were inhibited in the absence of any T cell help. A hapten cannot elicit a memory B cell response and needs to be coupled to a protein carrier to provide T cell help which in turn activates B cells. Therefore, the rules for memory B cell responses made by the Rajewsky lab follow the classical principles of primary B cell responses induced by model TD antigens.

Another study conducted by Hebeis and colleagues performed adoptive transfer experiments comparing memory B cell responses to intact virus and some soluble viral protein in RAG1 deficient mice (Hebeis et al., 2004). They claim, after adoptive transfer of memory B cells into RAG1 $^{-/-}$

recipient mice, the humoral response to virus mediated by the transferred memory B cells was independent of cognate as well as bystander T cell help. However, the detection of a humoral response in RAG1$^{-/-}$ mice may not be physiological, as B cells are transferred into an environment free of lymphocytes and free of secondary lymphoid organs with an appropriate structure (Mombaerts et al., 1992). The detection of a normal humoral immune responses in the absence of T_H may be explained by the ability of memory B cells to even differentiate into PCs when stimulated *in-vitro* (Arpin et al., 1995). With regards to physiological conditions, our experiments, where we transfer memory B cells into un-manipulated hosts, are therefore better suited to study memory B cell responses.

From a theoretical point of view, VLP-specific memory B cells may indeed respond to VLP re-encounter in the absence of T cell help, in particular since our model VLPs exhibit many feature characteristics of TI antigens. Indeed our experiments reveal new insights of the T cell help dependency in memory B cell responses. VLP specific memory B cells are able to differentiate into plasma cells and produce Abs after antigen re-challenge in the absence of cognate T cell help (CD40L is missing) and partly in the absence of CD4 T cells. Even when T cell help was completely absent, the humoral response was reduced early after challenge but almost normal at later time points (Fig. 4.3.5 D). It would be interesting to conduct functional assays to compare affinities of Abs obtained in WT or knockout mice e.g. MHC II$^{-/-}$

Although we observed that memory B cells could partly respond to their cognate Ag in the absence of CD4^{+} T cells, generation of a new pool of memory B cells which requires T cell help, however, is not established under these conditions. VLPs have a particulate structure which efficiently could cross-link the BCRs on memory B cells. In addition, the RNA-loaded VLP could further stimulate those experienced B cells via TLR 7/8 signaling. Therefore, BCR cross-linking as well as TLR stimulation could compensate the absent T cell help and induced a strong humoral response.

An interesting question will be whether responses of memory B cells require the presence of T_{FH} cells and whether presence of TLR7/8 ligands in VLPs as well as their repetitive structure are important. To assess the role of T_{FH} cells, IL-21-deficient mice will be used as recipients in adoptive transfer experiments and the other two questions will be studied by the immunization of RNA-free VLPs and soluble protein consisting of a dimer of 2 monomeric VLP-subunits instead of RNA-loaded VLPs.

5.4 Reduced naïve B cell activation in the presence of memory B cells

In our experiments we observed, that the specific *de-novo* activated naïve B cell response was reduced in the presence of memory B cells. This can be due to the rapid production of IgG Ab by secondary plasma cells after Ag re-encounter which may neutralize the Ag. In fact, ICs containing IgG and Qβ-VLP are trapped in the MZ and are quickly cleared by macrophages (Link et al., 2012). As a consequence, if the Ag is cleared from the system the specific immune response is stopped as no free Ag is available to bind to B cells to maintain the specific B cell response. In addition, in the presence of specific IgG the deposition of Qβ-VLP on FDCs is inhibited, which is essential to drive the GC reaction (Link et al., 2012). However, this reduced presence of Ag may also inhibit specific responses of memory B cells. As strong memory B cells are induced in the presence of specific IgG, this indicates that memory B cells are less susceptible to the presence of specific antibodies than naïve B cells, at least with respect to their ability to differentiate into plasma cells. Another possibility for the reduced responses of naive B cells in the presence of memory B cells is signaling via the FcγRIIb on B cells. FcγRIIb also called CD32 contains an inhibitory ITIM in the cytoplasmic domain. ICs can form and coengage FcγRIIb and BCR with high avidity that drive an inhibitory signal exclusively in cognate B cells and suppress their activation (Crowley et al., 2009; Heyman, 2003). Down-regulation of costimulatory factors also inhibits the B cell function as APC (Leibson, 2004). An interesting possibility will be to compare expression of CD32 on memory B cells versus naïve or newly activated B cells.

Why do memory B cells preferentially differentiate into highly potent secondary PCs? To this date, it was thought, that PCs always produce Abs at a constant rate. However, we now show here the opposite. Memory B cells differentiate quickly into PCs which produce increased amounts of Abs. Therefore, memory B cell responses exhibit two distinct advantages compared to primary B cell responses. The affinity as well as the amount of Abs produced is highly increased. From an evolutionary point it may be assumed that this process efficiently helps control pathogens during re-infections as antibodies are produced rapidly enough that replication of pathogens can be kept at bay right from the beginning.

Brink et al. showed in 2006, that early extrafollicular humoral responses were mediated by rather high affine B cells. Maybe this holds true for memory B cells, which exhibit increased affinities subsequent to the GC reaction, and therefore may be differentiating quickly into PCs while naïve low-affinity B cells get newly activated and supply the new pool of memory B cells (Phan et al., 2006). Maybe this high affinity is also the cause that memory B cell responses are rather independent of specific T cell help. In this case, the engagement of the hypermutated BCR seems to be sufficient to activate memory B cells as we observed almost normal levels of Abs in MHC II and CD40L deficient mice (Fig. 4.3.5. D). Gaining T cell help depends on Ag processing by professional APC. Liu and colleagues (Arpin et al., 1995) showed that GC B cells differentiated into PC when CD40 was down regulated. These results indicate that CD40 ligand co-stimulation directs the differentiation of germinal center B cells towards memory B cells rather than toward plasma cells. Thus high affine B cells differentiate quickly into PCs independent of CD40L – CD40 interaction. This may explain why memory B cells are able to differentiate into PCs and mediate a sufficient humoral response in mice deficient for MHC II and CD40L. Such a model also would explain why memory B cells are able to produce IgG at such rapid kinetics, as they do not need to find a cognate T_H cell.

5.5 Final remarks and outlook

Most prophylactic vaccines are based on the induction of long lived humoral B cell responses. VLPs exhibit many features comparable to natural viruses and are often used for vaccine development. In comparison to viruses VLPs are not infectious but still highly immunogenic due to certain structural characteristics 1) Viral-like particles with dimensions between 20 and 200 nm have an optimal size to drain freely from the site of injection to SLOs such as LNs or spleen. 2) Their highly repetitive surface can efficiently cross-link BCRs which drives a potent B cell activation. In addition, natural antibodies and components of the complement can also bind and opsonize the VLP, which can further enhance B cell activation as well as transport to and retention on FDCs in GCs. 3) Viral-like particles carry ligands for toll-like receptor 7/8 or optional 9 which activate B cells directly for isotype switching as well as dendritic cells for T cell priming.

VLPs are also a highly effective tool to study memory B cell responses. With the studies performed by us, we could provide further insights into the fate of memory B cells. In response to particulate and repetitive Ag such as VLPs memory B cells poorly proliferated but differentiated rapidly into highly effective secondary plasma cells capable of secreting higher amounts of Abs compared to their newly activated counterparts from naïve B cells (Figure 5.5.1). To mediate long lasting and protective humoral immune responses, usually multiple injections of recombinant vaccines are necessary especially in humans. Therefore, studies of memory B cell responses are indispensable to develop new vaccines as well as vaccine regimens.

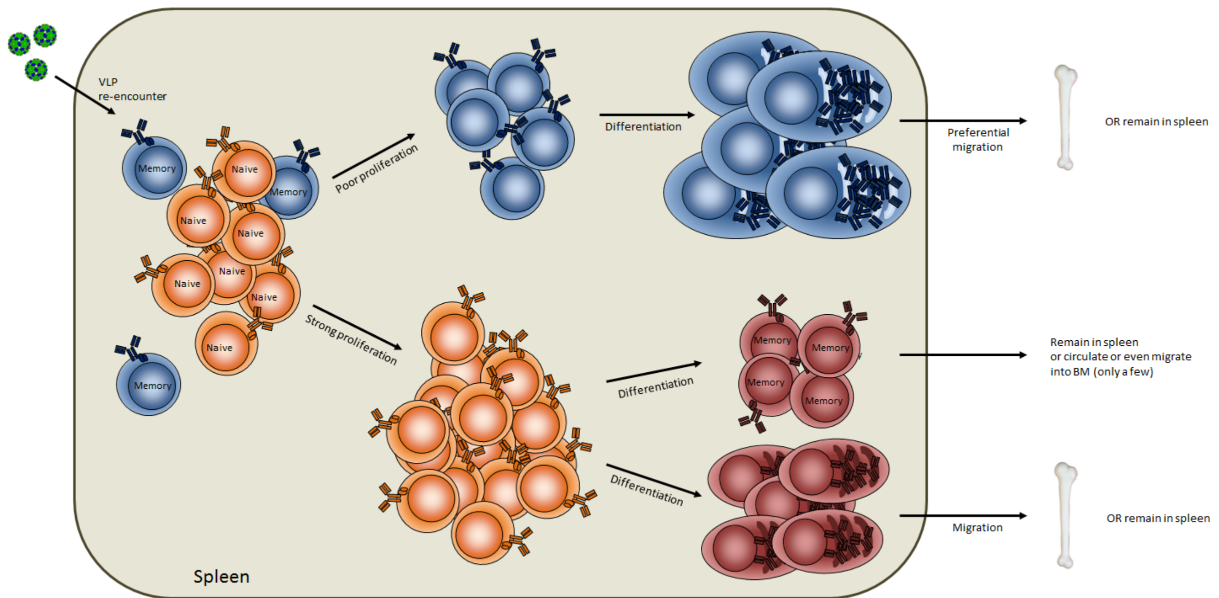


Figure 5.5.1 | Schematic presentation of VLP re-encounter and the fate of memory B cells.

Further studies

- It will be interesting to investigate the affinities of Abs derived from primary versus secondary PCs.
- To further compare recent studies assessed by different research groups using protein Ag, we should induce and challenge memory B cells with Qβ-VLPs and compare the response to those induced by Qβ-dimer exhibiting the same determinants. This would enable us to show differences of memory B cell responses mediated by particulate, repetitive Ag versus soluble protein.
- The role of TLR-ligands and T_{FH} cells will be an additional highly interesting aspect.

The sum of these experiments will allow us to map out the different pathways of memory B cell development and responses.

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Zotos, D., and D.M. Tarlinton. 2012. Determining germinal centre B cell fate. *Trends Immunol* 33:281-288.

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8 List of publications

Secondary plasma cell differentiation requires non cognate T cell help Zabel F, Kündig TM, Bachmann MF, In preparation

Viral particles drive rapid differentiation of memory B cells into secondary plasma cells producing increased levels of antibodies Zabel F, Mohanan D, Bessa J, Link A, Fettelschoss A , Saudan P, Kündig TM, Bachmann MF, In Revision at JI

Vaccination against Alzheimer`s disease: an update on future strategies Fettelschoss A, Zabel F, Bachmann MF, Hum Vaccin Immunother. 2014 Feb 17;10(4)

IgG-mediated down-regulation of IgE bound to mast cells: A novel mechanism of allergen-specific desensitization Uermösi C, Zabel F, Manolova V, Bauer M, Beerli RR, Senti G, Kündig TM, Saudan P, Bachmann MF, Allergy. 2013 Dec 19. doi: 10.1111/all.12327

Bacterially produced recombinant influenza vaccines based on virus-like particles Jegerlehner A, Zabel F, Langer A, Dietmeier K, Jennings G, Saudan P and Bachmann MF, PLoS One. 2013 Nov 18;8(11):e78947

Vaccine-induced humoral immunity: On How B cell responses are initiated by viral particles Zabel F, Kündig TM, Bachmann MF, Current Opinion in Virology, 2013 Jun;3(3):357-62

The antihistamines clemastine and desloratidine trigger apoptosis and inhibit STAT3 and c-Myc activities of human cutaneous T-cell lymphoma cells in vitro Döbbeling U, Waeckerle-Men Y, Zabel F, Graf N, Kündig TM and Johansen P, Exp Dermatol. 2013 Feb;22(2):119-24

Low-affinity B cells transport viral particles from the lung to the spleen to initiate antibody responses Bessa J, Zabel F, Link A, Jegerlehner A, Hinton HJ, Schmitz N, Bauer M, Kündig TM, Saudan P, Bachmann MF, PNAS Nov 2012, 109(50):20566-71

Innate immunity mediates follicular transport of particulate but not soluble protein antigen

Link A, Zabel F, Schnetzler Y, Titz A, Brombacher F, Bachmann MF; Journal of Immunology April 2012;188(8):3724-33.

Carrier induced epitopic suppression of antibody responses induced by virus-like particles is a dynamic phenomenon caused by carrier-specific antibodies Jegerlehner A, Wiesel M, Dietmeier K, Zabel F, Gatto D, Saudan P, Bachmann MF; Vaccine July 2010;28(33):5503-12

9 Scientific presentations

2013 Poster presentation at 11th German B cell Forum, February 25-27, 2013 in Schluchsee, Hochschwarzwald, Germany

2012 Poster presentation at the European Congress of Immunology, September 5-8, 2012 in Glasgow, Great Britain

Oral Presentation at the Joint Immunolgy Meeting of PhD students, August 8th 2012 in Zurich, Switzerland (45 min)

Oral presentation at XXIX Wolfsberg Meeting of Swiss PhD students in immunology, April 2-4, 2012, Ermatingen, Switzerland (10 min)

2011 Poster presentation at XXII Wolfsberg Meeting of Swiss PhD students in immunology, March 30 – April 1, 2011, Ermatingen, Switzerland

Poster presentation at Swiss society of allergology and immunology SSAI, March 17–18, 2011 in Lugano, Switzerland

2010 Poster presentation at 5th ENII EFIS EJI Immunology Summer School 2010, May 9-16, 2010 in Capo Caccia, Sardinia, Italy

Poster presentation at SSAI, April 15–16, 2010 in St. Gallen, Switzerland

10 Curriculum vitae

Franziska Zabel

Born on 9th February 1979 in Perleberg, Germany

German citizen

Since 01/12	University Hospital Zurich, Zurich, Switzerland Dermatology Department Group PD Dr. Thomas M. Kündig & Prof. Dr. Martin F. Bachmann PhD student under the supervision of Prof. Dr. M. F. Bachmann
03/10 – 12/11	Cytos Biotechnology AG, Schlieren, Switzerland Immunodrugs Department PhD student under the supervision of Prof. Dr. M. F. Bachmann
01/08 – 02/10	Cytos Biotechnology AG, Schlieren, Switzerland Immunodrugs Department Biologist
01/07 – 12/07	InVivo BioTech Services GmbH, Hennigsdorf near Berlin, Germany Customer and Marketing Management
09/01 – 11/06	Study of Biotechnology, University of Applied Sciences, Berlin, Germany Diploma Thesis for Diploma-Engineer of Biotechnology (FH) at InVivo BioTech Services GmbH, Hennigsdorf; Supervisor: Siegmund Karasch "Qualification of pyrolytical carbon carriers for culti-vation of adherent cell systems with the aim of transferring that cultivation to the bioreactor system BIOSTAT® TDC"
10/99 – 09/01	Study of Biotechnology, Technical University, Berlin, Germany
1992 - 1998	Gottfried Arnold Gymnasium, Perleberg, Germany High school degree