Localization and Characterization of Murine Memory B Lymphocytes

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Localization and Characterization of Murine Memory B Lymphocytes

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Contents

1 Introduction

1.1 Immunity and Immunological Memory

Immunity is the sum of protective properties of an organism against the invasive functions of pathogenic agents, e.g. bacteria, viruses, helminths or even toxins, or against their effective functioning within the organism. To achieve immunity, a large variety of molecular and cellular mechanisms has evolved, ranging from restriction enzymes to cleave foreign nucleic acids in bacteria [Stern and Sorek, 2011] to complex systems in higher vertebrates involving various cell types with immunological activity.

Immunological memory is one feature of complex immune systems as they are found in vertebrates. The term is used to describe the ability of the the immune system to react to an immunogen in an enhanced manner in recurrent encounters to that same or a structurally similar antigen. This quality of the adaptive immune system is conveyed by the retention of immune cells after the primary response to an antigen has ceased.

1.1.1 Innate and Adaptive Immunity

A prerequisite for the initiation of an adaptive immune response with the establishment of immunological memory in humans or rodents is the detection of a pathogen or foreign matter. One strategy for recognition is the utilization of receptors which can bind evolutionary conserved patterns associated with common pathogens. Best known among these pattern recognition receptors is the family of toll-like receptors, named after their homology to the toll proteins of *Drosophila melanogaster*, but there is a range of other proteins with functions in pattern recognition [Bryant and Monie, 2012, Buchmann, 2014]. Receptor activation leads to the initiation of inflammatory response mechanisms involving cells of the innate and the adaptive immune system. The cells of the adaptive immune system, T and B lymphocytes, deploy a different strategy for the creation of specific receptors which are composited from templates made up of randomly selected gene segments.

The cell types which are the primary actors in the innate defense against pathogens are derived from a common myeloid progenitor. They share the expression of evolutionary conserved pattern recognition receptors. Macrophages, natural killer cells (NK cells), and neutrophilic, basophilic and eosinophilic granulocytes are cells of the innate immune system. Especially macrophages play a prominent role in the removal of pathogens at early stages after infection and are able to clear most antigens without an adaptive immune response. They are key players in the initiation of inflammatory responses and tissue homeostasis [Gordon and Taylor, 2005] and are also responsible for the removal of

cellular debris and apoptotic cells [Mosser and Edwards, 2008]. Dendritic cells are the most prominent mediators at the interplay of innate and adaptive immune mechanisms. They act in the identification, ingestion and processing of antigens which are then presented to lymphocytes to initiate adaptive immune responses. Dendritic cells are found throughout almost all peripheral tissues. Murine dendritic cells are characterized by the expression of integrin-αX (CD11c), but there is extensive dendritic cell heterogeneity within and between different tissues [Mildner and Jung, 2014]. Dendritic cells play an important role in the maintenance of immunological tolerance to self-antigens [Lewis and Reizis, 2012]. Upon activation, dendritic cells present antigen-derived peptides on major histocompatibility complex class II (MHCII) together with the expression of co-stimulatory molecules such as B7-1 (CD80) and B7-2 (CD86) [Helft et al., 2010] to activate T cells reactive to the presented antigen which in turn can activate antigen-reactive B cells.

B and T cells are derived from a common lymphoid progenitor cell. Lymphocytes bind their cognate antigen by a randomly combined receptor which upon activation and clonal expansion of the activated cells is inherited by their daughter cells and is subjected to increased mutation to enhance the strength of antigen binding in a process called somatic hypermutation. Cells descending from the cells generated in such an antigen-specificity driven clonal expansion with subsequent fullfillment of cell type specific effector functions – or immune response – can be retained after the termination of a particular immune response in limited numbers as memory cells.

The Concept of Memory in the Immune System

The term *memory* refers to the ability of a system to conserve specific information in the absence of the original instruction [Yoshida et al., 2010]. In the context of immunity, *immunological memory* is a feature of the immune system which relates to the ability of an organism to respond to an antigenic stimulus which it has encountered before, in a more rapid and increased manner and by the production of antibodies of higher affinity [Tarlinton, 2006]. Traditionally, the notion refers to the improved responsiveness which is conveyed by the retention of cells from antigen-experienced B and T cell clones which have differentiated and expanded in the primary immune response[1]. Their survival after the termination of the initial immune response is independent from the continued presence of antigen in the system and signaling by their antigen-specific receptors[2]. Immunological memory is not dependent on proliferation, because the memory cells are, although not intrinsically long-lived, provided with external signals which mediate their longterm survival in dedicated cellularly defined niches by stromal an other cell types [Chu and Berek, 2013, Mueller and Germain, 2009, Tokoyoda et al., 2010].

1.1.2 Organs and Cells of the Lymphatic System

The focus of the work described here lies in the localization and phenotpyic characterization of memory B lymphocytes. Hence, the scope of the argumentation to cell types and tissues in which these cells are primarily found and only a limited part of their attributes und functionality is considered.

T Lymphocytes

T lymphocytes originate from hematopoietic progenitor cells in the bone marrow and mature in the thymus [Shah and Zúñiga-Pflücker, 2014]. Mature T cells display much heterogeneity and are involved in a wide variety of immunological functions. Cytotoxic T cells are characterized by the expression

[1] Although recently there have been suggestions to extend the term to cell types of the innate immune system, e.g. NK cells as reviewed by Min-Oo et al. [2013].

[2] In fact, plasma cells do not even possess surface B cell receptors.

of CD8 antigen (CD8) and restriction through major histocompatibility complex class I (MHCI). Their main function is the active elimination of pathogen-infected cells by the release of perforin and granzyme or by the induction of apoptosis in their target cells [Zhang and Bevan, 2011]. They shall not be considered here in further detail.

Helper T cells express Ly-4 (CD4) and underlie MHCII-restriction. Upon activation by cognate antigen presented by antigen-presenting cells such as dendritic cells, $CD4^+$ T cells can differentiate into various subsets, such as T helper 1, 2, 17, T follicular helper or regulatory T cells, depending on the cytokine milieu in which priming took place, antigen concentration and the accessory molecules of the antigen-presenting cells which act in co-stimulation [Zhou et al., 2009]. T helper 1 cells are mainly involved in the elimination of intracellular pathogens by the activation of macrophages [Martinez et al., 2008]. T helper 2 type cells play a role in immune responses directed against extracellular pathogens [Zeng, 2013]. T cell help is a prerequisite of the activation of B-2 cells and the formation of a germinal center response. By the production of cytokines T helper cells can also affect the isotype class-switch in the germinal center reaction and lead to the production of a particular isotype [Nguyen et al., 2012].

B Lymphocytes

B cells are generated in the mammalian bone marrow where the heavy and light chain genes of the B cell receptor are re-arranged in much the same way as T cell receptor α and β chains in T cell development [Mårtensson et al., 2010]. The theoretical range of B cell receptor diversity has been estimated to range up to 10^{18}, although the actual variation is observed to be much smaller due to differences in the probablity of certain re-arrangements [Jackson et al., 2013].

Based on phenotype, localization and function, B cells are commonly divided in three subsets: Marginal zone B cells, which reside in the marginal zones of secondary lymphatic organs, B-1 cells which are predominantly found in the peritoneal and pleural cavities, and B-2 cells which assert B cell receptor-mediated generation and maintenance of humoral immunity by the production of antibodies which are circulating freely in the blood stream or secreted on mucosal surfaces. B1 cells and marginal zone B cells can be activated in a T cell independent manner via toll-like receptor signaling and against repetitive structures or certain carbohydrate antigens [Garraud et al., 2012]. Conventional B cells, B-2 cells, are concentrated in the follicles of the secondary lymphoid organs and rely on T cell help for activation which is dependent on the interaction of their B cell receptor with an antigen. Following activation, B cells divide and undergo affinity enhancement of their antigen-binding B cell receptor by somatic hypermutation. Since the receptor encoding gene variants are propagated to the daughter cells and only cells with a receptor binding to antigen and signaling above a certain threshold can survive, this results in the clonal expansion of cells with B cell receptors of high antigen-binding capacity. Most of the surviving cells with high antigen-affinity receptors will then differentiate into antibody-secreting plasmablasts. Antibodies are released in the circulation or secreted and can bind pathogens or agents secreted by pathogens and mark them for phagocytosis [van Lookeren Campagne et al., 2007], lead to the activation of the compelement system [Daha et al., 2011], or block pathogen function by binding to functionally relevant molecules [Horiya et al., 2014].

Organs and Tissues of the Lymphatic System

Spleen The spleen is an organ with a range of functions centered on systemic circulation. It is prominently connected to the blood vessel system, but does not feature afferent lymphatic vessels [Cesta, 2006]. It is involved in a wide range of immunological chores from phagocytosis of aged erythrocytes, iron recycling and regulation of iron release to the elicitation and regulation of antigen-specific B and T cell responses and the production of various cell types [Bronte and Pittet, 2013]. The

rodent spleen, especially in fetal and newborn animals, is the site of hematopoiesis. Red and white pulp, two functionally distinct compartments, make up the morphology of the organ.

The red pulp serves as a blood filter. Specialized macrophages serve in host defense against encapsulated bacteria and in the removal of aged erythrocytes. The macrophages turn over iron from phagocytosed erythrocytes and free heme from the circulation, inhibiting bacterial growth by competition for iron and interference with the pathogens' ability to bind free iron. The red pulp is a site of residence for plasma blasts and plasma cells, allowing the immediate entry of newly produced antibodies into the circulation [Mebius and Kraal, 2005]. The white pulp surrounds the splenic central arterioles and is composed of periarteriolar lymphoid sheath, follicles and marginal zone. Mostly $CD4^+$ T cells with smaller numbers of $CD8^+$ T cells and interdigitating dendritic cells are present in the white pulp permanently, as are migrating B cells. In the follicular outer layers there are mostly small and medium lymphocytes, both B and T cells and, upon antigen-stimulation, plasmablasts and plasma cells. The follicles are continuous with the periarteriolar sheath. They are made up mainly of small recirculating naïve B cells co-expressing immunoglobulin M (IgM) and immunoglobulin D (IgD) [Steiniger et al., 2006]. Antigen-stimulation leads to formation of germinal centers which contain fewer cells and apoptotic B cells [Cesta, 2006]. The marginal zone is situated at the interface of the red pulp with the periarteriolar lymphoid sheath and follicles and serves to screen the circulation for antigens as well as processing of antigens. A band of metallophilic macrophages separates the marginal zone from periarteriolar sheath and follicles. Next to it is the marginal sinus which is continuous with capillary vessels. The sinus is neighbored by an outer ring of fibroblasts, specialized macrophages for clearance of microorganisms and other antigens, dendritic cells and marginal zone B cells [Cesta, 2006, Steiniger et al., 2006]. Marginal zone B cells are not migratory, although they are believend to originate from circulating precursors, and only leave the marginal zone upon massive stimulation. The cause for B cell retention is not fully clear: the binding of integrins [Lu and Cyster, 2002] and the balance of chemokine against sphingosine-1-phosphate receptor occupancy [Cinamon et al., 2004] are believed to play a role.

Even though splenectomy leads to a predisposition to ischemic heart disease and increases the risk of sepsis, most of the splenic function can be taken over by other tissues. This, however, is not the case for the defense against encapsulated bacteria such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Neisseria meningitidis* [Bronte and Pittet, 2013, Steiniger et al., 2006]. Moreover, the spleen has been proposed to play an important role in the maintenance of B cell memory to certain antigens such as *Vaccinia* virus [Mamani-Matsuda et al., 2008] and splenectomy has been associated with a loss in circulating memory B cells [Martinez-Gamboa et al., 2009] but not with a loss in the ability to mount a memory B cell response to a defined antigen such as tetanus toxoid [Giesecke et al., 2014].

Lymph Nodes and Peyer's Patches Lymph nodes and Peyer's patches together with the spleen are commonly called secondary lymphoid organs. In structure and function, lymph nodes resemble the splenic white pulp. Peyer's patches are not encapsulated as are lymph nodes, but otherwise structured similarly. Lymph nodes contain large numbers of lymphocytes as well as macrophages for the removal of pathogens and particulate matter as well as dendritic cells as professional antigen-presenting cells. They consist of multiple lymphoid lobules surrounded by lymph-filled sinuses enclosed by a capsular structure. Compartmentalization creates separate areas in which T and B lymphocytes are concentrated in follicles and where germinal centers are formed in acute immune responses [Buettner and Bode, 2012, Willard-Mack, 2006]. Lymph nodes are draing lymph from a limited part of an organism and serve as site of the presentation of antigens from the surrounding tissues to T and B cells [Thorek et al., 2014]. In mice, the number of lymph nodes is limited and the number of cells they contain is,

though variable depending on the immune status, generally much lower than in the spleen. In humans, the situation is somewhat different: total lymphocyte numbers in the lymph nodes are thought to be considerably higher than in the spleen[3] [Trepel, 1974].

Bone Marrow The bone marrow is the major hematopoietic organ and serves as the primary lymphoid tissue where erythrocytes, granulocytes, monocytes, lymphocytes, and platelets are generated [Travlos, 2006]. It is made up of hematopoietic parenchyma and a reticular stroma component. The stroma contains mutipotent non-hematopoietic progenitor cells which can differentiate into various mesenchymal tissues, such as osteoblasts, endothelial cells, reticular cells, fibroblasts and adipocytes [Zhao et al., 2012].

The bone marrow is the organ in which development and maturation of B cells takes place originating from hematopoietic progenitor cells. It is orchestrated by the interplay of signals provided by niches in which neighbouring cells extrinsically provide the signals required for survival, such as the chemokine CXCL12 or interleukin 7 (IL-7), as well as by signaling of the pre-B cell receptor [Clark et al., 2014, Tokoyoda et al., 2004]. Mature B cells egress from the bone marrow via the vasculature into the peripheral circulation to reach secondary lymphoid organs. Besides developing B cells, many other cell types of the immune system are present in the bone marrow in substantial numbers, such as T cells, dendritic cells, NK cells cells [Zhao et al., 2012]. But also mature B lineage cells like plasma cells [Manz et al., 1997] and memory B cells [Paramithiotis and Cooper, 1997] can be found. The latter two belong to the memory compartment of the adaptive immune system. Other members of the lymphocytic memory community reside in the bone marrow as well, such as $CD4^+$ T helper memory cells [Tokoyoda et al., 2009] and CD8-expressing cytotoxic T cells [Sercan Alp et al., 2015] which are resting in the bone marrow as will be further elaborated below. Altogether, the bone marrow is an organ of considerable size and cell numbers which holds high numbers of immune cells and lymphocytes among which memory cells are prominently present.

Besides the bone marrow and secondary lymphoid organs, there are also so-called tertiary or ectopic lymphoid tissues which can arise in the context of inflammation or infection in non-lymphoid tissues [Carragher et al., 2008, Ruddle, 2014]. These tissues are, however, beyond the scope of the work described here.

Survival of Memory Cells in Dedicated Cellular Niches

In adaptive immune responses, plasma cells and memory B and T cells are generated and retained after the antigen has been cleared from the system, and the accute immune response has ceased. Most of the immune system's effector cells undergo apoptosis due to lack of antigen-derived or co-stimulatory signals provided by accessory cells. Some cells, however, survive as long-lived memory cells. It has been shown that the bone marrow is the preferential place of residence of long-lived memory plasma cells [Manz et al., 1997, 1998], and even though they are not resting in regard to their vigorous antibody production, they are resting in terms of proliferation. These plasma cells depend on being provided with survival signals by surrounding cells, such as the cytokines interleukin 5 (IL-5), interleukin 6 (IL-6), tumor necrosis factor α (TNFα), and stromal cell-derived factor-1α, CXCL12 (SDF-1α) as well as signaling via HERMES (CD44) support the survival of plasma cells [Cassese et al., 2003, Manz et al., 2005]. 10-20% of the antigen-specific plasmablasts present at the peak of the immune response are stably retained in the bone marrow [Manz et al., 1997] where they may persist for the entire lifetime of the organism [Radbruch et al., 2006].

[3] It has been estimated that about 15% of total human lymphocytes are found in the spleen and more than 40% in the lymph nodes.

Similarly, also T helper as well as cytotoxic T cells relocate to the bone marrow in the contraction phase of an immune response where they contact IL-7-producing stroma cells in a resting state without proliferation, low transcriptional activity and the absence of the production of their effector cytokines. Upon confrontation with cognate antigen, memory T cells are rapidly re-activated and assert effector functions like the production of cytokines and co-stimulatory molecules and re-enter the cell cycle [Okhrimenko et al., 2014, Sercan Alp et al., 2015, Tokoyoda et al., 2009]. For human memory B cells, migration to [Paramithiotis and Cooper, 1997] and presence in the bone marrow [Giesecke et al., 2014] has been described. Whether there is a comparable dedicated survival niche for memory B cells in the bone marrow or which would be the factors required for their survival is so far not known.

1.1.3 Primary and Memory B Cell Responses

Memory B cells are derived from B cells which have divided in repsonse to antigens and remain stably present within an organism over long periods of time [Anderson et al., 2007]. In one report, it was found possible to re-activate memory B cells from the peripheral blood of survivors of the 1918 Spanish flu pandemic and differentiate them into antibody secreting cells by influenza virus particles of the 1918 H1N1 virus strain 90 years after infection [Yu et al., 2008]. Memory B cells ensure long-term survival by high expression of anti-apoptotic and pro-survival molecules, thereby providing the ability for quick recall antibody response by a lifetime persistence of a pool memory B cells in the host [Good et al., 2009].

Selection and Differentiation of B Cells in T Cell-dependent Immune Responses

Memory B cells are normally generated in a T helper cell dependent manner from the pool of naïve follicular B cells expressing IgM and IgD as well as complement receptor 2 (CD21) and Fcϵ receptor (CD23) [Radbruch et al., 2006, Tarlinton et al., 2008]. Generation of memory B cells in T cell-independent immune responses has also been described [Defrance et al., 2011].

T cell dependent activation of B cells in germinal centers involves a number of co-stimulatory molecules, and the loss of, e.g. TRAP (CD40) or ICOS (CD278) ligation results in decrease or total loss of germinal center activity and, subsequently, memory B cells [Yoshida et al., 2010]. An immune response to T cell dependent antigens leads to the formation of germinal centers in the B cell areas of secondary lymphoid organs. If the B cell receptor signal upon antigen presentation is sufficiently strong, activated B cells will migrate towards T cell zones in a chemokine (C-X-C motif) receptor 5 (CXCR5) dependent manner. Interaction with T cells leads to B cell proliferation and differentiation into either short-lived plasmablasts or cells which migrate back to the B cell follicle to re-encounter antigen-presenting cells and undergo the same cycle again [Tarlinton, 2006]. In the context of germinal centers B cell extrinsic signals from cytokines, toll-like receptors, integrins and other co-stimulatory molecules are integrated to induce isotype class-switch recombination resulting in the loss of IgD and IgM and lead to expression of e.g. immunoglobulin A (IgA) or IgG B cell receptors [Matthews et al., 2014, Pone et al., 2012a, Stavnezer and Schrader, 2014]. Eventually, selected high affinity B cells differentiate to either plasma cells which may become long-lived if they reach suited survival niches, or memory B cells.

It was long believed, that memory B cells require affinity maturation in germinal centers. However, mice deficicent for the transcription factor Bcl-6, which do not form germinal centers and express only low-affinity B cell receptor also can develop memory B cells independently from germinal centers even in response to T cell dependent antigens [Inamine et al., 2005]. Neither germinal centers nor somatic hypermutation or isotype class-switch are necessary for memory B cell formation [Dogan et al., 2009, Kaji et al., 2012, Longo and Lipsky, 2006, Takemori et al., 2014].

Memory B Lymphocyte Survival, Characteristics and Localization

Once generated, memory B cells can survive virtually indefinitely. Although the mechanisms by which their survival is mediated have not been clarified, some hypothetic mechanisms have been ruled out. It has long been suspected, that the continued presence of antigen in the organism would provide constant or periodically repeated B cell receptor signaling, e.g. by the presentation of antigen-antibody complexes on follicular dendritic cells. It has been shown otherwise experimentally : in mice, which had been immunized with one antigen to generate memory B cells, the conditional expression of a transgenic B cell receptor with another specificity was induced. This led to the loss of the B cell receptor for the immunizing antigen and expression of a new B cell receptor in the total absence of the antigen for which it was specific. This did not result in the loss of the memory cells nor their capacity to respond to their – now new – cognate antigen [Maruyama et al., 2000]. Another factor which had been widely suspected to be relevant for the maintenance of memory B cells, is the BLyS family of cytokines and receptors, because it also plays a well-known role in the homeostasis of mature naïve B cells [Benson et al., 2008b, Crowley et al., 2008, Gardam et al., 2008]. But the absence of neither family member, B cell activating factor, TNFSF13b (BAFF) or a proliferation-inducing ligand, Tnfsf13 (APRIL), has an impact on memory B cell maintenance [Benson et al., 2008a, Yoshida et al., 2010].

One of the difficulties of B cell memory research in mice has been the lack of a generally accepted memory marker. Instead, isotype-switch has been used as a proxy in combination with the lack of germinal center markers [Anderson et al., 2007, Klein et al., 1997, 1998]. One useful marker is CD38 antigen (CD38), which is expressed on memory B cells, but not in germinal centers, and upon re-activation of memory B cells remains stable *in vivo* even when undergoing intense proliferation [Benson et al., 2009]. In addition, some markers have been described which are useful, but heterogeneously expressed among memory B cells: CD80, Fas (CD95), L-selectin (CD62L), 5′ ectonucleotidase (CD73), and MHCII [Anderson et al., 2007]. The issue of heterogeneity within the memory B cell population has recently received much attention and led to the concept, that there are multiple, phenotypically distinct 'layers' of B cell memory which represent a continuum between more naïve-like cells destined to re-enter germinal center reactions and more differentiated cells, which rapidly differentiate further into antibody-secreting cells after re-activation by antigen [Bergmann et al., 2013, Kurosaki et al., 2015, Weill et al., 2013]. These are reported to be distinguishable by the differential expression of varying combinations of the surface markers CD73, CD80, CD86, PD-Ligand 2 (CD273), PD-Ligand 1 (CD274) [Tomayko et al., 2010, Zuccarino-Catania et al., 2014].

Previous work in mouse models has focused almost exclusively on memory B cells located in the spleen[4]. Numbers of antigen-specific memory cells per mouse spleen were estimated to 5,000 to 50,000 in an immune response to the model antigen p-azophenylarsonate [Liu et al., 1996]. In the spleen, they were reported to reside in the marginal zones [Dunn-Walters et al., 1995, Liu et al., 1988] or, according to more recent works, between follicles and marginal zone [Anderson et al., 2007] close to contracted germinal centers [Aiba et al., 2010]. In humans, memory phenotype cells have also been described as recirculating in peripheral blood, e.g. by Anderson et al. [2006], Klein et al. [1998], Weller et al. [2004]. Also in mice, isotype-switched B cells with a memory-like phenotype can be observed in the peripheral blood. Memory B cells express many molecules related to migration, e.g. integrins, CD62L, and also sphingosine-1-phosphate receptor 1, Edg1 (S_1PR1), a surface molecule essential for lymphocyte egress from thymus and secondary lymphoid tissues [Bhattacharya et al., 2007]. Antigen-specific memory

[4]This is demonstrated by the works of Anderson et al. [2007], Bhattacharya et al. [2007], Chappell and Jacob [2006], Dogan et al. [2009], Dunn-Walters et al. [1995], Good et al. [2009], Hikida et al. [2009], Inamine et al. [2005], Liu et al. [1996, 1988, 1991], McHeyzer-Williams et al. [1991], Ridderstad and Tarlinton [1998], Schittek and Rajewsky [1990], Tomayko et al. [2008].

B cells following intranasal challenge of mice with influenza virus vaccination, memory IgG B cells were found evenly distributed over the secondary lymphoid organs, spleen and lymph nodes, as well as in the blood [Joo et al., 2008, 2010]. These findings suggest a role for recirculating memory B cells in the mouse as well.

The role of the spleen is highlighted by a decrease in peripheral blood memory phenotype B cells in spelenectomized patients [Martinez-Gamboa et al., 2009], which is, however, not associated with a full loss in antigen-specific memory to defined antigens as tetanus toxoid [Giesecke et al., 2014]. The spleen has also been shown to be a reservoir of memory B cells induced by vaccinia virus or respective vaccines and to control memory B cell maintenance and homeostasis as analyzed in splenectomized patients [Yoshida et al., 2010]. On the other hand, studies show that functional B cell memory can be functional even in animals lacking spleen, lymph nodes, and Peyer's patches (SLP mice) where the B cell response was induced in ectopic lymphatic tissues [Moyron-Quiroz et al., 2006].

Secondary B Cell Responses

Signaling capacity of isotype-switched B cell receptors is different from that of non-switched B cell receptors [Surova and Jumaa, 2014] by the recruitment of an amplification mechanism [Engels et al., 2009], suggesting that isotype-switched B cells require less or no co-stimulatory signals for B cell receptor-mediated activation. It is still unclear whether this is true in all cases. Viral antigens, which contain highly repetitive structural elements, can initiate memory B cell re-activation independently from T cell help [Hebeis et al., 2004b, Weisel et al., 2010]. Possibly, T cell help is only a strict requirement for memory B cell re-activation by monomeric protein antigen, as anti-CD4 treatment effectively blocks the secondary B cell response against 4-hydroxy-3-nitrophenylacetyl (NP)-chicken γ globulin (CGG) [Vieira and Rajewsky, 1990]. One experiment suggested, that re-activation of memory B cells even required the help of memory T helper cells primed with the same protein antigen but not a different one [Aiba et al., 2010].

Canonical memory B cell responses would, therefore, require CD40 ligation before they can re-enter germinal center reaction, which theoretically could also be provided by other CD40 ligand expressing cells like macrophages or dendritic cells [Tarlinton et al., 2008]. The classical notion conceived, that re-activated memory B-cells do not undergo class-switch or somatic hypermutation after re-activation [Askonas et al., 1970, Benson et al., 2009, Siekevitz et al., 1987]. It has recently been demonstrated convincingly by B cell receptor sequencing of cells from secondary response germinal centers that class-switched memory B cells are able to form germinal center reactions and do undergo somatic hypermutation upon re-activation [McHeyzer-Williams et al., 2015].

Memory B cells do possess an antigen-specific receptor of enhanced antigen-specificity which has been proven already in a previous immune response. For effective re-activation by monomeric protein antigen, they are reported to require T cell help from antigen-experienced T cells. Based on the finding, that memory T cells reside in the bone marrow and are, therefore, not scanning the periphery for their cognate antigen, it is an interesting, yet speculative, question whether antigen-presentation to re-activate memory T cells in secondary immune reponses could be one of the functions of isotype-switched B memory cells. After engaging and re-activating memory T helper cells with a corresponding specificity to the same immunogen, an effective secondary antibody response could be mounted.

1.2 Scope and Objectives

It is known that memory plasma cells as well as cytotoxic and helper T cells are maintained within survival niches in the bone marrow, where they are provided with molecular signals required for their

long-term survival. Resting memory CD4 and CD8 T cells retain their antigen-specificity and are kept available to rapidly reassume their effector functions after antigenic re-stimulation. It has been known, though not widely considered, that B cells can also relocate to the bone marrow after being generated in an immune response. It is widely accepted that memory B cells are concentrated in the secondary lymphoid organs with a special role for the spleen and that they are recirculating in the periphery. With consideration to the more recent findings on the resting T cell memory in the bone marrow, it was the focus of the work described here to re-investigate the lifestyle and phenotypic properties of memory B cells in mice.

Memory T cells and plasma cells survive as resting cells in specialized niches in the bone marrow after the retraction phase of an immune response. The work described here was guided by the question whether the concept of survival as resting cells with defined molecular requirements also holds true for memory B cells. To this end, memory B cells in spleen, bone marrow, peripheral blood, and lymph nodes were quantified in various controlled immunizations with experimental antigens in laboratory mice as well as in mice with less confined immunological background obtained at commercial pet shops or by catching free-living mice. In addition, the phenotype of memory B cells in the different organs was compared in order to identify differences in maintenance and function of memory B cell subsets isolated from distinct environments. Moreover, the B cell receptor repertoire in spleen and bone marrow were compared to address the hypothesis that B cell clones elicited in particular immune responses are distributed evenly between the organs or that, alternatively, preferential localization of clonally-related memory B cells to spleen or bone marrow could be observed. The latter suggests a role for the type of antigen or other specific features of the immune response in preferentially directing the resulting memory cells to a particular organ.

Ultimately, the requirements for the reactivation of memory B cells in secondary immune reactions should be addressed. It was examined whether dendritic cells, which are required as antigen presenting cells for the initiation of primary immune responses, are also necessary to elicit the memory B cell response in a secondary antigen challenge.

2 Materials and Methods

2.1 Mice and Immunizations

2.1.1 Mice

All animal experiments were performed according to institutional guidelines and animal protection laws and by permission of the responsible agencies of the German federal states of Berlin and Brandenburg as applicable. All mice were housed under specific pathogen-free (SPF) conditions in the Deutsches Rheuma-Forschungszentrum (DRFZ) animal facility unless otherwise stated.

Laboratory Animals

C57Bl/6J C57Bl/6J mice were obtained from Charles River.

B1-8i NP-specific B cell receptor transgenic mice which express the heavy chain V gene variant 186.2 producing B cell receptor with high NP-affinity when combined with a λ1 light chain [Sonoda et al., 1997]. B1-8i mice were crossed to Ig light chain $\kappa^{-/-}$ mice so that mature B cells pair the V_H186.2 with a λ light chain leading to nearly 100% NP-specific B cells. The mice were bred at DRFZ facility at the Bundesinstitut für Risikobewertung in Berlin and kindly provided by Anja Hauser from DRFZ.

Cγ1-switch reporter Mice expressing Cre recombinase upon expression of the Cγ1 transcript [Casola et al., 2006] crossed to mice with a stop codon flanked by LoxP sites in the ubiquitously expressed R26 locus, leading to expression of enhanced yellow fluorescent protein (YFP) in all cells after Cγ1 transcription and their descendants. The mice were bred at the DRFZ facility at the Bundesinstitut für Risikobewertung in Berlin and kindly provided by Anja Hauser from DRFZ.

CD11c-DTR-GFP Transgenic Mice Expression of the simian diphteria toxin receptor (DTR)-green fluorescent protein (GFP) fusion protein under the control of the murine CD11c promotor leads to susceptibility to diphtheria toxin-mediated killing of cells expressing the construct [Hebel et al., 2006, Jung et al., 2002]. The mice were bred at the DRFZ facility at the Bundesinstitut für Risikobewertung in Berlin.

Non-laboratory Bred Mice

For quantification of memory B cells in different organs as well as for transcriptional profiling and sequencing of the B cell receptor repertoire, mice held under non-SPF conditions were used. The mice were obtained at pet shops in Berlin. Care was taken to select older mice on purchase, as assessed by animal size and weight.

Wild Mice

For quantification of memory B cells in different organs, free-living mice were caught using non-lethal traps. The traps were placed at non-residential buildings of a farm in Altlandsberg, Brandenburg, near Berlin. Traps were set up and controlled within 12 hours by a trained veterinarian and mice were anesthetized with isofluran prior to sacrification. Initial classification was performed by visual inspection.

Classification For classification of the mice to ensure that only *Mus musculus* were included for analysis, ploymerase chain reaction (PCR) amplification of the Gpr33 gene was performed.

Target	Primer forward	Primer reverse
Gpr33	5′-GGCCACCAAGATGAAAGAGA-3′	5′-TCAGGGTTTGTGTCCTTTCTG-3′

Mus musculus Gpr33 contains a Tat1 restriction site, whereas Gpr33 genes of *Apodemus* species do not. Restriction fragment presence and lengths were assessed by agarose gel separation.

2.1.2 Immunizations and Infection Models

Immunizations were generally carried out in adult mice (aged 12-14 weeks) with the antigenic hapten NP covalently bound to Keyhole Limpet hemocyanin (KLH) (NP-KLH, Biosearch) using lipopolysaccharide (LPS) as an adjuvant. For primary immunizations 100µg 4-hydroxy-3-nitrophenylacetyl-Keyhole Limpet Hemocyanin (NP-KLH) and 10µg LPS (from *Escherichia coli* K12, Invivogen) and for boost immunizations 10µg NP-KLH without adjuvant were used. Antigen administration was subcutaneous (SC) at the tailbase or intraperitoneal (IP) for primary immunizations as specified and SC, intravenous (IV), or per os (PO) for boost immunizations. Immunizations were generally carried out in 200µl of phosphate-buffered saline (PBS) for SC, IP, and PO immunization and 100µl PBS for IV application.

For quantification of memory B cell numbers in laboratory mice, infections with 2×10^5 plaque-forming units (PFU) Armstrong strain of lymphocytic choriomeningitis virus (LCMV) IP [Sercan Alp et al., 2015] and IV infection with 10^6 colony-forming units (CFU) attenuated *Salmonella typhimurium* strain SL7207 [Shen et al., 2014] were used. Mucosal immune response was provoked by immunization with 100mg ovalbumin (OVA) (Sigma-Aldrich) and 10µg cholera toxin (List Biologicals) using a feeding needle by oral gavage. Gastric acid was neutralized by application of 50% saturated sodium bicarbonate solution via the feeding needle prior to immunization [Lemke et al., 2015].

Infection with *Salmonella typhimurium* was performed by Vasiliki Lampropoulou from DRFZ and mice were kept under SPF conditions at Max-Planck-Institut für Infektionsbiologie in Berlin. Özen Sercan and Sibel Durlanik carried out LCMV infection experiments at Berlin-Brandenburg Center for Regenerative Therapies. PO immunizations with OVA were performed by Anja Lemke and Magdalena Kraft at DRFZ. Immediately after sacrificing the animals, organs or freshly prepared cell suspensions were provided for analysis.

2.1.3 CD11c-DTR-GFP Bone Marrow Chimera

Wildtype mice were subjected to a lethal γ-ray dose (7.25Gy) in a ^{137}Cs source and on the next day received 2-6 $\times 10^6$ CD90-depleted (MACS technology, Miltenyi Biotech) bone marrow cells in 100µl PBS by IV injection to reconstitute hematopoetic stem cells lost to radiation-induced damage. Mice received antibiotic treatment PO in the drinking water (Baytril, Bayer) for two weeks following cell transfer. Primary immunizations were performed 6 weeks after reconstitution.

2.1.4 Acquistion of Human Samples

Human blood was obtained from leukocyte filters after blood donations by anonymous healthy donors or together with bone marrow material from patients undergoing hip joint replacement surgery and tonsillar material from tonsillectomized patients. Written informed consent of the patients was obtained and research in accordance with provisions of the ethics committee at Charité Universitätsmedizin Berlin.

2.2 Experimental Procedures

2.2.1 Cell Isolation

Splenocytes and lymph node cells were isolated by passing whole organs through a 70µm mesh and washing with PBS with bovine serum albumin (BSA). For bone marrow isolation, bones were opened by use of mortar and pestle. Cells were passed through a 70µm cell strainer (BD Biosciences) and washed with PBS with BSA. Peyer's patches from the small intestine were separated using a stereomicroscope, cell isolation was performed as for lymph nodes. For spleen and bone marrow cells erythrocyte lysis was performed by resuspenion in erythrocyte lysis buffer for one minute at room temperature and washing cells afterwards in PBS with BSA.

Mononuclear cells from human blood and bone marrow or tonsils were isolated by density gradient centrifugation using Ficoll-Hypaque (Sigma-Aldrich) as described in Giesecke et al. [2014], Okhrimenko et al. [2014].

2.2.2 Cytometry and Cell Sorting

For lymphocyte staining for multicolor flow cytometry, cells were resuspended in PBS with 0.5% BSA and 5µg per ml Fcγ receptor IIB-blocking antibody (DRFZ, clone 2.4G2) at up to 5×10^8 cells per ml for surface staining with the desired antibodies conjugated to fluorescent dyes. Intranuclear staining was done using Foxp3 staining kit (eBioscience).

Cell separation by fluorescence-activated cell sorting (FACS) was done at BD FACSAria II. For cytometric analyses Canto, Fortessa (BD) or MACSQuant (Miltenyi) were used. Compensation was done with single staining positive controls and fluorescence minus one (FMO) [Roederer, 2002]. For appropriate negative controls, e.g. non-immunized animals, were used as background controls. Data were analysed by FlowJo (version 8, TreeStar) unless otherwise stated.

Enrichment of B cells was done by magnetic separation using anti-mouse CD19 antigen (CD19) microbeads (MACS technology, Miltenyi Biotech) following incubation of the cells with Fcγ receptor IIB blocking antibody (DRFZ, clone 2.4G2).

2.2.3 ELISA

Quantification of NP-specific serum IgG was done by enzyme-linked immunosorbent assay (ELISA). High binding 96-well plates (Nunc) were coated with NP-conjugated BSA in PBS. Sera in varying dilutions in PBS were applied to the plates and incubated at 37°C. After washing with PBS with BSA and Tween-20 (0.05%, Sigma-Aldrich), ploYclonal goat anti-mouse IgG conjugated with alkaline phosphatase in PBS was applied. After washing alkaline phosphatase activity was detected by incubation with p-nitrophenyl phosphate (Sigma-Aldrich), the enzymatic reaction was stopped by addition of 3M NaOH and absorbance was measured at 405nm.

2.2.4 EdU Detection by Flow Cytometry

Mice were fed with 0.5 mg/ml 5-Ethynyl-2′-deoxyuridine (EdU) (Life Technologies) via drinking water over 12 days after boost immunization. To ensure individual intake and fresh supply, EdU was changed every 3 days. EdU was detected by Click-it Alexa 647 kit (Life Technologies) according to the manufacturer's protocol following surface staining as described in chapter 2.2.2.

2.2.5 Gene Expression Analysis

Gene transcription analysis on the global level was assayed using Mouse Genome 430A 2.0 GeneChips (Affymetrix). Cells were sorted by FACS at 4°C and immediately afterwards washed and resuspended in lysis buffer and ribonucleic acid (RNA) was isolated by RNA MiniPrep kit (Zymo Research). Further processing of the samples was performed as specified by Affymetrix protocols.

Microarray data were normalized by robust multi-array average (RMA) [Irizarry et al., 2003] and differential transcript expression assessed by linear models for microarray data (LIMMA) [Smyth, 2004] using the package `limma` [Ritchie et al., 2015] in the R statistical programming environment [R Core Team, 2014]. Computation was performed by Pawel Durek from DRFZ.

2.2.6 Antibody Libraries for High-throughput Sequencing

Bone marrow cells from tibiae, femora, pelvic bones, humera, and sterna or splenocytes were enriched for CD19 expression by magnetic cell sorting (MACS) (Miltenyi) and sorted by FACS – immunoglobulin G subclass 2b (IgG_{2b}) memory B cells: $CD19^+$ $CD38^+$ $IgG_{2b}{}^+$ IgM^- IgD^- $CD93^-$ $CD138^-$ $CD11c^-$ $GL7^-$ PI^-; naïve B cells: $CD19^+$ $CD38^+$ IgM^+ IgD^+ $IgG2b^-$ $CD93^-$ $CD138^-$ $CD11c^-$ $GL7^-$ PI^-. After sorting, cells werde lyzed in Trizol reagent (Sigma-Aldrich) and stored at −80°C.

Total RNA was extracted using the TRIzol Plus RNA Purification Kit (Life Technologies) according to the manufacturer's protocol. First-strand copy DNA (cDNA) was synthesized with AccuScript High-Fidelity Reverse Transcriptase (Agilent Technologies) using total RNA and Oligo(dT) primers (Thermo Scientific). cDNA was pooled and used as template for PCR amplification of the variable heavy chain genes. For one mouse, RNA was split and reverse transcription and library amplification was performed in technical duplicates in order to determine lower thresholds of reliable clonal detection [Greiff et al., 2014, Menzel et al., 2014]. PCR was performed similar to Menzel et al. [2014] using a set of 19 forward primers with the gene-specific regions annealing to framework 1 of the VDJ-region [Krebber et al., 1997] in combination with a reverse primer with the gene-specific region binding to the IgG constant region 1 for the IgG_{2b} memory B-cell samples or a reverse primer with the gene-specific region binding to the IgM constant region 1 for the naïve B-cell samples.

Primers also contained a GC-rich overhang for second-step PCR (PCR2). PCR1 was performed with Q5 Hot Start High-Fidelity DNA polymerase (NEB). Conditions for PCR1 are shown in table 2.1. Number of cycles in last annealing step was varied depending on cell numbers.

Target	Primer reverse
IgG constant region 1	5′-CARKGGATRRRCHGATGGGG-3′
IgM constant region 1	5′-CGAGGGGGAAGACATTTGGG-3′

Table 2.1: Conditions in PCR1.

No cycles	Temperature	Time
	98°C	30s
4	98°C	10s
	50°C	20s
	72°C	30s
4	98°C	10s
	55°C	20s
	72°C	30s
8-15	98°C	10s
	63°C	30s
	72°C	30s
	72°C	2min
	4°C	storage

PCR cleanup was performed to reduce the sample volume of parallel reactions, followed by gel-excision and purification of bands of appropriate size (450 bp) from 1% agarose gels. Products were used as template for the second round PCR (PCR2), in which one forward and one reverse primer was used resulting in the addition of full-length universal and index adapter sequences to the antibody library. Conditions for PCR2 are shown in table 2.2.

Table 2.2: Conditions in PCR2.

No cycles	Temperature	Time
	98°C	30s
2	98°C	10s
	40°C	20s
	72°C	30s
5-10	98°C	10s
	65°C	20s
	72°C	30s
	72°C	2min
	4°C	storage

Final products were gel-purified from 1% agarose gels. All amplicon libraries were submitted for a quality control step on a Fragment Analyzer (DNF-486 High Sensitivity NGS Fragment Analysis Kit, Advanced Analytical). Only libraries showing a single clear peak of appropriate fragment length (≈550 bp) were used for sequencing. A 10 nM library pool was prepared from all samples and concentration of the pool was measured by qPCR using the KAPA Library Quantification Kit (KAPA Biosystems). Based on qPCR results, the final pool was diluted to 4 nM concentration. Antibody libraries were prepared by Ulrike Menzel at the Department for Biosystems Science and Engineering of the ETH Zürich in Basel.

2.3 Materials

2.3.1 Buffers and Cell Culture Medium

Buffers and cell culture medium were prepared at DRFZ and autoclaved or sterile-filtered as required.

Buffer/Medium	Contents
Erythrocyte lysis buffer	8.3 g/l NH_4Cl 0.1mM EDTA 10mM $NaHCO_3$
PBS	4.3 mM Na_2HPO_4
RPMI medium	GIBCO RPMI + GlutaMAX 1640 with 10% FCS and β-mercaptoethanol

2.3.2 Antibodies

For flow cytometry a variety of antibodies conjugated with fluorescent proteins at DRFZ Berlin or obtained from different commercial suppliers were used. A list of the antibodies used is provided in table 2.3. Additionally, a commercially available screening kit of phycoerythrin (PE)-conjugated antibodies against murine surface markers (LEGENDScreen Mouse Cell Screen (PE) Kit, BioLegend) was used according to manufacturer's directions.

2.4 Data Presentation and Statistical Analysis

2.4.1 Calculation of Absolute Cell Numbers per Organ

Absolute numbers of mouse and human B cell subsets per organ were calculated based on their frequency in a given sample. For mouse spleen, total organs were prepared and the total numbers of B cell populations were calculated based on the numbers in a defined volume determined by flow cytometry (MACSQuant, Miltenyi). Mouse bone marrow cell numbers were calculated analogously based on cell numbers in a single femur of a mouse which is estimated to harbor 6.3% of total bone marrow leading to a conversion factor of of 15.87 for one or 7.9 for two femora for total mouse bone marrow [Benner et al., 1981]. Consequently, femora were prepared separately and cell numbers counted and used to calculate bone marrow cell numbers.

B cell numbers in human bone marrow and blood were based on the caculations human of lymphocyte numbers in different organs by Trepel [1974] and calculated on the basis of the proportion of the subset in question among lymphocytes in the sample.

2.4.2 Similarity Index for Automated and Unbiased Comparison of Marker Expression by Flow Cytometry

For the analysis of comparative surface marker expression screening of murine B cell subsets by PE-labeled antibodies a composite index was devised. The index integrates a set of statistic measures to allow comparison of a single variable between populations with extreme differences in cell number. The necessity of a more complex approach was based on the number of comparisons following the use of the LEGENDScreen kit (BioLegend) in which ultimately more than 430 comparisons were to be made. The traditional approach of visually comparing histograms produces observer-biased results, comparisons of mean fluorescence intensity lack definitve measure of difference between populations, whereas simpler statistical measures for comparative statistics can be overly sensitive and identify even

Table 2.3: List of Antibodies

Antigen	Clone	Supplier
human CD3	UCHT1	DRFZ
human CD14	TM1	DRFZ
human CD19	BU12	DRFZ
human CD27	2E4	DRFZ
human CD21	Bu32	BioLegend
human CD62L	DREG-56	BioLegend
human CD138	DL-101	BioLegend
human IgA	rabbit polyclonal	BioLegend
human IgD	IA6-2	BioLegend
human IgG	11F6D3	BioLegend
human IgM	MHM-88	BioLegend
human/murine Ki-67	B56	BD Biosciences
murine CD11c	N418	DRFZ
murine CD19	1D3	DRFZ
murine CD20	AISB12	eBioscience
murine CD38	90	BioLegend
murine CD62L	MEL-14	DRFZ
murine CD80	16-10A1	BioLegend
murine CD86	GL-1	BioLegend
murine CD93	AA4.1	BioLegend
murine CD127	A7R34	DRFZ
murine CD138	281-2	BD Biosciences
murine CD273	TY25	BioLegend
murine CD274	10F.9G2	BioLegend
murine IgA	C10-3	BD Biosciences
murine IgD	11.26c	DRFZ
murine IgG_1	A85-1	BD Biosciences
murine IgG_{2a}	R19-15	BD Biosciences
murine $IgG_{2a/b}$	R2-40	BD Biosciences
murine IgG_{2b}	A95-1	BD Biosciences
murine IgG_{2b}	MRG2b-85	BioLegend
murine IgM	M41	DRFZ

rather small differences between groups and, depending on the test, be obstructed by vast differences in the size of the populations to be compared.

The composite *similarity index* was developed by evaluation of a test set of markers, which based on previous measurements were known to be or not be differentially expressed in subsets included in the assay – either between naïve and memory B cells or between the IgG_{2b} memory B cells in spleen and bone marrow.

Similarity Index Composition

The *similarity index* is mapped between 0 and 1 and is calculated by adding the partial scores depending on statistical tests performed on PE-channel values for all events in the populations to be compared. The index is attributed an initial value of 0. Test results suggesting a higher degree of conformity are rewarded by adding to the score whereas indications for difference are punished by substraction from index score.

Kolmogorov-Smirnov Test The Kolmogorov-Smirnov test is a statistical test for the equality of two probability distributions [Haz, 2001]. If the calculated p-Values by the test are $p < 0.05$,

the difference between the distribution of PE values in both samples are not considered statistically significant and a value of 0.7 is added to the index score.

Equal Quantile Distribution To test whether for the probability distributions of PE-values from both populations quantile distribution is highly similar, the 5^{th} and 95^{th} as well as 10^{th} and 90^{th} percentiles are determined. If the 10^{th} percentile of one population is less than the 20^{th} percentile and greater than the 5^{th} percentile as well as 90^{th} percentile of the same population is less than the 95^{th} percentile and greater than the 80^{th} percentile and vice versa, a value of 0.1 is added to the index score.

Deviation from Equal Distribution To quantify differences in probability distributions of PE-values between two populations X and Y of different sizes m and n, where $m > n$, n randomly selected values from Y are drawn, making up the set Y'. X and Y are cropped, so that the upper and lower 5^{th} percentiles are removed. The remaining values are normalized, so that $Min(X \cup Y') = 0$ and $Max(X \cup Y') = 1$.

If probability distributions of Y' and X are equal, then for every member y' and x of Y' and X,

$$x = y'$$

For Y' and X, the distance Δ of 5^{th}, 20^{th}, 35^{th}, 50^{th}, 65^{th}, 80^{th}, and 95^{th} percentiles from the function $x = y'$ is calculated, which is then weighted by

$$\text{dist} = 1 - (4\Delta + 8|Var(\Delta)|)^2 \tag{2.1}$$

A value of $0.8 \times$ dist is added to the index score.

Shift in Probability Distribution If a large shift in distributions is detected, in which the 75^{th} percentile of one population is less than the other popluation's 25^{th} percentile, a value of 0.3 is substracted from the index score.

Difference in Means If a difference in PE fluorescence means is detected, 0.1 is subtratced from the score. Means are considered different, if the modulus difference of means is greater than the sum of variances .

$$|\bar{x} - \bar{y}| > Var(X) + Var(Y) \tag{2.2}$$

where x and y are the PE fluorescence values of populations X and Y.

Number of Peaks in Frequency Distribution If for the frequency distributions of PE-channel values in both populations an unequal number of peaks is detected, a value of 0.1 is subtracted from the index score.

Similarity Index The final *similarity index* is set to 0 if the calculated value is less than 0 and to 1 if greater than 1.

Software

Initial gating was performed in FlowJo (version 8, TreeStar), further processing and computation of similarity index was done in the R statistical programming environment [R Core Team, 2014]. Non-base R packages used for analyses were: `ggplot2` Wickham [2009], `WRS` [Wilcox and Schönbrodt,

2014], `flowCore` [Ellis et al., 2014b], `flowViz` [Ellis et al., 2014a], `flowWorkspace` [Finak and Jiang, 2011], `flowFlowJo` [Gosink, 2010].

The similarity index was developed together with and R scripts were written by Stefan Kröger from Institut für Informatik, Humboldt-Universität zu Berlin.

2.4.3 B Cell Receptor Repertoire Analysis

Reliable detection analysis Filtered and IMGT-detected 3rd complementarity-determing regions (CDR3s) were ranked in decreasing order of frequency and tested for simultaneous presence in the corresponding replicate from the same mouse. The number of clones which belonged to the highest frequency set of clones with 95% of the contained clones present in the replicate dataset are given in Table 2.4 below:

Table 2.4: Number of clones which belonged to the highest frequency set of clones with 95% of the contained clones present in the replicate dataset.

Cell population	Replicate 1 (CDR3 cutoff)	Replicate 2 (CDR3 cutoff)
Bone marrow memory	837	948
Spleen naïve	6.3858×10^4	4.9286×10^4
Spleen memory	1074	916

From all naïve B cell datasets only 49,286 and from memory B cells 837 clones, ranked from high to low frequency, were retained for all downstream analyses as reliably detected in all downstream data analyses [Greiff et al., 2014, Menzel et al., 2014]. For all high-throughput dataset comparisons, replicates 1 of the samples from mouse 3 were used if not mentioned otherwise.

Shannon Evenness The Shannon evenness is a measure for the extent of clonal expansion. It is given by the exponential of the quotient of Shannon entropy [Shannon and Weaver, 1963] and the number of unique CDR3 sequences

$$\text{evenness} = \frac{-\sum f_i \log f_i}{N} \tag{2.3}$$

where f_i is the frequency of the i^{th} clone in a given antibody dataset and N is the number of species (e.g. unique CDR3s).

Morisita-Horn dissimilarity index The Morisita-Horn dissimilarity index is a distance estimate to allow for the comparison of data with uneven sample size [Horn, 1966] and is given by

$$d_{\text{MH}} = 1 - \frac{2\sum_{i=1}^{N} x_i y_i}{\left(\frac{\sum_i^N x_i^2}{X^2} + \frac{\sum_i^N y_i^2}{Y^2}\right) XY} \tag{2.4}$$

where N is the number of species, X and Y are the size of each population, x_i and y_i are numbers of the i^{th} clone in a given antibody dataset.

Data analyses were performed by Victor Greiff at the Department for Biosystems Science and Engineering of the ETH Zürich in Basel and Ulrik Stervbo at DRFZ.

Software

Starting from IMGT/HighV-QUEST output, data analyses were performed using the R statistical programming environment [R Core Team, 2014]. Non-base R packages used for analyses were: `ggplot2` Wickham [2009], `lattice` [Sarkar, 2008], `ShortRead` [Morgan et al., 2009], `hexbin` [Carr et al., 2014], `VennDiagram` [Chen, 2014], and `circlize` [Gu, 2014].

2.4.4 Comparative Statistics and Correlation Analysis

For all analyses not yet referred to, statistical tests were calculated with Graph pad prism version 5. For numerical data, Mann-Whitney test for unpaired samples was used. For correlation analyses of non-parametric data, Spearman correlation coefficient r was determined. Differences between groups were considered statistically significant, where $p \leq 0.05$ unless otherwise stated. Values were rounded to three significant digits.

3 Results

3.1 Murine Isotype-switched Memory B Lymphocytes Accumulate in Spleen and Bone Marrow

3.1.1 Localization of Isotype-switched Memory B Lymphocytes in Mice

Antigen-driven B cell activation via the B-cell receptor results in clonal expansion and selection of B lymphocytes for differentiation into antibody-secreting cells or the memory B cell compartment. The process may but not necessarily has to include isotype class switch and antigen-affinity maturation through somatic hypermutation. Although some markers have been suggested, e.g. by Anderson et al. [2007] and Tomayko et al. [2010], no single marker or simple set of markers has been described to selectively and comprehensively identify memory B cells and discriminate them from naïve B cells in mice[1]. Therefore, the experimental work carried out in mice, which is described here, was concentrated on isotype-switched memory B cells. Since, *in vivo*, antigen binding by the B cell receptor is required for the activation of a B cell which may consequently result in isotype class-switch, isotype-switch can be considered a surrogate marker for memory B cells.

$\mathbf{IgG_{2b}}$ Memory B Cells Accumulate in Spleen and Bone Marrow

In order to investigate the localization of memory B cells in mice, spleen, bone marrow, peripheral and mesenteric lymph nodes, and Peyer's patches were examined and probed for the presence of isotye-switched B memory lymphocytes. To obtain comprehensive insight, mice were submitted to different immunization protocols at hand:

- one single tail base SC immunization with NP-KLH and LPS
- prime and boost (day 21) tail base SC immunization with NP-KLH and LPS (2× NP-KLH/ LPS)
- IP infection with Armstrong strain of LCMV
- IV infection with attenuated *Salmonella typhimurium* strain SL7207

[1] Contrastingly, for human memory B cells TNFRSF17 (CD27) has beed described as comprehensive memory B cell marker which allows to identify non-class-switched B cells as memory B cells [Klein et al., 1998].

At late timepoints between 4 months and up to one and a half years after immunization the distribution of IgG_{2b} memory B cell to different organs was quantified by flow cytometry. IgG_{2b} memory B cells were defined as low scatter lymphocytes with expression of surface-IgG_{2b}, CD19, and CD38 in the absence of Syndecan 1 (CD138), IgD, or IgM. Gating was performed as exemplarily shown for the bone marrow in fig. 3.1C, (for spleen gating see fig. S1, p. 92).

A summary of all experiments is shown in figures 3.1A for absolute numbers and B for frequencies among CD19-expressing cells. Absolute numbers of IgG_{2b} B cells in spleen and bone marrow varied between individual mice and between experiments in which the animals had been immunized (fig. 3.1C,D). Whereas the total number of IgG_{2b} memory B cells was generally higher in the spleen, in single experiments, namely LCMV infection and more than one year after two tailbase SC immunizations with NP-KLH, in which a similar (LCMV) or even higher (NP-KLH) number of IgG_{2b} memory B cells was located in the bone marrow than in the spleen.

The frequencies of IgG_{2b} memory B cells in peripheral lymph nodes were generally comparable to spleen and higher than in the bone marrow. Absolute numbers of B cells in peripheral lymph nodes of mice held under SPF conditions was generally at least ten-fold lower than in spleen or bone marrow[2] (see fig. 3.3C). This was equally true for one experiment in which IgG_{2b} frequency among B cells in mesenteric lymph nodes and Peyer's patches was determined (2× NP-KLH/ LPS) where IgG_{2b} memory frequencies were, although with greater variance, comparable to spleen and periperal lymph nodes, but total cell numbers remained small due to the limited overall organ size (fig. 3.3D,E).

IgG_1 and IgA Memory B Cells Accumulate in Spleen and Bone Marrow

As for IgG_{2b} memory, the distribution of IgA and immunoglobulin G subclass 1 (IgG_1) memory B lymphocytes was determined by flow cytomtry. Gating was performed analogously for IgG_1 or IgA surface expression, respectively, as demonstrated for IgG_{2b} in fig. 3.1C. Data were obtained in the same exeriments with samples from the same animals.

IgG_1 memory B cells were found in spleen, peripheral lymph nodes, mesenteric lymph nodes, Peyer's patches, and peripheral blood at comparable frequencies, which were about ten-fold lower than those of IgG_{2b} memory B cells from the same experiments. The frequencies in the bone marrow were, again, lower than in the secondary lymphoid organs. Absolute numbers of IgG_1 memory B cells in the bone marrow, however, were considerably higher than in peripheral lymph nodes, mesenteric lymph nodes, and Peyer's patches due to the high absolute number of B cells in the bone marrow. Absolute numbers were generally in the same order of magnitude as in the spleen, with IgG_1 numbers in the spleen two to five times higher than in the bone marrow (fig.3.2A,B,C).

Similarly, IgA memory B cells numbers and frequencies in the organs were ten-fold lower than for IgG_{2b}. In the experiment, in which mice had been immunized with NP-KLH and LPS twice, more IgA memory B were found in the spleen, with numbers twice as high as in the bone marrow. In contrast, after *Salmonella* infection and 18 months after a single immunization with NP-KLH and LPS comparable numbers of IgA memory B cells were found in spleen and BM (fig. 3.2D,E.F).

Interestingly, the ratio of memory B cells of IgA isotype located in the spleen as compared to the bone marrow was generally lower than for IgG_1. The picture for the distribution of IgG_{2b} memory B lymphocytes is less consistent. Yet, in one case – 400 days after prime and boost immunizations with NP-KLH and LPS – even higher absolute numbers of IgG_{2b} memory B cells were detected in the spleen in contrast to preferential localization of either IgG_1 or IgA memory B cells in the single experiments.

[2]The actual number of B cells in the lymph nodes of a mouse can vary greatly with lymph node size and is obviously dependent on the extent in which a particular animal is subjected to immunological challenges at a given timepoint.

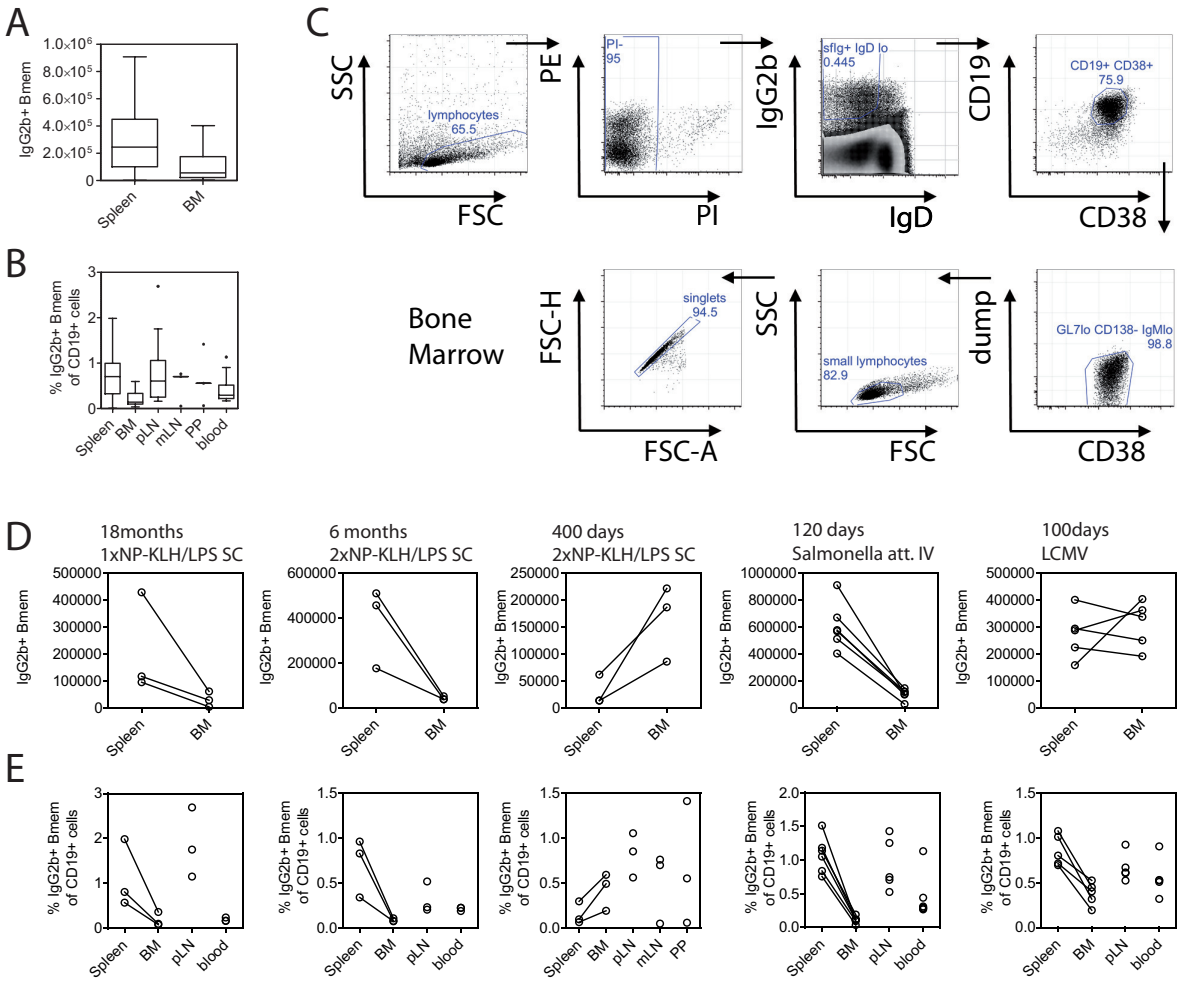

Figure 3.1: Murine IgG_{2b} memory B lymphocytes accumulate in spleen and bone marrow in high numbers.

A: Quantification of IgG_{2b} splenic and bone marrow (BM) memory B cells isolated from C57BL/6 mice from SPF housing (pooled data from D). Cell numbers were determined by flow cytometry. Shown are absolute numbers of surface-$IgG_{2b}^+CD19^+CD38^+CD138^-$ small lymphocytes per organ. For A and B, whiskers span 1.5 interquartile ranges where outliers are shown, else from min to max; n=28. **B:** Frequency of IgG_{2b}-memory B cells isolated from C57BL/6 mice from SPF housing in spleen, bone marrow (BM), peripheral lymph nodes (pLN), mesenteric lymph nodes (mLN), Peyer's patches (PP), and peripheral blood (pooled data from E). Cell numbers were determined by flow cytometry. Shown are frequencies among B cells per organ of surface $IgG_{2b}^+CD19^+CD38^+CD138^-$ small lymphocytes. **C:** Gating strategy for analysis of flow cytometric quantification of murine IgG_{2b}-switched memory B lymphocytes. Shown here is an example of bone marrow cells which were sorted by magnetic labeling for expression of CD19 and then analysed by flow cytometry. Live cell measurements excluded dead cells by PI incorporation, cell doublets by scatter signal height and included $IgG2b^+IgM^-IgD^-CD19^+CD38^+CD138^-$ small lymphocytes, the dump channel contained GL7 and CD11c markers. **D:** Quantification of IgG_{2b} splenic and bone marrow (BM) memory B cells isolated from C57BL/6 mice which were immunized or infected according to different protocols. Cell numbers were determined by flow cytometry. Shown are absolute numbers of surface-$IgG_{2b}^+CD19^+CD38^+CD138^-$ small lymphocytes per organ in spleen or bone marrow (BM): 18 months after a single immunization with 100µg NP-KLH and 10µgLPS SC, 6 months and more than 1 year (400 days) after prime (100µg NP-KLH/10µgLPS) and boost (10µg NP-KLH/10µgLPS) SC immunizations, 4 months (120 days) after IV infection with attenuated *Salmonella typimurium* SL7207 and 110 days after IP infection with LCMV Armstrong. **E:** Frequency of IgG_{2b}-memory B cells among $CD19^+$ cells in in spleen, bone marrow (BM), peripheral lymph nodes (pLN), mesenteric lymph nodes (mLN), Peyer's patches (PP), and peripheral blood as determined. Graphs refer to experiments presented in D accordingly.

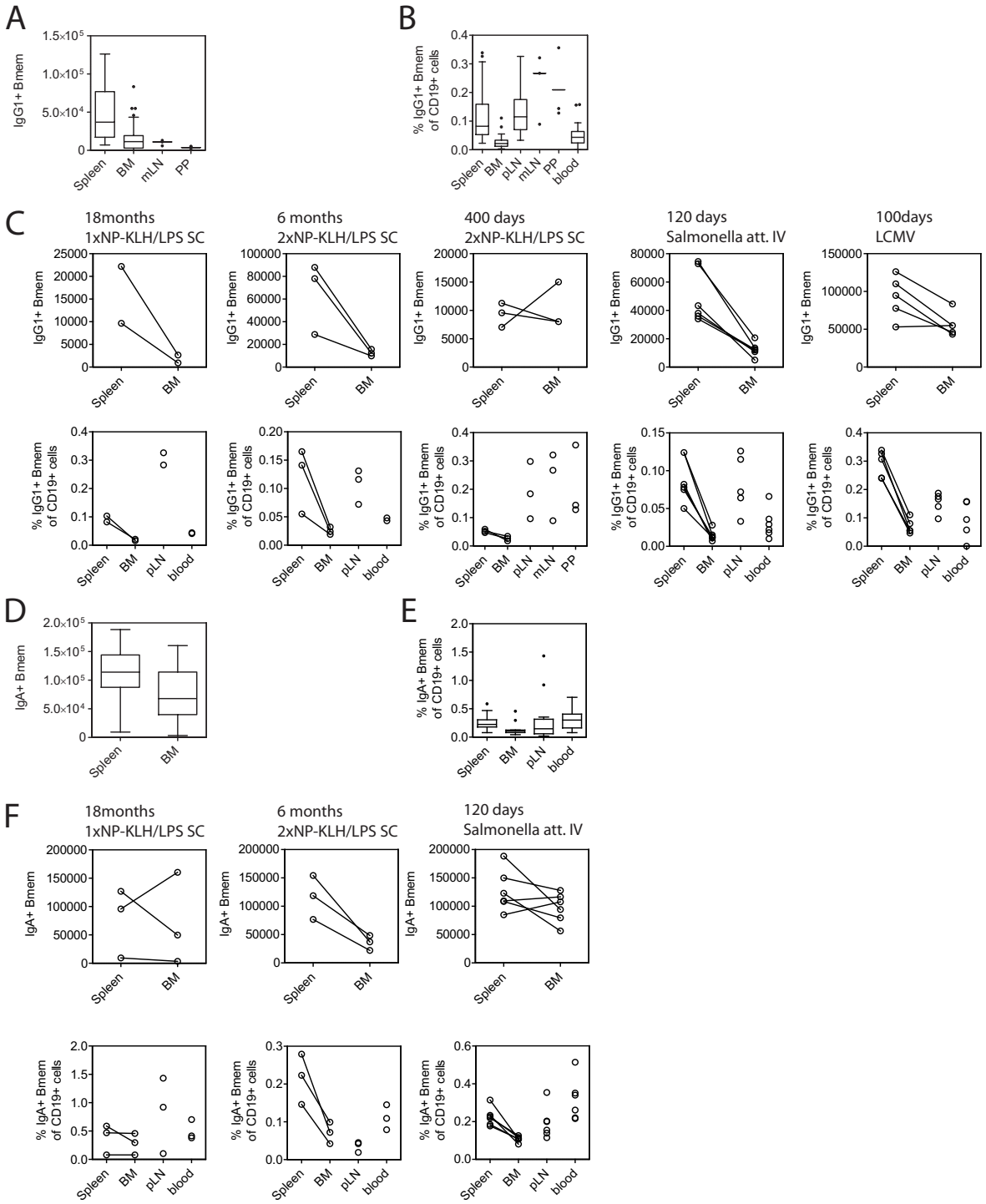

Figure 3.2: Murine IgA and IgG_1 memory B lymphocytes accumulate in spleen and bone marrow. **A:** Quantification of IgG_1 splenic and bone marrow (BM) memory B cells isolated from C57BL/6 mice from SPF housing (pooled data from C upper row). Cell numbers were determined by flow cytometry. Shown are absolute numbers of surface-$IgG_1{}^+CD19^+CD38^+CD138^-$ small lymphocytes per organ. For A and B, whiskers span 1.5 interquartile ranges where outliers are shown, else from min to max; n=24. **B:** Frequency of IgG_1-memory B cells isolated from C57BL/6 mice from SPF housing in spleen, bone marrow (BM), peripheral lymph nodes (pLN), mesenteric lymph nodes (mLN), Peyer's patches (PP), and peripheral blood (pooled data from C lower row). **C:** Quantification of IgG_1 splenic and bone marrow (BM) memory B cells isolated from C57BL/6 mice which were immunized or infected according to different protocols corresponding to data for IgG_{2b} memory in figure 3.1. Cell numbers were determined by flow cytometry. *(continued on p. 25)*

Spleen and Bone Marrow Hold Memory B cells Spleen and bone marrow are organs which harbor cells of the immune system in great numbers. Even though memory B cell frequency may be lower in the bone marrow compared to lymph nodes or spleen, overall high numbers of memory B cells of all isotypes, but IgA and IgG_{2b} in particular, were found in murine bone marrow after confrontation with various antigens.

3.1.2 Localization of Isotype-switched Memory B Cells in non-SPF Mice

By intent, the extent and nature of immunological challenges to which laboratory animals are subjected is very limited. Therefore, it was hypothesized that mice not held under SPF conditions may mount and accumulate immunological memory to a very different scale. Hence, the quantification of B cell memory was extended to mice which had not been shielded from contact to various pathogens. Mice in commercial pet shops can be confronted with a variety of infectious micro-organisms [Dammann et al., 2011]. The same is likely to be true for wild mice in their natural habitat.

For comparison to the results with respect to memory B cell quantification and distribution in C57BL/6J inbred laboratory mice held under SPF conditions, wild mice were extracted by live-mouse traps from their natural environment at a farm in Altlandsberg, Brandenburg, near Berlin. Additionally, mice were bought at commercial pet shops in Berlin. The animals were sacrificed and cell numbers and frequencies were determined as described above. To ensure that the wild mice caught were indeed *Mus musculus*, in addition to visual inspection PCR for amplification of the Gpr33 gene [Xiang et al., 2008] was performed for classification (see fig. S2, p. 92). One mouse was classified as *Apodemus agrarius* and subsequently excluded from the analysis.

The laboratory mice were held at three different SPF facilities in Berlin: DRFZ for all NP-KLH immunized animals, Max-Planck-Institut für Infektionsbiologie for *Salmonella*-infected mice, and Berlin-Brandenburg Center for Regenerative Therapies for LCMV infection. Pet mice were obtained at two different pet shops in Berlin at five different timepoints over the course of more then one and a half years from early spring of 2012 to late autumn of 2013. Wild mice were caught in the same period in farm stables and non-residential buildings at Altlandsberg.

By visual comparison, one obvious distinction between the mice of different origin was fur color which was white for all mice from pet shops, greyish for all wild mice included for analysis and dark grey for C57BL/6 laboratory mice. Body size and, consequently, weight was considerably smaller for wild mice than for both, laboratory and pet shop mice (see fig. 3.4A). Immediately after sacrificing the animals, organs were prepared for analysis. At this step further differences became apparent. Wild mice displayed a distinctly and significantly smaller spleen size ($p < 0.001$) than both, laboratory and pet mice. This was represented by size, weight, and numbers of total PTPRC $(CD45)^+$ leukocytes as

Figure 3.2: *(continued)* Shown are absolute numbers of surface-$IgG_1{}^+CD19^+CD38^+CD138^-$ small lymphocytes per organ in spleen or bone marrow (BM). Immunization schemes were: 18 months after a single immunization with 100µg NP-KLH and 10µgLPS SC, 6 months and more than 1 year (400 days) after prime (100µg NP-KLH/10µgLPS) and boost (10µg NP-KLH/10µgLPS) SC immunizations, 4 months (120 days) after IV infection with attenuated *Salmonella typimurium* SL7207 and 110 days after IP infection with LCMV Armstrong. **D:** Quantification of IgA splenic and bone marrow (BM) memory B cells isolated from C57BL/6 mice from SPF housing (pooled data from F upper row). Data presented as in A for IgG_1 accordingly. For D and E, whiskers span 1.5 interquartile ranges where outliers are shown, else from min to max; n=12. **E:** Frequency of IgA memory B cells in different organs, data presented as in B for for IgG_1 accordingly (pooled data from F lower row). **F:** Quantification of IgA splenic and bone marrow (BM) memory B cells isolated from C57BL/6 mice which were immunized or infected according to different protocols. Data presented as in C for IgG_1 accordingly.

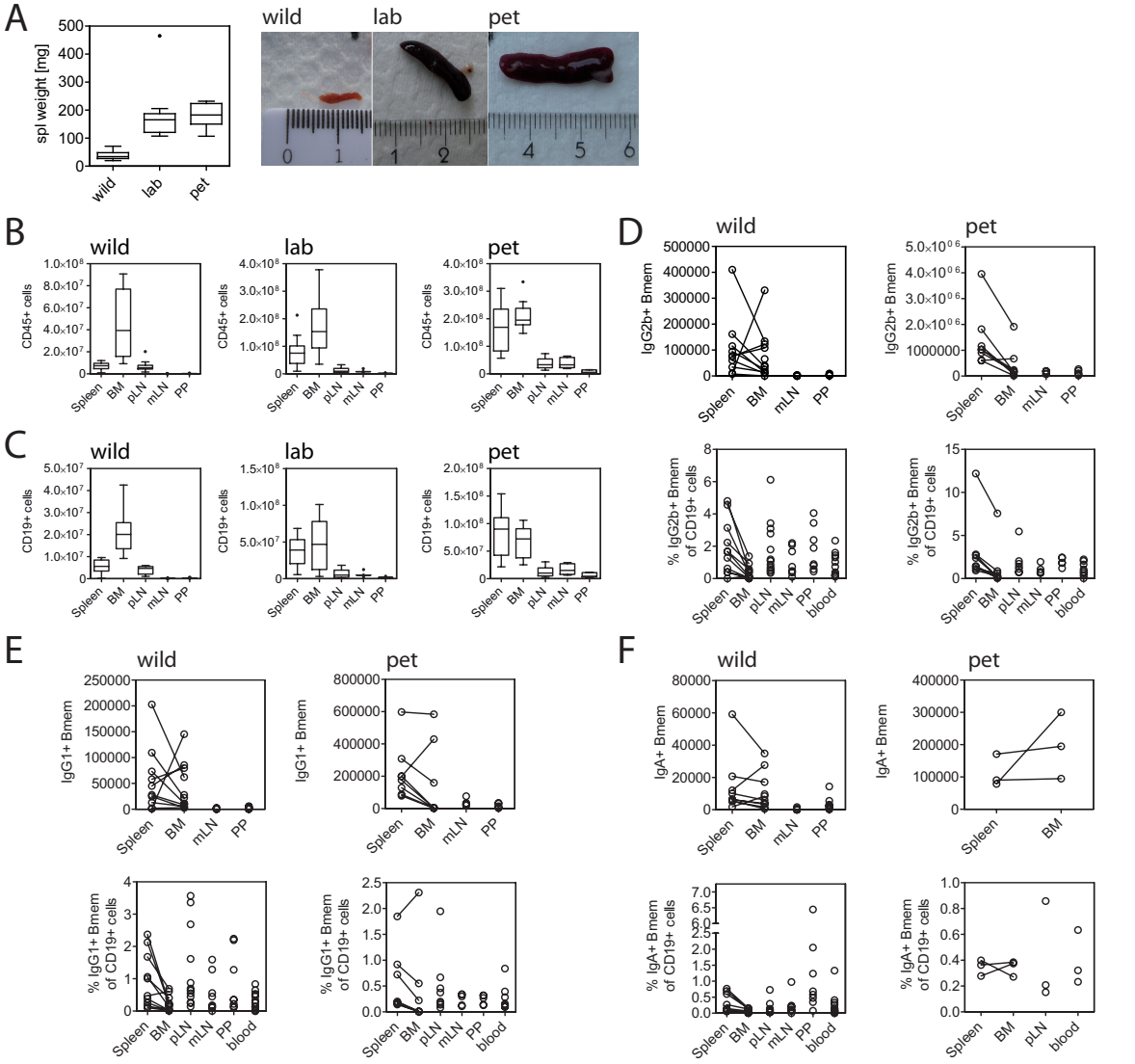

Figure 3.3: Isotype-switched memory lymphocytes accumulate in the spleen and bone marrow of mice from non-SPF conditions.
A: Spleen weight of wild mice (wild), C57BL/6 mice held under SPF conditions (lab), and mice obtained at a pet shop (pet). **B:** Absolute numbers of $CD45^+$ leukocytes per organ in spleen, bone marrow (BM), peripheral lymph nodes (pLN), mesenteric lymph nodes (mLN), Peyer's patches (PP), and peripheral blood. **C:** Absolute numbers of $CD19^+$ B cells per organ in spleen, bone marrow (BM), peripheral lymph nodes (pLN), mesenteric lymph nodes (mLN), Peyer's patches (PP), and peripheral blood. **D:** Quantification of IgG_{2b} splenic and bone marrow (BM) memory B cells isolated from wild mice (wild) and mice obtained at a pet shop (pet). Cell numbers were determined by flow cytometry. Shown are absolute numbers of surface-$IgG_{2b}{}^+CD19^+CD38^+CD138^-$ small lymphocytes per organ (upper row). Frequency of IgG_{2b}-memory B cells cells isolated from wild or pet mice in spleen, bone marrow (BM), peripheral lymph nodes (pLN), mesenteric lymph nodes (mLN), Peyer's patches (PP), and peripheral blood (lower row). Cell numbers were determined by flow cytometry. Shown are frequencies among B cells per organ of surface $IgG_{2b}{}^+CD19^+CD38^+CD138^-$ small lymphocytes. **E:** Quantification of IgG_1 splenic and bone marrow memory B cells from wild and pet mice. Data presented as for IgG_{2b} in D accordingly. **F:** Quantification of IgA splenic and bone marrow memory B cells from wild and pet mice. Data presented as for IgG_{2b} in D accordingly.

well as $CD19^+$ B cells present within the organ as represented in fig. 3.3 (A,B,C), all being reduced to about one fifth as compared to lab and pet mice. Additionally, the size of the lymph nodes as determined by total leukocyte and B cell content was also reduced by a factor of about three as compared to laboratory and about five to pet mice (fig. 3.3B,C).

The pattern for the distribution of isotype-switched memory B lymphocytes to the organs examined followed along the same lines that have been described above for laboratory mice. Generally, frequencies of a given isotype among $CD19^+$ B cells were similar within one particular group of mice in spleen, peripheral lymph nodes, mesenteric lymph nodes, Peyer's patches, and blood. Frequencies in the bone marrow were lower but, due to the high absolute cell numbers within the organ, total numbers were highest in spleen and bone marrow. IgG_{2b} memory B cells in wild mouse spleen and bone marrow were on the same level. In three out of 14 individual animals, more IgG_{2b} memory B cells were located in the bone marrow than in the spleen. In pet mice, the spleen harbours from equal distribution in one mouse up to ten times the number of IgG_{2b} memory B cells than the bone marrow.

The organ distribution of IgG_1 memory B cells in wild mice was very similar to that of IgG_{2b} memory, albeit numbers were about two-fold lower. This was similarly true for pet mice, with a reduction as compared to IgG_{2b} to about one fifth. For IgA, distribution was somewhat different: for both, pet and wild mice IgA memory B lymphocytes accumulated in spleen and bone marrow at a near 1:1 ratio. Interestingly, the number for IgA memory B cells was similar in the Peyer's Patches of wild mice as in spleen and bone marrow (fig. 3.3F upper left), for pet mice no data are available.

IgG_{2b} is the most abundant of the class-switched isotypes or subclasses which were quantified. In SPF laboratory mice the median (1st quartile,3rd quartile) IgG_{2b} memory B cell number in spleen was 2.5×10^5 $(1 \times 10^5, 4.5 \times 10^5)$ and in the bone marrow 5.6×10^4 $(2.2 \times 10^4, 1.8 \times 10^5)$. For IgG_1 the corresponding values were in spleen 3.7×10^4 $(1.7 \times 10^4, 7.7 \times 10^4)$ and in the bone marrow 1.2×10^4 $(3000, 3.2 \times 10^4)$ and 1.1×10^5 $(8.8 \times 10^4, 1.44 \times 10^5)$ in spleen and 6.8×10^4 $(4 \times 10^4, 1.1 \times 10^5)$ in the bone marrow for IgA, respectively.

Memory B Cells in Spleen and Bone Marrow Accumulate with Mouse Age

The difference in animal size and weight can partly be attributed to differences in mouse age between the groups of wild, laboratory and pet mice. Certain knowledge of mouse age is only possible in the case of laboratory mice. The size of the femoral bone, however, necessarily is a function of mouse age[3] and is not subject to reduction in times of nutritional deficiency. It was therefore considered a valid proxy variable for mouse age, suitable at least within a particular group of mice. A linear relationship between femur length and age is not expected, but especially for young mice, which are less likely to have yet encountered as many immunological challenges as older mice, it is assumned to provide reasonably good resolution to substitute for age. Femur length correlated well with mouse weight within the groups of wild ($r = 0.882, p < 0.001$) and pet mice ($r = 0.926, p = 0.002$; fig. 3.4A)[4]. This had not been expected, since periods of insufficient nutrition are quite conceivable for wild mice, whereas laboratory and pet mice are provided with water and feed *ad libitum*. Given the observed correlation, weight was used to replace femur length as a proxy for age since weight measurements were available for more individual mouse datasets.

When mouse weight is plotted against numbers of IgG_{2b}, IgG_1, or IgA memory B cells in the spleen or bone marrow (fig. 3.4B,C,D), high numbers of isotype-switched memory B cells in either

[3]That is not to imply that the relation between mouse age and femur length is entirely the same for laboratory, pet shop and wild mice.

[4]For laboratory mice no correlation was found. Presumably, because no experiments were included to generate data on younger mice, whereas mouse age for wild and pet mice was, more or less randomly, sampled from mouse populations including animals of various ages.

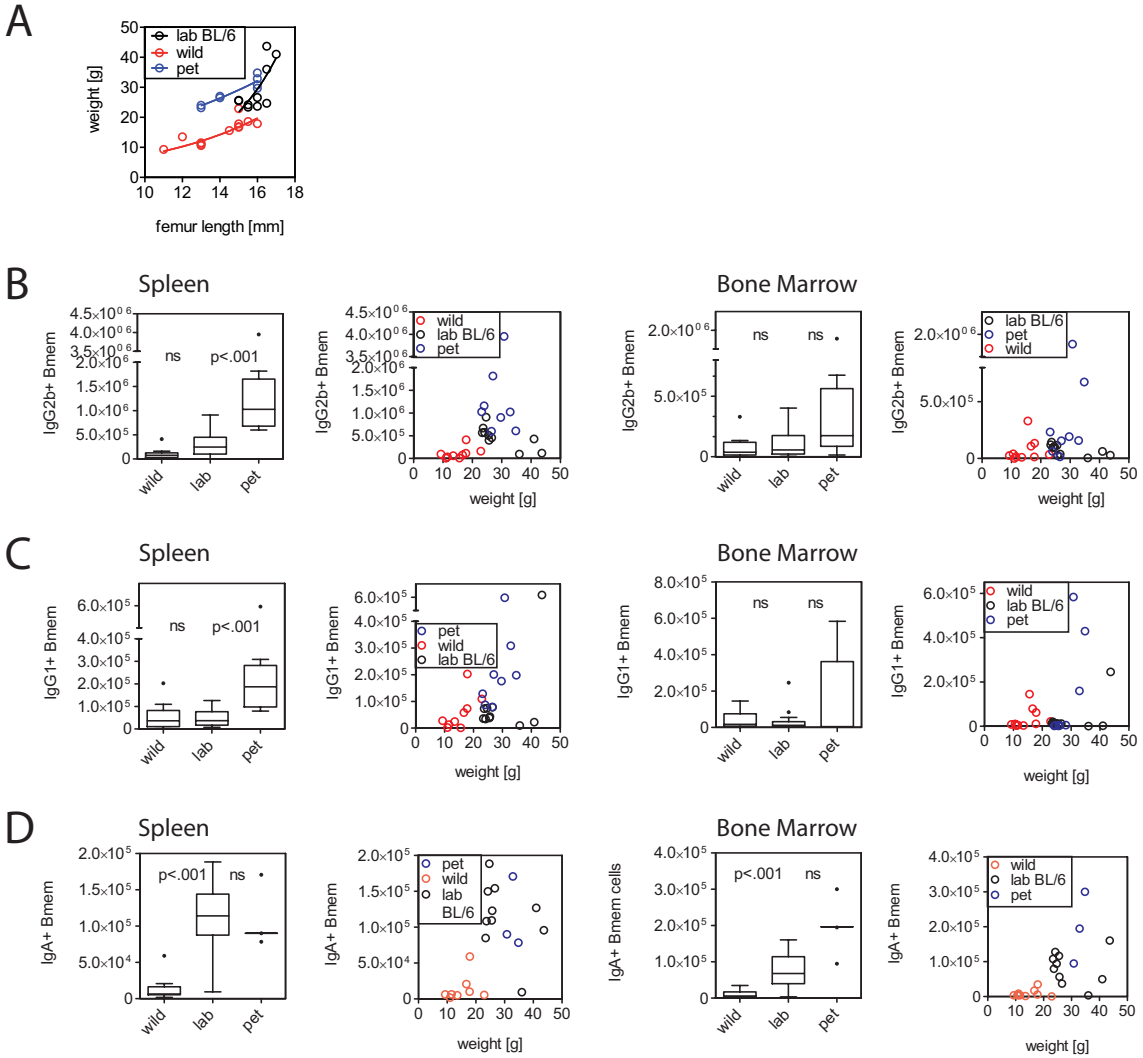

Figure 3.4: Memory B cells accumulate in spleen and bone marrow with increasing mouse age. **A:** Spleen weight against length of femoral bone of wild mice (wild), C57BL/6 mice held under SPF conditions (lab), and mice obtained at a pet shop (pet). Regression lines are semi-log. **B:** Quantification of IgG_{2b} splenic and bone marrow memory B cells isolated from wild mice (wild), C57BL/6 mice held under SPF conditions (lab), and mice obtained at a pet shop (pet). Cell numbers were determined by flow cytometry. Shown are absolute numbers of surface-$IgG_{2b}{}^{+}CD19^{+}CD38^{+}CD138^{-}$ small lymphocytes per organ (left) and plotted against mouse weight (right). In boxplots, whiskers span 1.5 interquartile ranges where outliers are shown, else from min to max; n(wild)=12, n(lab)=28, n(pet)=8. **C:** Quantification of IgG_1 splenic and bone marrow memory B cells isolated from wild, SPF lab, and pet mice. Data representation as in B for IgG_{2b}. n(wild)=12, n(lab)=25, n(pet)=8. **D:** Quantification of IgA splenic and bone marrow memory B cells isolated from wild, SPF lab, and pet mice. Data representat!ion as in B for IgG_{2b}. n(wild)=11, n(lab)=12, n(pet)=3.

organ are associated with high weight, representing mouse age, regardless whether IgA, IgG_1, or IgG_{2b} are considered. However, high mouse weight alone was not sufficient for an association with high absolute numbers of switched B memory lymphocytes within spleen or bone marrow. Significant positive correlation with mouse weight was found for:

- spleen IgA memory B cell frequencies ($r = 0.817, p = 0.004$) in pet mice
- spleen IgG_1 memory B cell numbers ($r = 0.717, p = 0.037$) and frequencies ($r = 0.783, p = 0.017$) in wild mice
- spleen IgG_1 memory B cell frequencies ($r = 0.762, p = 0.037$) in pet mice
- bone marrow IgG_1 memory B cell numbers in pet mice ($r = 0.857, p = 0.011$)

In the comparison between laboratory, pet, and wild mice based on group alone, the pet mice which were included in the study, displayed the highest memory B cell numbers of all three isotypes (considering mean as well as maximum values). Significant differences were found for:

- higher IgG_{2b} numbers in spleen of pet as compared to lab mice ($p < 0.001$)
- higher IgG_1 numbers in spleen of pet as compared to lab mice ($p < 0.001$)
- lower IgA numbers in spleen($p < 0.001$) as well as bone marrow ($p < 0.001$) of wild as compared to lab mice

The fact, that the spleen size was significantly smaller in wild than in laboratory mice, but spleen memory IgG_1 and IgG_{2b} B cell numbers were not, is reflected by significant differences in frequencies of IgG_1 ($p < 0.001$) and IgG_{2b} ($p = 0.035$).

Spleen and Bone Marrow are Sites of Memory B Cell Accumulation In mice not held under SPF conditions, isotype-witched memory B cells in spleen and bone marrow could be found in higher maximum numbers than in laboratory mice from SPF housing. The numbers of memory B cells in wild or pet mice were found to increase with mouse age. The bone marrow could harbor as many IgA or IgG_{2b} memory B cells than the spleen in individual mice, particularly in wild mice which had much smaller spleens.

3.1.3 Tracking Murine Memory B Cells in Defined Immune Responses

NP-specific Memory B Cells in an Immune Response to NP-KLH Localize to Spleen and Bone Marrow

A majority of the isotype-switched memory B lymphocytes in mice relocate to spleen or bone marrow in variable proportions. In the previous section, memory cells have been defined only by isotype-switch and absence of markers associated with recent activation such as GL-7 antibody or peanut agglutinin (PNA)[5]-binding, absence of plasmablast and plasmacell marker CD138 but with high CD38 expression[6]. To assess the localization of B memory lymphocytes generated in a defined immune response, C57BL/6J mice were immunized with NP-KLH and the adjuvant LPS. Following immunization, isotype-switched surface-B cell receptor expressing cells capable of binding the hapten NP were tracked and their numbers within different organs were quantified.

[5]In germinal center (GC) context, GL-7 antibody and PNA is used interchangeably to mark for GC B cells [Butcher et al., 1982, Laszlo et al., 1993]. GL-7, however, also marks bone marrow developing B cell stages from pro-B cells to immature B cells [Cervenak et al., 2001] and was therefore preferred here for discrimination.

[6]Surface CD38 expression is inversely correlated with GC phenotype, e.g. Inamine et al. [2005].

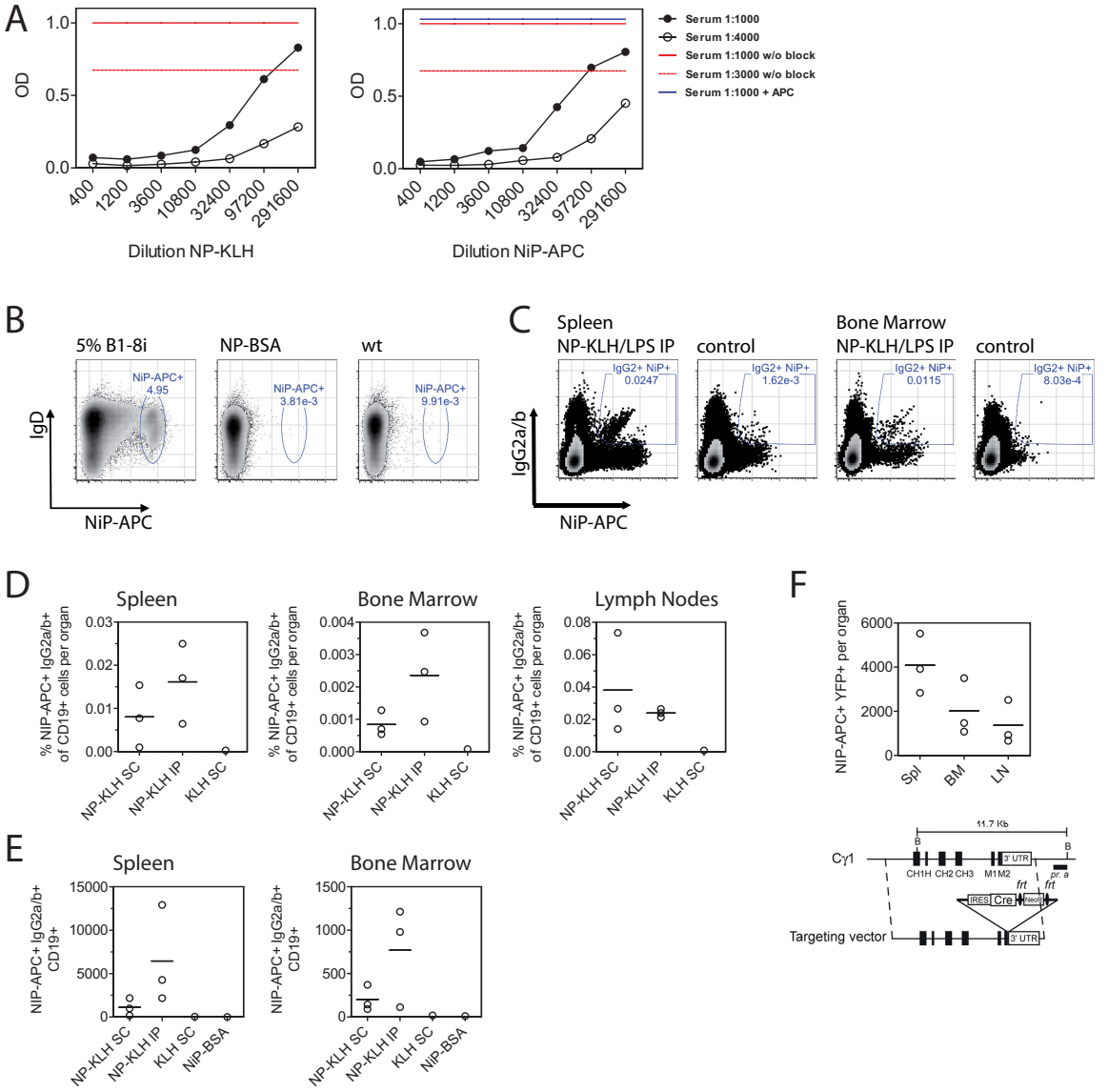

Figure 3.5: Antigen-specific IgG_{2b} memory B cells are localized in spleen and bone marrow of mice immunized with NP-KLH and LPS.
A: ELISA for NP-specific serum IgG in C57BL/6 mice on day 85 after NP-KLH/LPS immunization. Serum dilutions of 1:1000 (closed symbols) and 1:4000 (open symbols). Plots show the blocking effect of the immunizing antigen NP-KLH (left) and NiP-APC, which was used for flow cytometric detection of NP-reactive B cells (right). Dotted red line: serum 1:4000 without blocking reagent. Red line: serum 1:1000 without blocking reagent. Blue line: serum 1:1000 with APC alone. **B:** Surface staining of NP-binding B cells with NiP-APC by flow cytometry. Wild type spleen cells were mixed with 5% spleen cells from NP-specific B cell receptor transgenic B1-8i× $Ig\kappa^{-/-}$ mice (5% B1-8i, left), blocking control with wild type spleen with 5% B1-8i× $Ig\kappa^{-/-}$ spleen cells incubated with NiP-BSA before staining (NiP-BSA, middle) and wildtype spleen cells (wt, right panel). **C:** Detection of NP-specific $IgG_{2a/b}$-switched B cells on day 85 after IP immunization with NP-KLH and LPS in spleen and bone marrow by flow cytometry. Controls are day 85 spleen and bone marrow of mice immunized with KLH without NP. **D:** Frequency of NP-binding $IgG_{2a/b}{}^{+}$ of B cells in spleen, bone marrow and peripheral lymph nodes of mice immunized with NP-KLH/LPS IP or SC or controls immunized with KLH/LPS on day 85 after immunization. **E:** Absolute numbers of NP-binding $IgG_{2a/b}{}^{+}$ B cells in spleen and bone marrow of mice immunized with NP-KLH/LPS IP or SC on day 85 after immunization. Controls were mice immunized with KLH/LPS or NP-BSA blocking controls from immunized mice. **F:** Quantification of NP-binding $YFP^{+}CD19^{+}CD38^{+}CD138^{-}IgD^{-}$ cells from Cγ1-Cre×R26-stop-eYFP switch reporter mice on day 35 after NP-KLH/alum SC. Representation of targeting construct in cγ1-reporter mice from Casola et al. [2006]. Lines in D-F denote mean values.

To determine whether an NP-specific B cell response had occured in C57BL/6 mice immunized with 100µg NP-KLH and 10µg LPS, an ELISA for the presence of NP-specific IgG in the serum was performed. The presence of NiP-binding[7] IgG antibodies was confirmed. Binding of NP-IgG in the serum of NP-KLH-immunized mice could be reduced by pre-incubation of the serum with high concentrations of the immunizing antigen NP-KLH or NiP-APC, the reagent used for detection of NP-binding B cell receptor in flow cytometry (fig. 3.5A). ELISA detection of NP-binding can be efficiently blocked to background level by the immunizing as well as the staining reagent.

By use of NiP, a moleclule which closely resembles the immunizing hapten but has higher affinity to NP-binding B cell receptors[7], conjugated to the fluorescent protein APC, NP-specific B cells could be detected in flow cytometry. The reagent allows for reliable detection of NP-specific B cells by specific surface staining which is fully blockable by pre-incubation with NiP-BSA and produces minimal background in non-immunized wildtype mice (fig. 3.5B). To confirm staining specificity and recovery rates of antigen-specific cells by the staining, spleen cells from mice expressing the transgenic heavy chain V gene variant 186.2 which produces B cell receptor with high NP-affinity when combined with a λ1 light chain [Sonoda et al., 1997] on Igκ$^{-/-}$ background (B1-8i× Igκ$^{-/-}$) were mixed with wildtype splenocytes in a 1:20 ratio. As expected, 5% of surface-NP-staining cells were detected.

In mice immunized with NP-KLH and LPS, on day 85 after SC or IP immunization NP-binding isotype-switched B cells were detectable in spleen, bone marrow and lymph nodes (fig. 3.5C,D,E). NP-binding B cells mostly expressed IgG_{2b} (fig. S3). Therefore, quantification and localization was restricted to IgG_{2b} B cells. NP-binding IgG_{2b} memory B cells were detectable by flow cytometry in spleen, bone marrow and lymph nodes of mice immunized with NP-KLH and LPS whereas no NP-binding cells were found in mice immunized with the carrier protein KLH alone. Absolute numbers of NP-binding IgG_{2b} B cells were calculated from their frequencies in spleen and bone marrow. Here, as was the case for the quantification of total numbers of isotype-switched B cells, the absolute number of NP-specific IgG_{2b} B cells is dependent on organ size: Although higher frequencies of NP-binding cells were detected in spleen and lymph nodes, about one fifth of total NP-specific IgG_{2b} expressing B cells were found in the bone marrow on day 85 after one single immunization with NP-KLH and LPS. After IP immunization about 5,000 NP-binding IgG_{2b} memory B cells were found in the spleen and about 800 in the bone marrow, for SC immunization the distribution was similar with numbers being about one third of those in IP immunization. The pattern of memory B cell distribution to the differennt organs was confirmed in Cγ1-switch reporter mice, expressing eYFP in all cells which at one time have expressed the Cγ1 transcript [Casola et al., 2006] and their descendants (figs. 3.5F,S4), where half as many YFP-expressing NiP-APC-staining cells were found in the bone marrow as in the spleen.

Memory B Cells Generated During a Primary Mucosal Immune Response to OVA Relocate to the Bone Marrow

B cell isotype and the expression of homing receptors and adhesion molecules is subject to context-dependent regulation which integrates signals such as cytokines and other milieu-dependent factors, e.g. the presence of retinoic acid in the gut. Relative differences in the distribution of IgA compared to IgG_1 isotypes to spleen and bone marrow have been elaborated in section 3.1.1. IgA is strongly associated with mucosal immune respones [Brandtzaeg and Johansen, 2005]. An elevated propensity of IgA memory B cells to localize to the bone marrow could depend not primarily on their isotype but rather on the circumstances under which they were primed. Therefore, the localization of memory

[7]NiP was used for detection of NP-binding antibodies in ELISA as well as flow cytometry to exploit the long-known property of heteroclicity of anti-NP antibodies to bind the hapten NiP with higher affinity than the structurally related NP [Cumano and Rajewsky, 1985, Imanishi and Mäkelä, 1974].

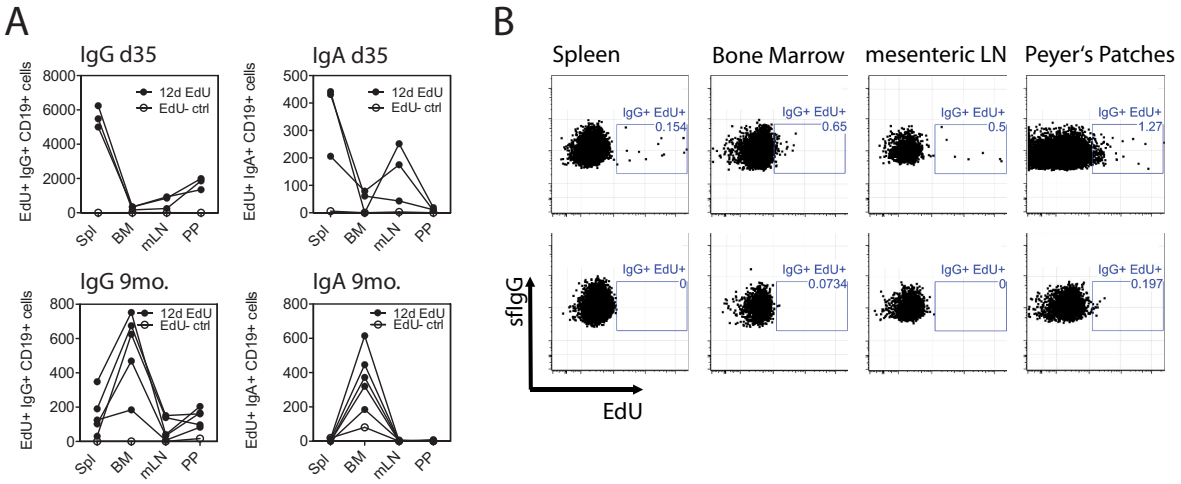

Figure 3.6: IgA and IgG memory B cells generated in OVA/cholera toxin PO immunization accumulate in the bone marrow.
C57BL/6 mice were immunized with OVA/cholera toxin PO and received EdU PO for 12 days after immunization. EdU^+ IgA^+ and IgG^+ B cells were quantified by flow cytometry 35 days (d35) and 9 months (9mo.) after immunization.
A: Absolute numbers of EdU^+ IgG^+ (left) and IgA^+ (right) memory B cells per organ in spleen (Spl), bone marrow (BM), mesenteric lymph nodes (mLN), and Peyer's patches (PP) 35 days (upper row) and 9 months after immunization (lower row). Lines connect samples from same aninmal. **B:** Flow cytometric detection of EdU^+ cells 9 months after immunzation: surface-IgG plotted against EdU-staining in $CD19^+CD38^+CD138^+IgD^-$ cells in mice which were administered EdU (upper row) and EdU^- control (lower row) for spleen, bone marrow, mesenteric lymph nodes (LN) and Peyer's patches.

B cells generated in a defined gut-restricted immune response was examined.

A mucosal immune response in the gut was induced by PO immunization with OVA together with cholera toxin which serves as a potent adjuvant in mucosae [Lavelle et al., 2004]. For twelve days folllowing the immunization EdU was administered with the drinking water. Subsequently, cells which had incorporated EdU into the deoxyribonucleic acid (DNA) in cell divisions during the period in which EdU was ingested, could be tracked for up to nine months after immunization. By flow cytometry IgG and IgA-expressing memory B cells in the gut-associated lymphatic tissues of Peyer's patches and mesenteric lymph nodes as well as spleen and bone marrow were detected and probed for the presence of EdU. Cells with detectable levels of EdU were found on day 35 as well as nine months after immunization in all organs tested. Their numbers within the respective organs, however, were very different. Additionally, the distribution of EdU^+ cells was dependent on the isotype: whereas IgG memory B cells were found mostly in the spleen and Peyer's patches on day 35 after immunization and after 9 months were found preferentially in the bone marrow, IgA memory B cells were found in the spleen at about ten-fold lower numbers than IgG on day 35 and to a comparable extent in mesenteric lymph nodes. At the late timepoint, nine months after immunization, EdU^+ IgA memory B cells were only detectable in the bone marrow (fig. 3.6).

Memory B Cells from Defined Immune Responses Relocate to Spleen and Bone Marrow
Isotype-switched memory B cells from defined immune responses, identified by their capacity to bind the immunizing antigen NP or by the incorporation of EdU administered in the period in which a mucosal immune response had been initiated could be detected in the bone marrow as well as in the spleen. IgA memory B cells which had incorporated EdU in a mucosal immune response could exclusively be retrieved from the bone marrow 9 months after immunization, whereas EdU^+ IgG memory B cells were found preferentially in the bone marrow but in lower numbers also in Peyer's

patches and the spleen. NP-specific IgG_{2b} memory B cells were found preferentially in the spleen but also in the bone marrow after a single IP or SC immunization with NP-KLH/LPS.

3.2 Divergent Properties of Isotype-switched Memory B Cells in Spleen and Bone Marrow

In the recent past a new interest has arisen in the heterogeneity within the memory B cell compartment and its functional significance [Anderson et al., 2007, Dogan et al., 2009, Tomayko et al., 2010, Weill et al., 2013, Zuccarino-Catania et al., 2014]. There has, however, not been much attention to bone marrow-resident B cell memory. In order to gain insight into immunological features such as cytokine and homing receptor distribution, cell adhesion molecules, B cell function-related molecules as well as the B cell receptor repertoire, B cells from spleen and bone marrow were purified for phenotypical characterization of isotype-switched B memory lymphocytes in general as well as the identification of differences between spleen and bone marrow memory in particular.

3.2.1 Gene Expression Profiles of Spleen Naïve and Memory B Cells

For the generation of gene expression profiles murine memory B cells from pet as well as C57BL/6 laboratory mice from the DRFZ SPF facility were isolated by FACS after pre-enrichment of CD19-expressing cells by magnetic cell sorting. Since B cell numbers are dependent on mouse age as demonstrated in chapter 3.1.2, mice with comparatively higher age were used. The samples were pooled cells from groups of five female C57BL/6 mice aged 14 months and two to four mice obtained at a pet shop with careful selection of larger mice with high body weight. Cells were FACS-sorted and purity was controlled as shown exemplarily in fig. s5 (p. 94). Cells were obtained at a purity of generally 98% or higher. Gene expression profiles were measured by microarrays prepared and analysed as described in chapter 2.2.5.

It should be noted, that for the comparative analysis of gene transcription microarray of spleen naïve, spleen $IgG2_2$ memory and bone marrow IgG_2 memory B cells, data from C57BL/6 and pet mice were combined. Spleen naïve, spleen IgG2 memory[8] and bone marrow memory B cells. Pooling data for analysis which were derived from different mouse strains increases inter-sample variance, but can on the other hand aid in the identification of meaningful differences in gene transcription between the cell populations compared.

Differential Gene Expression in Spleen and Bone Marrow Memory B Lymphocytes

A comparison of the gene expression profiles of IgG_2 memory B cells from spleen versus bone marrow revealed significant differences in the detection by 1438 probesets for gene transcripts (limma $p < 0.1$) (fig. s6, p. 94). Detectable messenger RNA (mRNA) was found for 18385 probes, 7.85% of which were differentially transcribed, the vast majority of them with limited regulation between 1.3- and two-fold. The calculation of trees based on the similarity in gene expression demonstrates that the greatest similarities existed between the profiles of either memory or naïve cells from mice of the same group. The similarity between cells of the same subset, memory or naïve cells from the same organ, for all comparisons was so extensive that the profiles of memory or naïve cells from pet and wild mice are clustered in the same branch of a tree. The difference between memory subsets was greater for

[8] In the memory B cell sorts from pet mice, an antibody specific for $IgG_{2\ a\ and\ b}$ was used. IgG_{2a} frequencies, however, are very low: in all mice tested the number of IgG_{2b} exceeded that of IgG_{2a} by at least ten-fold. Moreover, there is evidence, that the difference between isotype-switched memory B cells is less dependent on isotype than on their localization (see chapter 3.2.2).

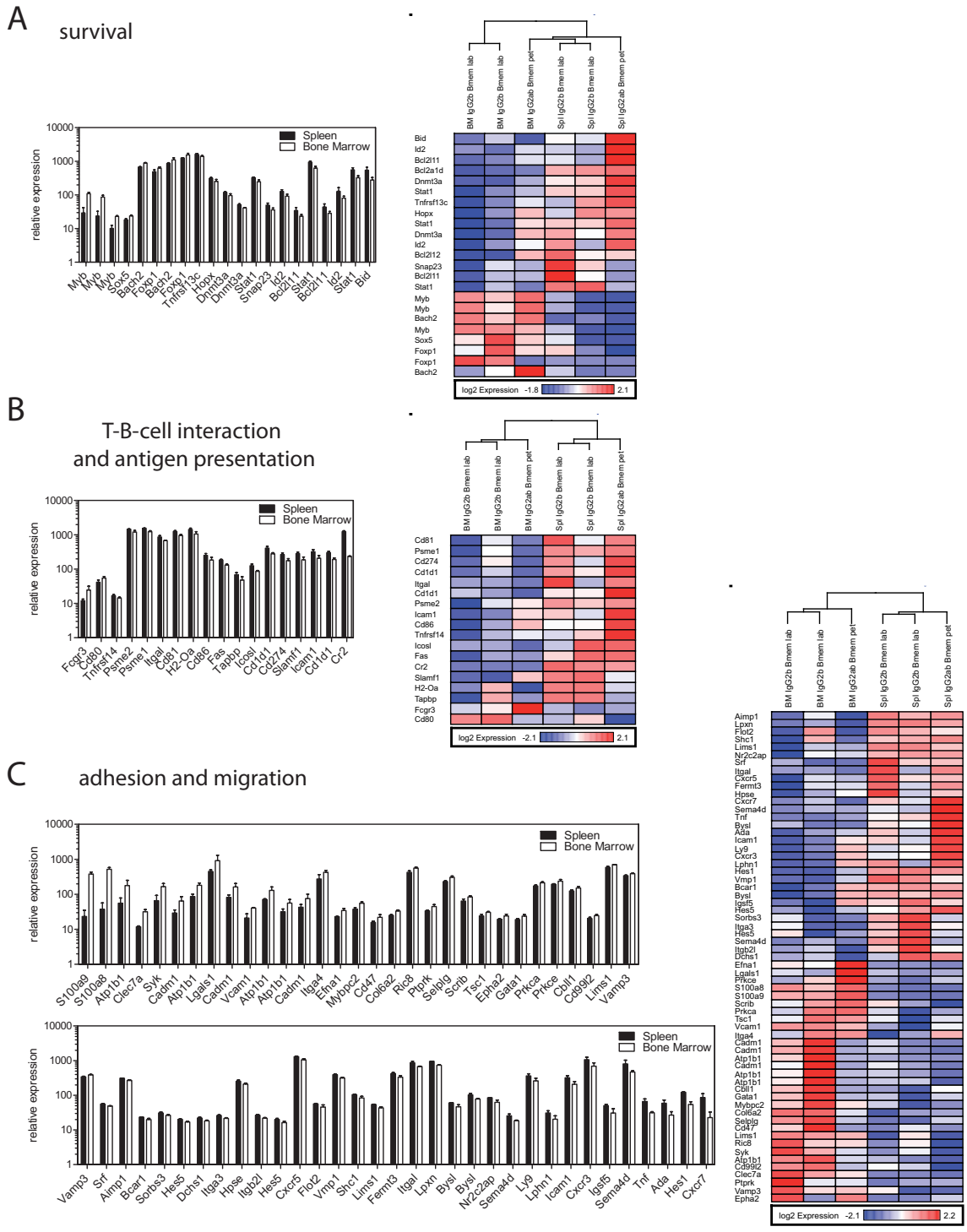

Figure 3.7: Differential gene expression between spleen and bone marrow IgG_2 memory B lymphocytes. Heatmaps of significantly differentially expressed genes in spleen (Spl) and bone marrow (BM) memory B cells (Bmem). Red denotes relative up-, blue down-regulation of gene expression. In the barplots mean expression values are drawn for all probesets represented in the heatmaps; genes appear in order of fold-change from lowest (up in bone marrow) to highest (down-regulated in bone marrow). Cells isolated from pooled C57BL/6 SPF (lab) or pet mouse spleen or bone marrow. For some genes more than one probesets indicated significant differences; in these cases, values for all of these were included. **A:** Differentially expressed genes with known function in cell survival of lymphocytes. **B:** Differentially expressed genes with known function in cellular adhesion. **C:** Differentially expressed genes with known function in T-B-cell-interaction or antigen presentation. The bar graphs show up- (top) or down-regulated genes (bottom) in the bone marrow.

the transcripts consiedered than that between C57BL/6 and pet shop mice. However, the variance between cells of the same subset between laboratory and pet mice did remain considerable.

In addition to a number of transcripts which can be attributed to contaminating cells[9], three sets of genes associated with known cellular functions were particularly prominently present among the differentially expressed genes:

- cell survival and the regulation of apoptosis,
- cellular adhesion and migration, and
- T-B-cell interaction and antigen presentation (see fig. 3.7).

Genes with a role in apoptosis or the regulation of cell survival which were up-regulated in bone marrow memory B cells compared to the spleen were most prominently the proto-oncogene *Myb*, the transcription factors *Bach2* and *Foxp1*, whereas the pro-apoptotic Bcl2-familiy genes *Bid* and *Bim* (*Bcl2l11*) and the transcription factor *Id2* were down-regulated (fig. 3.7A). Notably, differentially expressed genes with a role in the interaction with T cells and in antigen presentation were, with the exception of *Cd80*[10], less expressed in the bone marrow, e.g. *Cd81*, *Cd86*, *Fas*, *Icosl*, *Cd274*, *Slamf1*, *Icam1*, *Cd1d1*, and *Cr2*. For migration and adhesion-related genes, the situation was more balanced: a number of genes were differentially regulated in memory B cells in either organ: *Itga4* and integrin-associated protein gene C*d47*, *Cadm1*, *Vcam1*, *Selplg*, *Cd99l2* were up- and *Itga3*, *Itgal*, *Icam1* and the chemokine receptor genes *Cxcr3*, *Cxcr5* and *Cxcr7* down-regulated in bone marrow compared to spleen memory B cells.

Differential Gene Expression in Spleen Naïve and Memory B Lymphocytes

The comparison of naïve and memory B cells, both isolated from spleen, was concentrated on two areas of interest since extensive studies comparing the transcriptional profiles of splenic memory and naïve B cells have been carried out elsewhere before [Bhattacharya et al., 2007, Luckey et al., 2006, Tomayko et al., 2008]. Here, the analysis was therefore concentrated on two areas of particular interest for further comparison of naïve B cells to spleen and bone marrow memory B cells with respect to migration and cellular adhesion as well as molecules involved the interaction between T and B cells and in antigen presentation (see fig. 3.8). In splenic memory B cells the transcription factor genes *Foxo1*, *Foxp1*, *Bach2*, *Satb1*, *Maf*, *Runx1* and the anti-apoptotic genes *Pten*, *Xiap*, but also the pro-apoptotic *Bcl2l11* were down-regulated compared to naïve B cells. Expression of *Runx2*, *Myb*, *Batf*, *Tcf7*, *Id2*, *KLf2*, *Tnfrsf13b* (*Taci*), and *Ahr* were increased in memory compared to naïve cells. The comparison of transcription of genes involved in the interaction with T cells and in antigen presentation shows that, except for *Cd40* the following genes were up-regulated in spleen memory compared to naïve B cells[11]: *Cd80*, *Cd86*, *Fas*, *Pvr*, PD-ligand 1 and 2 genes, *Fcgr2b*, *Fcerg1*, *Itgal*, *Icam1*, *Cd59b* and *Cd81*.

For the comparison between spleen naïve and memory B cells, differential expression was detected for a total of 3219 probesets (fig. S7), 244 genes were found more than two-fold up- and 107 down-regulated (limma $p < 0.05$, tables S3 and S4, p. 99ff.). Lists of genes which were differentially

[9]The cellular composition of the spleen and bone marrow – the organs from which memory B cells were isolated – is very different, especially when considering the B cell compartment: The bone marrow is the site of B cell development, whereas naïve B cells are held ready for mounting immune responses in the spleen where also B cell activation, isotype-switch, somatic hypermutation and the generation of antibody-secreting cells take place. Hence, genes were prominently found, which are very likely expressed in recently activated B cells in the spleen (e.g. *Prdm1*) or related to B cell development in the bone marrow (e.g. *Cd93*, *Rag1*, *Vpreb2*)

[10]The *Fcgr3* gene is expressed in myeloid cells, detection is likely due to contaminating cells.

[11]This is true disregarding the expression of the Igμ constant region gene *Ighm*, which was naturally down-regulated in isotype-switched memory B cells.

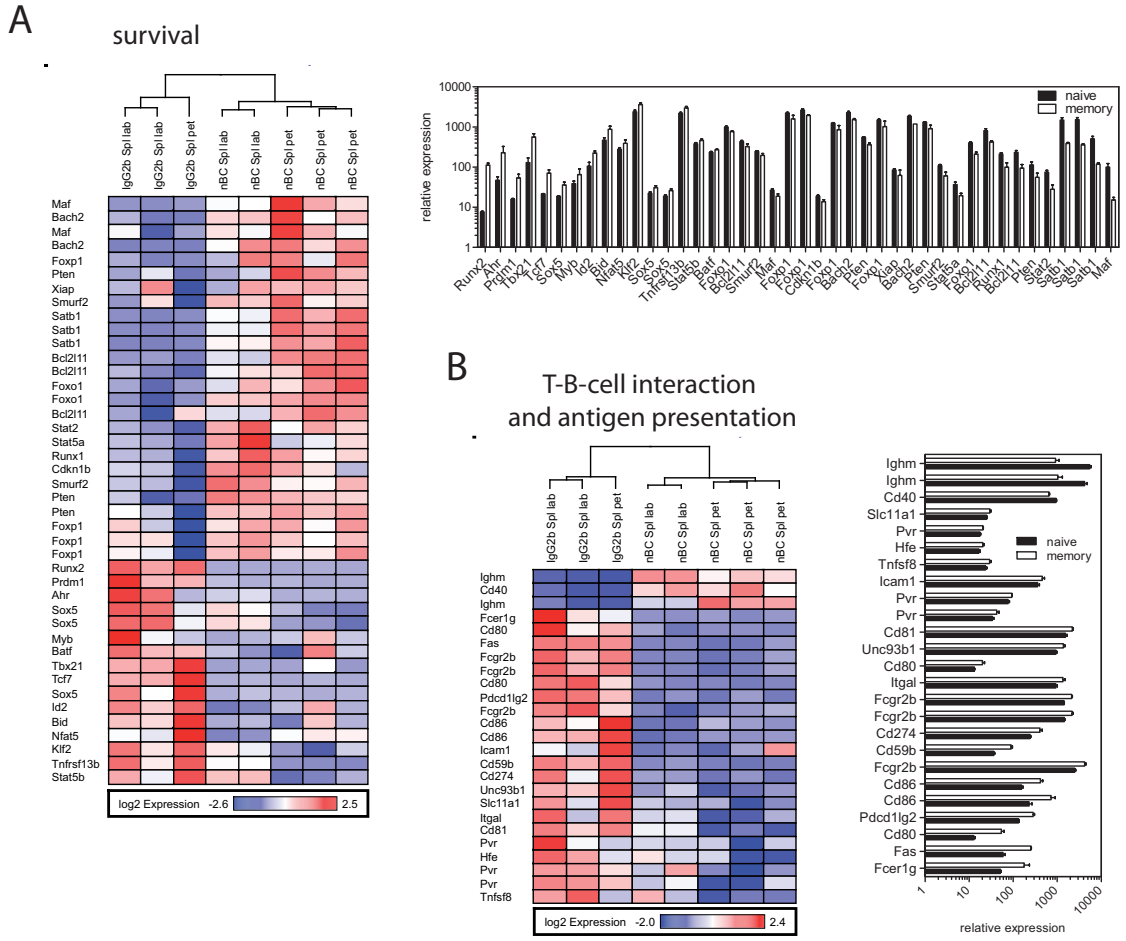

Figure 3.8: Differential gene expression between spleen naïve and IgG_2 memory B lymphocytes. Heatmap of significantly expressed genes in spleen (Spl) naïve (nBC) and memory B cells (Bmem). Red denotes relative up-, blue down-regulation of gene expression. In the barplots mean expression values are drawn for all probesets represented in the heatmaps; genes appear in order of fold-change from lowest (up in memory cells) to highest (down-regulated in memory cells). Cells isolated from pooled C57BL/6 SPF (lab) or pet mouse splenocytess. For some genes more than one probesets indicated significant differences; in these cases, values for all of these were included. **A:** Differentially expressed genes with known function in cell survival ofs lymphocytes. **B:** Differentially expressed genes with known function in T-B-cell-interaction or antigen presentation.

transcribed and more than two-fold up- or down-regulated in IgG_2 memory B cells are shown in tables S1 and S2 (p. 97f.). Merely 40 genes were found to be up-regulated and 39 were down-regulated with fold-change values more than two and limma $p < 0.05$ in bone marrow compared to spleen memory B cells. In general, the number of genes which were highly differentially expressed between spleen and bone marrow memory B lymphocytes was limited.

Similar Memory B Cell Transcription Profiles with Few but Distinct Differences The transcriptional profiles between IgG_2 memory B cells isolated from spleen or bone marrow were similar and the number of genes with highly differential expression in both subsets very limited. The differences between naïve and memory B cells from the spleen were more pronounced. In the spleen, naïve B cells showed the hallmark of resting cells and memory B cell profiles resemble a somewhat more activated phenotype. Memory B cells from the bone marrow in turn, showed less signs of activation than their splenic counterparts and had lower expression of genes encoding molecules involved in interaction between B and T cells. Memory B cells from spleen and bone marrow did differ in the expression of migration related genes, such as adhesion molecules and integrins.

3.2.2 Comparative Surface Marker Expression in Naïve and Spleen and Memory B Cells

Beyond transcript level analyses, memory B cells from spleen and bone marrow as well as splenic naïve B cells were subjected to quantitative comparative analysis of surface marker expression by flow cytometry. Spleen and bone marrow cells from aged ex-breeder C57BL/6J mice were isolated and pooled from 25 mice, and B cells enriched by magnetic labeling of CD19-expressing cells. Isolated B cells were surface stained with a cocktail of antibodies for the identification of naïve and IgG_{2b} memory B cells and in a large-scale assay screened for the expression of more than 200 surface markers by staining with PE-conjugated antibodies (LEGENDScreen Mouse Cell Screen (PE) Kit, BioLegends; see chapter 2.2.2). Gating was performed analogously to the strategy demonstrated in fig. 3.1C.

Comparison of Surface Marker Expression by Similarity Index

For the unbiased automated comparison of surface marker expression a composite index for the evaluation of the degree of similarity between two sets of frequency distributions of PE fluorescence signals was devised. The index allows ranking the distribution of different markers between two populations of cells based on the degree of similarity taking into account not only mean fluorescence values or the range of a frequency distribution. For detailed composition of the similarity index[12], refer to chapter 2.4.2 (p. 16). The similarity index ranges from 0 to 1, where 1 denotes a high degree of similarity and a score converging 0 indicates high dissimilarity. In figure 3.9 similarity indices for comparative surface marker expressions between spleen naïve and IgG_{2b} memory B cells (fig. 3.9A) and spleen and bone marrow IgG_{2b} memory B cells (fig. 3.9A) are shown ordered from low similarity index, for a high degree of difference, to higher similarity. Shown are the 40 comparisons with the lowest similarity scores, where the dashed lines in the graphs indicate the similarity index below which the distribution of a given marker in two populations was considered to be different[13].

[12] Conventionally, the comparison of expression levels of a given marker between different cell populations is done by visual comparison of histograms or two-dimensional dotplots or the comparison of mean fluorescence intensities for the whole population of cells. A calculated score has the advantage of producing numerical values as output which can be used e.g. for ranking differences in similarities of different markers based on scrutable grounds.

[13] The cut-off was defined by the similarity index computed for markers which by definition, e.g. anti-hamster IgG isotype control, or gating, e.g. AA4.1 (CD93), CD138, should not be present nor, therefore, be differentially expressed by naïve or memory B cells.

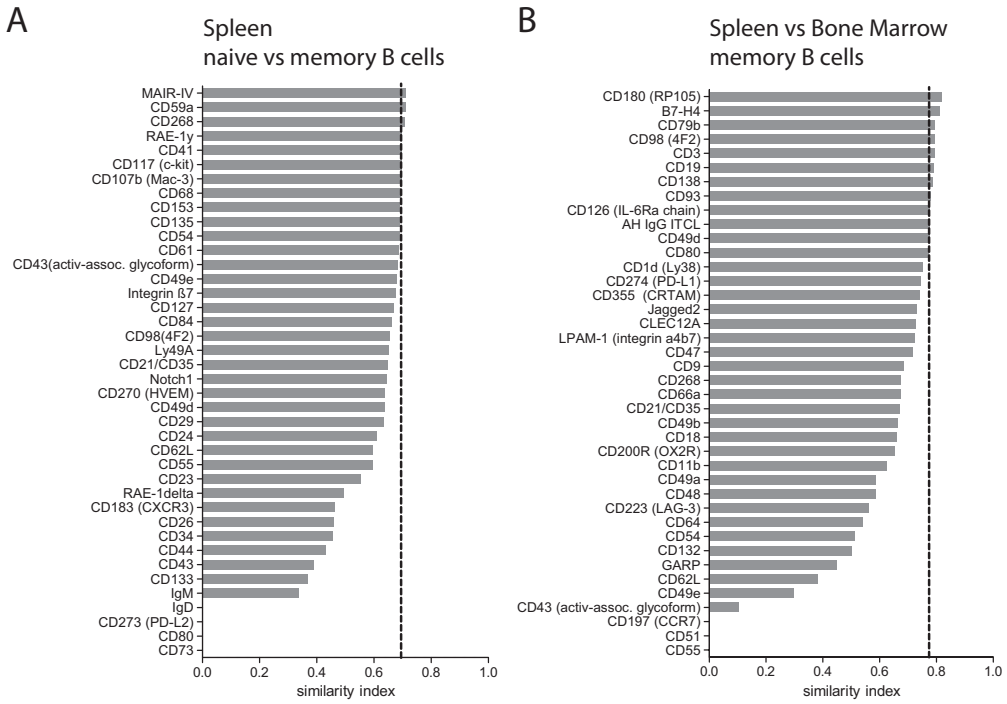

Figure 3.9: Differential surface marker expression in splenic naive and IgG_{2b} memory B cells from spleen and bone marrow.
A: Similarity index for 40 lowest-ranked flow cytometric comparisons for surface marker expression by spleen naïve and IgG_{2b} memory B cells. Similarity index of 0 denotes low similarity in comparative marker expression. **B:** Similarity index for 40 lowest-ranked flow cytometric comparisons for surface marker expression by spleen and bone marrow IgG_{2b} memory B cells. The dashed line indicates the similarity index below which the expression of a given marker is considered not to be equally distributed in the compared cell populations; the value is dependent on the respective comparison.

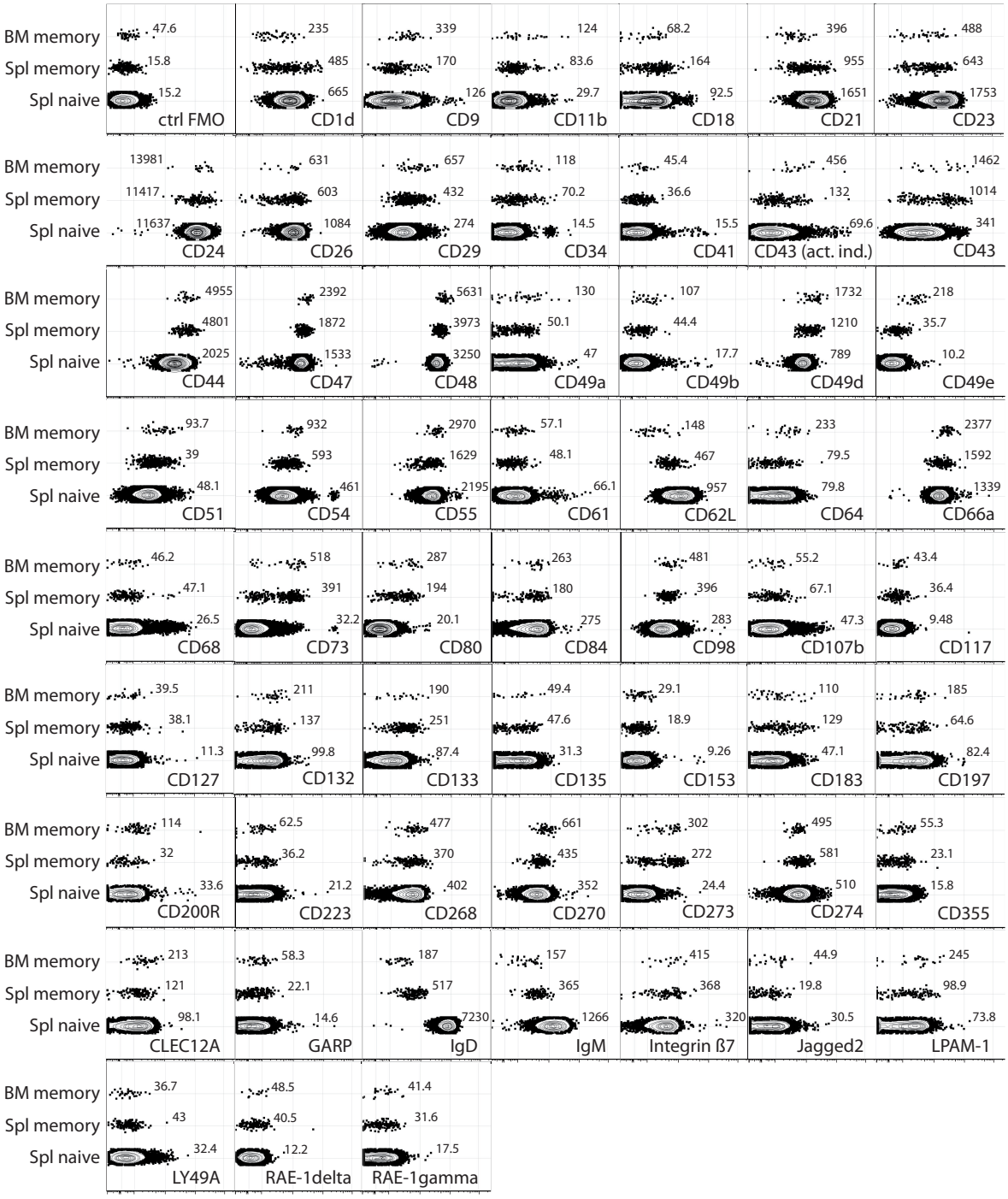

Figure 3.10: Surface expression of markers with low simlarity index for spleen naïve against IgG_{2b} memory B cells or spleen against bone marrow IgG_{2b} memory B cells.
Quantification of surface marker expression by flow cytometry. Staining with PE-conjugated antibodies for given marker was performed in spleen and bone marrow cells enriched for expression of CD19 by magnetic labeling. Gated for $PI^{-}CD19^{+}CD38^{+}CD138^{-}GL7^{-}$ small lymphocytes either $IgG_{2b}{}^{hi}IgD^{lo}IgM^{lo}$ for spleen and bone marrow memory or $IgG_{2b}{}^{lo}IgD^{hi}IgM^{hi}$ for splenic naïve follicular B cells. FMO: background control without PE-antibody added (upper left panel). Numbers on plots indicate geometric mean of PE signal area.

For spleen and bone marrow memory B cells, 33 comparisons with similarity indices below the cut-off were found and 38 for spleen naïve and memory B cells. Adding both sets of markers gives a total of 58 surface markers for which the index points to differential expression in one of either comparisons. In fig. 3.10 frequency distributions for the PE signals are displayed for all three populations: spleen naïve and IgG_{2b} memory B cells and bone marrow memory IgG_{2b} B cells. Mean fluorescence intensities for each population are indicated on the plots. A number of the markers with high dissimilarity in at least one of the comparisons was not detected with signal intensities notably different from FMO controls. These were therefore not included for further consideration. Surfaces markers which consistently displayed a high degree of dissimilarity for at least one comparison are summarized in tables 3.2 for the comparison of spleen and bone marrow resident and 3.1 for naïve and memory B cells from the spleen with the ratio of fluorescence intensities and accompanying gene expression microarray data.

Extent and Distribution of Differential Surface Marker Expression in Spleen and Bone Marrow B Cells

As was to be expected, in the comparison between naïve and memory B cells, IgM and IgD were strongly down-regulated and staining intensity reduced in IgG_{2b}-switched memory cells. Markably differentially expressed were also CD73, CD80, and CD273 which have been described as molecules with a role in B cell memory before [Tomayko et al., 2010, Zuccarino-Catania et al., 2014]. In addition, sialoporin (CD43), CD44, CXCR3 (CD183) were found strongly up- and CD62L, CD23, and CD21 down-regulated in memory B cells.

When comparing IgG_{2b} memory B cells isolated from the spleen with those from bone marrow, the extent of heterogeneity is smaller than between naïve and spleen memory B cells as it was the case for gene expression analyses. There were, however, some notable differences in the expression of integrins, e.g. integrin α V (CD51), integrin α6 (CD49e), integrin α 1 (CD49a), CD18 antigen (CD18), acCD49b and other migration-related molecules such as ICAM-1 (CD54) and CCR7 (CD197) and to a high extent the activation-induced glycosliation isoform of CD43 and CD62L. Moreover, CD21, SLAMF-2 (CD48), CD274 and CD80 were found to be differentially regulated between the two memory B cell populations in spleen and bone marrow.

Altogether, some extent of heterogeneity in the expression of surface molecules within the memory B cell subset could be identified by flow cytometry. Notably, a number of markers which were found at different levels in the comparison between naïve and memory cells in the spleen, were

- further up-regulated in bone memory B cells: integrin β1 (CD29), the activation-induced glycoform of CD43, CD47 antigen, integrin-associated signal transducer (CD47), integrin α 4 (CD49d), CD49e, CD73, CD80, 4F2 (CD98), integrin β7, whilst other markers

- which were found down-regulated in spleen memory compared to naïve B cells were even further down-regulated in the bone marrow: CD1d antigen (CD1d), CD21, CD23, CD62L.

Some surface molecules were detected to be expressed differentially from naïve B cells on memory B cells, in both, spleen and bone marrow:

- with higher expression levels: Tspan29 (CD9), CD43, CD73, HVEM (CD270), CD273 or

- with lower expression for Dpp-4 (CD26).

[14] CD43 isoform which in T cells glycosilated upon activation, the modification is post-translational [Alcaide et al., 2007].

[15] Surface L-selectin levels are not only regulated on transcription level, but also by ectodomain shedding [Smalley and Ley, 2005].

Table 3.1: Differential surface marker expression between spleen naïve (nBC) and IgG_{2b} memory B cells (Bmem).
Included are markers found to be differentially expressed by similarity index on a level above FMO controls. Up- or down-regulation is indicated by ratio of geometric mean fluorescence intensities (MFI) by flow cytometry or foldchange values for microarray; values > 1 indicate higher expression in memory B cells.

Marker	Gene name	Similarity Index	MFI ratio (Bmem / nBC)	Microarray foldchange (Bmem vs nBC)
CD80	*Cd80*	0	9.65	2.34
CD73	*Nt5e*	0	12.14	5.35
CD273	*Pdcd1lg2*	0	11.15	2.11
IgD	NA	0	0.07	NA
IgM	*Ighm*	0.339	0.29	0.289
CD133	*Prom1*	0.371	2.87	ns
CD43	*Spn*	0.39	2.97	4.1
CD44	*Cd44*	0.432	2.37	2.96
CD34	*Cd34*	0.458	0.48	ns
CD26	*Dpp4*	0.459	0.56	ns
CD183	*Cxcr3*	0.465	2.74	4.30
CD23	*Fcer2a*	0.554	0.37	0.45
	Fcer1g			3.47
CD55	*Cd55*	0.598	0.74	0.55
CD62L	*Sell*[14]	0.598	0.49	0.54
CD29	*Itgb1*	0.636	1.58	4.15
CD49d	*Itga4*	0.638	1.53	1.80
CD270	*Tnfrsf14*	0.638	1.24	ns
CD21/35	*Cr2*	0.648	0.58	ns
CD98	*Slc3a2*	0.655	1.40	ns
Integrin β7	*Itgb7*	0.678	1.15	ns
CD49e	*Itga5*	0.68	3.50	ns
CD43 (activation-induced)	NA[15]	0.685	2.97	NA
CD268	*Tnfrsf13c*	0.708	0.92	ns

Table 3.2: Differential surface marker expression between spleen (Spl) and bone marrow (BM) IgG_{2b} memory B cells.
Included are markers found to be differentially expressed by similarity index on a level above FMO controls. Up- or down-regulation is indicated by ratio of geometric mean fluorescence intensities (MFI) by flow cytometry or foldchange values for microarray; values > 1 indicate higher expression in bone marrow.

Marker	Gene name	Similarity Index	MFI ratio (BM / Spl)	Microarray foldchange (BM vs Spl)
CD51	*Itgav*	0	2.40	ns
CD55	*Cd55*	0	1.82	ns
CD197	*Ccr7*	0	2.87	ns
CD43 (activation-induced)	NA[14]	0,103	3.45	NA
CD49e	*Itga5*	0.299	6.11	ns
CD62L	*Sell*[15]	0.382	0.32	ns
CD132	*Il2rg*	0.501	1.54	ns
CD54	*Icam1*	0.514	1.57	0.65
CD64	*Fcgr1*	0.541	2.93	ns
CD48	*Cd48*	0.585	1.41	ns
CD49a	*Itga1*	0.586	2.59	ns
CD200R	*Cd200r1*	0.652	3.56	ns
CD18	*Itgb2*	0.661	0.42	ns
CD49b	*Itga2*	0.664	2.41	ns
CD21/35	*Cr2*	0.67	0.41	0.19
CD268	*Tnfrsf13c*	0.674	1.29	0.86
CD66a	*Caecam1*	0.674	1.49	ns
CD9	*Cd9*	0.687	1.99	ns
CD47	*Cd47*	0.717	1.28	1.39
LPAM-1	*Itga4*	0.724	2.48	1.55
	Itgb7			ns
CLEC12A	*Clec12a*	0.727	1.76	NA
CD274	*Cd274*	0.746	0.85	0.67
CD1d	*Cd1d1*	0.753	0.4	0.64
CD80	*Cd80*	0.774	1.48	1.32
CD49d	*Itga4*	0.777	1.43	1.55

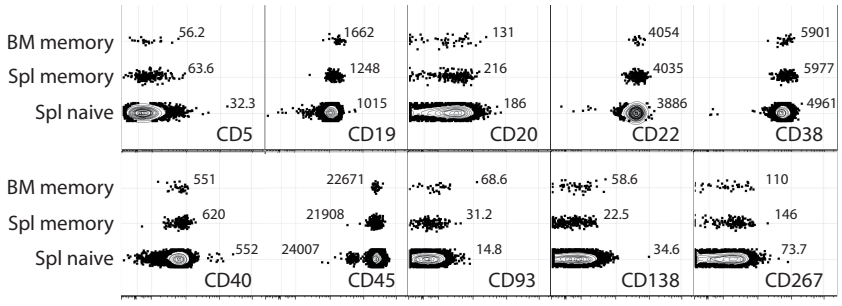

Figure 3.11: Surface expression of selected markers with high simlarity index for spleen naïve against IgG_{2b} memory B cells or spleen against bone marrow IgG_{2b} memory B cells.
Quantification of surface marker expression by flow cytometry. Staining with PE-conjugated antibodies for given marker was performed in spleen and bone marrow cells enriched for expression of CD19 by magnetic labeling. Gated for $PI^{-}CD19^{+}CD38^{+}CD138^{-}GL7^{-}$ small lymphocytes either $IgG_{2b}{}^{hi}IgD^{lo}IgM^{lo}$ for spleen and bone marrow memory or $IgG_{2b}{}^{lo}IgD^{hi}IgM^{hi}$ for splenic naïve follicular B cells. Numbers on plots indicate geometric mean of PE signal area.

The expression of CD48, CD49a, integrin α 2 (CD49b), CD51, CD54, Fcγ receptor I (CD64), CAECAM-1 (CD66a), IL-2 receptor γ chain (CD132), CD197, OX2R (CD200R), c-type lectin 12a (CLEC12A), $\alpha 4\beta 7$-integrin (LPAM-1) was only up-regulated in bone marrow but not in spleen IgG_{2b} memory B cells. GPI-DAF (CD55) was found down-regulated in spleen and up in bone marrow memory B cells compared to naïve B cells.

High Degree of Uniformity in Surface Marker Expression in B Cell Populations

As on transcriptional level, for most markers – irrespective whether they were expressed in detectable quantities on the cell surface or not – no significant differences between memory and naïve subsets for over 200 of the markers which were included in the assay. For those molecules the similarity indices converge to 1. In particular, the plasmablast and plasma cell marker CD138, B1 cell marker Ly-1 (CD5), or pro- and pre-B cell marker CD93 were not expressed. For B cell specific or B cell function-associated e.g. CD19, Ly-44 (CD20), CD22 antigen (CD22), CD38, B-220 (CD45R), TACI (CD267), MHCII or the leukocyte marker CD45 were not found to be present in different levels on the surface of either memory IgG_{2b} B cells or naïve B cells (some of which are exemplarily shown in fig. 3.11).

Comparative Surface Marker Expression in IgA, IgG_1, IgG_{2a} and IgG_{2b} Memory B Cells

For a limited range of surface molecules which had been found to be differentially expressed between either naïve and IgG_{2b} memory B cells in the spleen or between IgG_{2b} memory B cells in spleen and bone marrow, expression levels were measured for comparison between memory B cells of different isotypes and IgG subclasses. CD21, CD80, CD86, CD273, CD274 were not found to be substantially differentially expressed by IgA, IgG_1, IgG_{2b} cells. CD80 and CD274 were found elevated in bone marrow IgG_{2a} memory B cells as compared to all other class-switched memory B cells. CD21 was found at lower levels in spleen IgA and IgG_{2b} memory B cells than in IgG_1 and IgG_{2a} memory B cells or naïve B cells. These cases, in which differences in mean fluorescence intensities were detected, were not so much due to absolute differences in expression levels: rather there were two populations present with higher or lower expression of the respective marker. Differences in fluorescence intensity were based in all cases in shifts in frequency distribution, e.g. loss of the higher or lower expressing population for a marker (fig. 3.12B). It should be noted, that in the experiment presented here, CD86 was also found to be up-regulated in memory B cells as compared to naïve B cells, which had not been detected in the screening experiment.

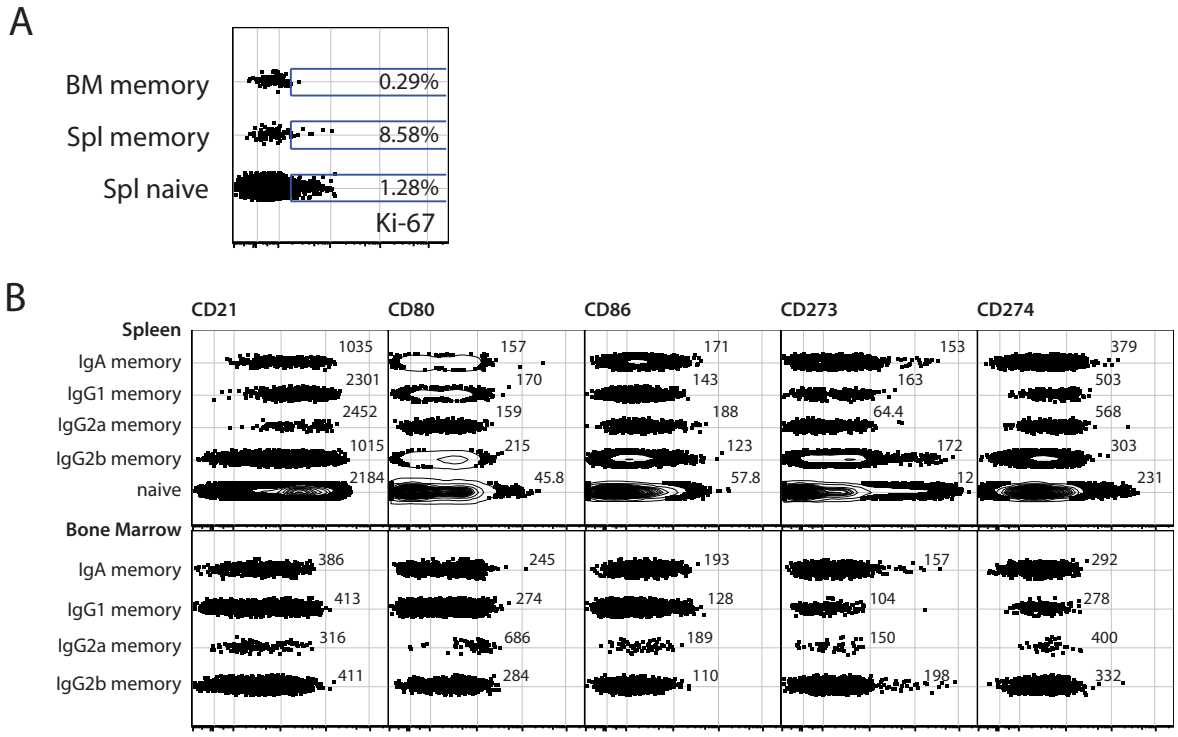

Figure 3.12: Bone marrow IgG_{2b} memory B cells are resting in G_0 phase of cell cycle. No differential surface expression of CD21, CD86, CD273 in IgA, IgG_1, IgG_{2a}, and IgG_{2b} memory B cells localized within the same organ, spleen or bone marrow.
A: Quantification of Ki-67 expression of naïve and IgG_{2b} memory B cells by flow cytometry. Intracellular staining of Ki-67 in spleen and bone marrow cells enriched for expression of CD19 by magnetic labeling. Gated for $CD19^+CD38^+CD138^-GL7^-$ small lymphocytes either $IgG_{2b}{}^{hi}IgD^{lo}IgM^{lo}$ for spleen and bone marrow memory or $IgG_{2b}{}^{lo}IgD^{hi}IgM^{hi}$ for splenic naïve follicular B cells. **B:** Quantification of surface marker expression by flow cytometry. Staining with antibodies for given marker was performed in spleen and bone marrow cells enriched for expression of CD19 by magnetic labeling. Gated for $PI^-CD19^+CD38^+CD138^-GL7^-$ small lymphocytes either $IgG_{2b}{}^{hi}IgD^{lo}IgM^{lo}$ for spleen and bone marrow memory or $IgG_{2b}{}^{lo}IgD^{hi}IgM^{hi}$ for splenic naïve follicular B cells. Numbers on plots indicate geometric mean signal area for the respective marker.

Differential Ki-67 Expression between Spleen and Bone Marrow Memory B cells

The bone marrow has been described as the organ in which plasma cells, memory T helper cells, and, most recently, cytotoxic T cells survive as long-lived cells independent of proliferation [Manz et al., 1997, 1998, Sercan Alp et al., 2015, Tokoyoda et al., 2009]. To test, whether memory B cells in spleen or bone marrow are proliferating, nuclear staining for the Ki-67 antigen was performed which is expressed in all phases of the cell cycle, except G_0 [Gerdes et al., 1983, 1984]. B cells from spleen and bone marrow were isolated by magnetic labeling for CD19 expression. Equal numbers of purified B cells were surface stained, fixed and intranuclear staining for mouse Ki-67 performed. Whereas Ki-67 was detectable in 5-10% of spleen IgG_{2b} memory B cells and 1-3% of naïve B cells, the fraction of Ki-67 expressing bone marrow IgG_{2b} memory B cells was generally below 0.5%. Exemplary flow cytometry data are shown in fig. 3.12A.

Bone Marrow Memory B Cells are Resting and Differ from Spleen Memory B Cells in the Expression of a Limited Set of Surface Markers Memory B cells isolated from the bone marrow in contrast to those from the spleen were negative for Ki-67, which is expressed in all phases of the cell cycle exceptG_0. With respect to surface marker expression differences between different subsets of memory B cells, were not primarily dependent on isotype but more on localization in spleen or bone marrow. Memory B cells showed strong up-regulation of CD80, CD73, and PD-Ligand 2 compared to naïve cells, whereas CD23, CD26 and L-selectin were down-regulated from high expression in naïve B cells to lower levels in memory B cells. In memory B cells from the bone marrow CR2 and L-selectin were further down-regulated compared to splenic memory cells as well as molecules which are involved in T helper cell interaction as ICAM-1, PD-Ligand 1. A number of molecules involved in migration and cellular adhesion such as the activation-associated glycosilation variant of CD43 as well as several integrins were also found to be present on the surface of spleen and bone marrow IgG_{2b} memory B cells in different levels.

3.2.3 Differences in B Cell Receptor Repertoires in Spleen and Bone Marrow Memory

Transcriptional profiles and the expression of cell surface markers show that, even though the above sections have concentrated on differences between spleen and bone marrow memory B cells, there is much conformity between both populations with limited but distinct differences. In respect to memory B cell functionality in the context of providing immunity against pathogens, B cell receptor sequence and specificity arguably is the decisive aspect, however. The determinants of antibody-antigen interactions have not yet been sufficiently understood to allow prediction of B cell receptor specificity solely based on antibody sequence information [Sela-Culang et al., 2013, 2014]. Therefore, conclusions about the antigens towards which the B cell responses were directed is not possible. Still, antibody sequence data can provide insights on the extent and history of B cell reponses by the assessment of clonal relationship between spleen and memory B cells and, thus, whether both organs harbor memory cells which were likely raised against the same antigens in the same immune responses.

To generate information about the receptor repertoire of the memory B cell subsets in spleen and bone marrow, IgG_{2b} memory B cells from spleen and bone marrow were isolated from three mice obtained at a pet shop. The animals were kept for 8 months after purchase under non-SPF conditions. The exact age of the animals was not known, but was at minimum 11 months. Bone marrow cells and splenocytes were magnetically enriched for surface expression of CD19. Sorting by FACS and control of purity was determined as shown in fig. s5 (p. 94). The number of cells isolated from both organs for individual mice is listed in table 3.3. It should be noted, that cells could be isolated from

Table 3.3: Numbers of spleen naïve follicular B cells and spleen and bone marrow IgG_{2b} memory B cells sorted by FACS from 3 individual pet mice.

Mouse	Organ	Cell population sorted	Cell no sorted
1	Bone marrow	IgG_{2b}^+ memory	19,000
	Spleen	IgG_{2b}^+ memory	29,000
		IgM^+IgD^+ naive	817,000
2	Bone marrow	IgG_{2b}^+ memory	48,000
	Spleen	IgG_{2b}^+ memory	70,800
		IgM^+IgD^+ naive	636,000
3	Bone marrow	IgG2b$^+$ memory	20,300
	Spleen	IgG_{2b}^+ memory	27,400
		IgM^+IgD^+	560,000

the whole spleen, whereas bone marrow could only be extracted from well accessible bones – tibiae, femora, pelvic bones, sterna, and humeri. Irrespective of the limitation to the most accessible bones, the number of IgG_{2b} memory cells isolated from the mice was for all three animals about two thirds of that isolated from the spleen.

For the analysis, IgM or IgG heavy chain genes were amplified and sequenced as described in chapter 2.2.6. Clonal analyses were performed on the level of amino acid sequence in the heavy chain CDR3s. The cut-off for the number of reliably detected CDR3 sequences was defined as laid out in chapter 2.4.3. Equal cutoffs were used, so that 837 top-ranked clones by frequency for memory subsets from spleen and bone marrow and the 49,286 most abundant CDR3 sequences from naïve cell samples were included for downstream analysis. The comparative analysis of the clonal repertoire as presented in fig. 3.13A,B and frequency distribution in 3.13C was based on the incidence of fully identical CDR3 amino acid sequences.

Limited Clonal Overlap Between Spleen and Bone Marrow IgG_{2b} B Cells

The Venn diagrams in fig. 3.13A show the extent of clonal overlap by representation of the proportion of identical CDR3 sequences which were present among the 837 top-ranked clones from spleen and bone marrow for each mouse individually. Interestingly, mouse 2 for which most memory B cells could be retrieved from both samples showed the highest number of CDR3s to be present in both spleen and bone marrow memory B cells. 20% of the sequences present among the top-ranked next generation sequencing (NGS) reads were found in both spleen and bone marrow of mouse 2, in mouse 1 the figure was 15.1%, and 11.5% for mouse 3. The comparison of CDR3 sequences between memory subsets of the different mice revealed virtually no overlap with less than 1% of the sequences present in two different animals (3.13B).

Besides overlap, e.g. mere presence of particular CDR3 sequences in one or more populations, information on the frequency and relative distribution to different organs or individuals can also be considered. In fig. S8A (p. 95) rank information is included: among the sequences found in both organs of one mouse, the most frequently present CDR3 sequences in the spleen also ranked highly in the bone marrow. Interestingly, in mouse 2 from which the highest number of memory B cells could be isolated, one single clone made up for almost 15% of the sequencing reads among 837 top-ranking unique CDR3s, thereby also markably reducing Shannon evenness[16] for both memory populations in that particular mouse. In mice 1 and 3 the top-ranking clones in spleen and bone marrow were much

[16]Shannon evenness is a measure of clonal expansion in a population. It is given by equation 2.3, p. 19.

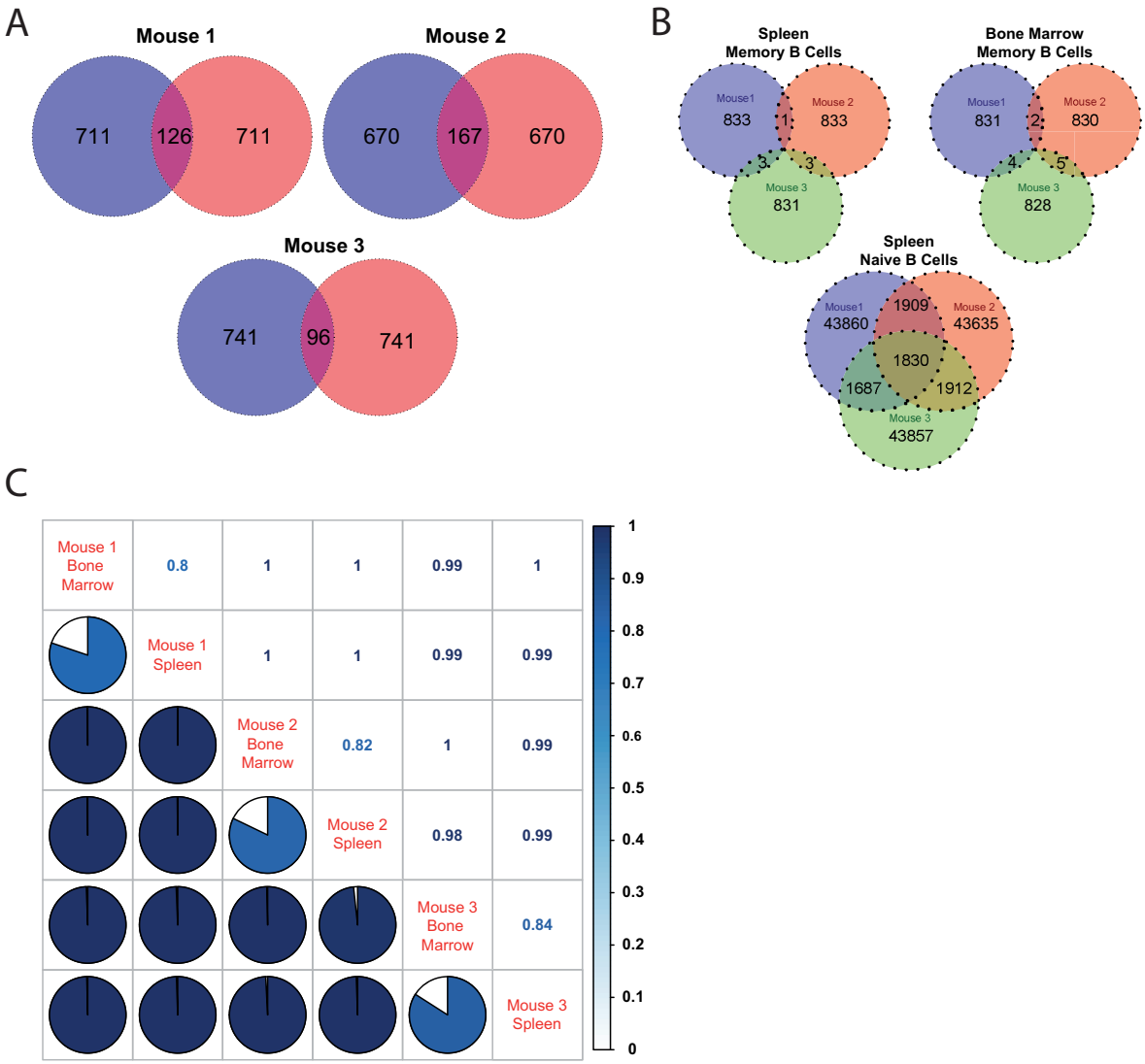

Figure 3.13: The B cell receptor repertoire of IgG_{2b} memory B cells localized in the bone marrow is different from spleen IgG_{2b} memory cells.
A: Overlap of CDR3 sequences between spleen and bone marrow IgG_{2b} memory cells. Overlapping part of the circles represents the proportion of the 837 most abundant CDR3 sequences at amino acid level which were present in spleen as well bone marrow of each mouse. **B:** Overlap of CDR3 sequences between spleen IgG_{2b} memory cells (top left), bone marrow IgG_{2b} memory cells (top right), and spleen naïve B cells of the three individual mice (lower panel). Overlapping part of the circles represents the proportion of the 837 most abundant IgG_{2b} memory CDR3 sequences at amino acid level which were present in spleen or bone marrow of more than one mouse or of the 49286 most abundant CDR3 amino acid sequences for naïve B cells. **C:** Morisita-Horn dissimilarity matrix for comparison the extent of clonal diversity between spleen and memory IgG_{2b} B cells and naïve B cells from all mice. A value of 1 for the index denotes a high degree of dissimilarity between two populations and 0 high similarity.

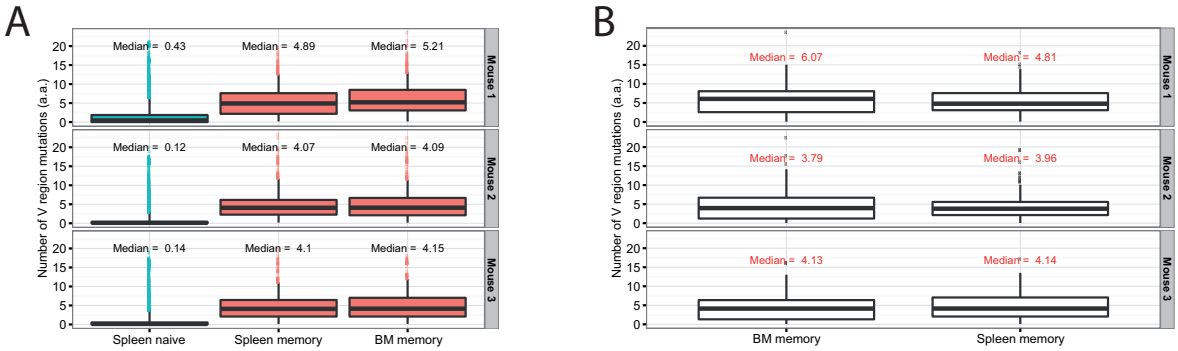

Figure 3.14: The numbers of V region somatic hypermutations do not differ between spleen and bone marrow IgG_{2b} memory B cells.
A: Number of V region somatic hypermutations on the amino acid level across all sequencing reads per cell population for spleen naïve and spleen and bone marrow (BM) IgG_{2b} memory B cells. **B:** Number of V region somatic hypermutations on the amino acid level across all sequencing reads with CDR3 sequences found in both, spleen and bone marrow (BM) IgG_{2b} memory B cells.

less frequent than in mouse 2 with a maximum of about 2.5% (fig. S8B, p. 95). In fig. 3.13C a matrix for comparison of the Morisita-Horn dissimilarity index between all memory populations in the three animals is presented. The index is a measure which integrates presence of a given CDR3 sequence with its frequency (equation 2.4, p. 19). The index shows that in spite of being lower than for the comparison of memory B cell populations among different mice, dissimilarity in the distribution of unique CDR3s to spleen and bone marrow of the same animal was still high and the influence of the presence of a single highly expanded clone in mouse 2 has been compensated in comparison with the other two individuals.

The distribution of differential heavy chain gene usage by the different antibody clones is somewhat less informative, due to the limited overall number of heavy chain genes. However, in fig. S9 (p. 96) heavy chain usage in the different memory populations has been quantified. The analysis demonstrates that the different variable heavy chain genes distributed unevenly to the different organs, suggesting that clonal distribution was also differential.

No Differences in the Extent of Somatic Hypermutation between Spleen and Bone Marrow IgG_{2b} Memory Cells

It has been reported elsewhere that phenotypic differences in memory B cell subsets are reflected by the extent of somatic hypermutation [Anderson et al., 2007]. For B cells isolated from spleen and bone marrow of aged pet mice, somatic hypermutation was determined using IMGT/HighV-QUEST and quantified by the number of amino acid changes compared to the respective heavy chain germline V gene. The comparison of V region somatic hypermutations across all sequencing reads between spleen naïve and spleen and bone marrow IgG_{2b} memory B cell populations, showed consistently higher somatic hypermutation numbers in memory compared to naïve B cells. No significant differences were found between spleen and bone marrow memory B cell populations (fig. 3.14A). When the latter analysis was restricted to those sequences for which CDR3 overlap had been found (see fig. 3.13A) the result was not changed: there were no significant differences in the extent of somatic hypermutation between spleen and bone marrow memory B cells (fig. 3.14B).

Spleen and Bone Marrow Memory B Cells Differ in Clonal Composition Memory B cells in the spleen and in the bone marrow bear B cell receptors with different CDR3 sequences and, thus,

very likely different specificities. They do not differ significantly in the number of hypermutations per heavy chain gene and show moderate differences in heavy chain use.

3.2.4 Localization and Characterization of Human Memory B Cells

The relocalization of memory B cells to the bone marrow in humans has been described before [Paramithiotis and Cooper, 1997] and was estimated to be, although numbers were found in the bone marrow were somehat lower, in the same order of magnitude in the bone marrow as in the spleen [Giesecke et al., 2014]. The ratio between spleen and bone marrow memory B cell numbers was similar to those found for mice in chapter 3.1.1. After extensive characterization of murine memory B cells in different anatomical compartments, some aspects were also examined in human memory B lymphocytes, namely the distribution of isotype-switched cells and the expression of two of the surface molecules found to be differentially expressed between murine spleen and bone marrow. Bone marrow samples were obtained from patients undergoing hip replacement surgery, tonsils originated from scheduled tonsillectomy and peripheral blood was isolated from leukocyte filters from healthy donor blood donations or was provided, in some cases, by the same patients who underwent hip replacement.

Human Bone Marrow Holds Substantial Numbers of Isotype-switched Memory B cells

Memory B cells from human peripheral blood, tonsils and bone marrow, were surface-stained and quantified by flow cytometry. Isotype-switched memory B cells were identified by expression of CD19, CD27 and absence of IgD and IgM as demonstrated in fig. 3.15B. Numbers of IgA- and IgG-switched memory B cells as well as for naïve $CD27^{-}sIgM^{+}IgD^{+}$ B cells were determined. Although the majority of the B cells in all three organs compared were naïve B cells, a substantial proportion of the B cells in either organ were IgG or IgA memory B cells. Taking into account the total organ size, even though the frequency of memory B cells of either isotype was found to be lower in the bone marrow, the absolute number of memory B cells by far exceeded that in tonsils or circulating in the peripheral blood. For IgA a mean total number of 1.1×10^8 ($SD = 7.1 \times 10^7$) was calculated for the bone marrow, with 4.3×10^6 ($SD = 3.5 \times 10^6$) in peripheral blood and 3.6×10^7 ($SD = 1.4 \times 10^7$) in the tonsils. IgG numbers is the bone marrow were 7.8×10^6 ($SD = 9.2 \times 10^7$), 4.5×10^6 ($SD = 5.5 \times 10^6$) in the blood and 2.0×10^7 ($SD = 1.2 \times 10^7$) in the tonils.

Isotype-switched Human Bone Marrow Memory B cells Express lower Levels of CD21 and CD62L than those from Blood or Tonsils

The surface levels of CD21 and CD62L were quantified, results are presented in fig. 3.15C,D. The expression of CD21 was reduced for all memory B cells in the bone marrow compared to tonsils and periperal blood. Consistently expression was reduced between corresponding blood and bone marrow samples from the same patients. CD62L was likewise reduced in blood samples compared to the corresponding bone marrow samples for IgA and IgG memory B cells, whereas expression in tonsils was found at even lower levels than in the bone marrow.

3.3 Efficient Secondary B Cell Resonse to Systemic Antigen Challenge is not Dependent on $CD11c^{hi}$ Dendritic Cells

To clarify whether a secondary antibody response is depedendent on the presence of dendritic cells as professional antigen presenting cells, an experiment was conducted in which dendritic cells were depleted prior to the adminsitration of a boost immunization. The experiment is outlined in 3.16A.

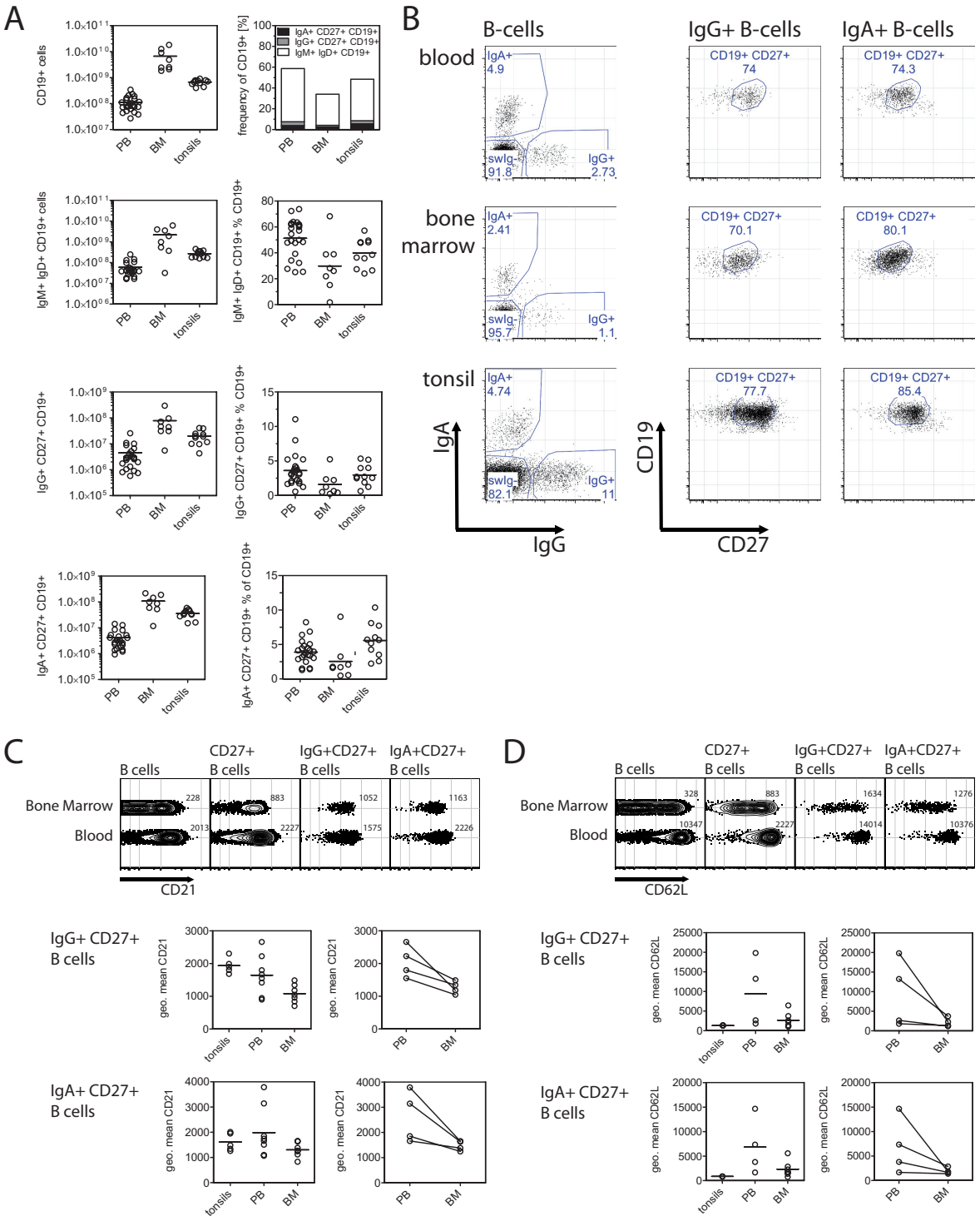

Figure 3.15: Human isotype-switched memory B lymphocytes accumulate in the bone marrow in high numbers and show differential expression of CD21 and CD62L as compared to memory B cells in peripheral blood and tonsils.
A: Quantification of total B cell numbers and absolute numbers (left) and frequencies among B cells (right) of naïve IgM^+IgD^+ and IgA and IgG memory B lymphocytes in human peripheral blood (PB), bone marrow (BM), and palatinal tonsils (tonsils). **B:** Flow cytometric quantification of B cells from human peripheral blood, bone marrow and palatinal tonsils. B cells gated for $PI^-CD19^+CD3^-CD138^-$ small lymphocytes (left panels). Right panels show CD27 expression for IgG^+ and IgA^+ B cells. Numbers on plots indicates percentage of cells in gate. **C:** Surface CD21 expression in human IgG and IgA memory B cells by flow cytometry. Cells gated for $PI^-CD19^+CD3^-CD138^-$ small lymphocytes (B cells), $CD27^+$, and IgG or IgA. *(continued on p. 51)*

Wildtype mice were lethally irradiated and reconstituted with bone marrow from CD11c-DTR transgenic mice, resulting in bone marrow chimeric mice in which all cells descending from bone marrow progenitor cells bear the transgene. The mice were immunized with NP-KLH and LPS. Before boost immunization with NP-KLH without adjuvant, cells expressing the diphtheria toxin receptor, which is not normally present in mice, are susceptible to the toxin and can be efficiently depleted [Jung et al., 2002].

Depletion by diphtheria toxin was highly efficient and depleted 94% of the $CD11c^{hi}GFP^{+}$ cells in the spleen and 95% in the bone marrow at the time of the boost immunization (fig. 3.16C). To initiate a recall response, mice SC primed with NP-KLH and LPS 200 days before, were immunized with NP-KLH without adjuvant either IV or SC at the tailbase and serum samples were taken over 24 days after boost. The secondary NP-specific serum antibody response was measured by ELISA specific for NP-binding IgG (fig. 3.16B). The comparison of serum titers of NP-specific IgG of CD11c-depleted and non-depleted PBS-treated control animals showed that the serum response was not different between both groups. In contrast, a delay in the kinetics of the increase of serum IgG specific for NP was observed in CD11c-depleted mice compared to PBS controls after having been boosted by SC application of the antigen. Thus, the extent to which dendritic cells had been depleted was obviously sufficient to cause observable effects on the secondary B cell response but did, on the other hand, have no effect on the efficacy of the recall antibody response in the systemic IV application of the antigen.

Figure 3.15: *(continued)* Numbers on dotplots indicate CD21 geometric mean signal area. Lower rows show geometric mean for CD21 signal for IgG and IgA memory B cells in tonsils, peripheral blood (PB), and bone marrow (BM) for all samples measured (left) and for corresponding blood and bone marrow samples isolated from the same patient. **D:** Surface CD62L expression in human IgG and IgA memory B cells by flow cytometry. Data representation as in C for CD21 respectively.

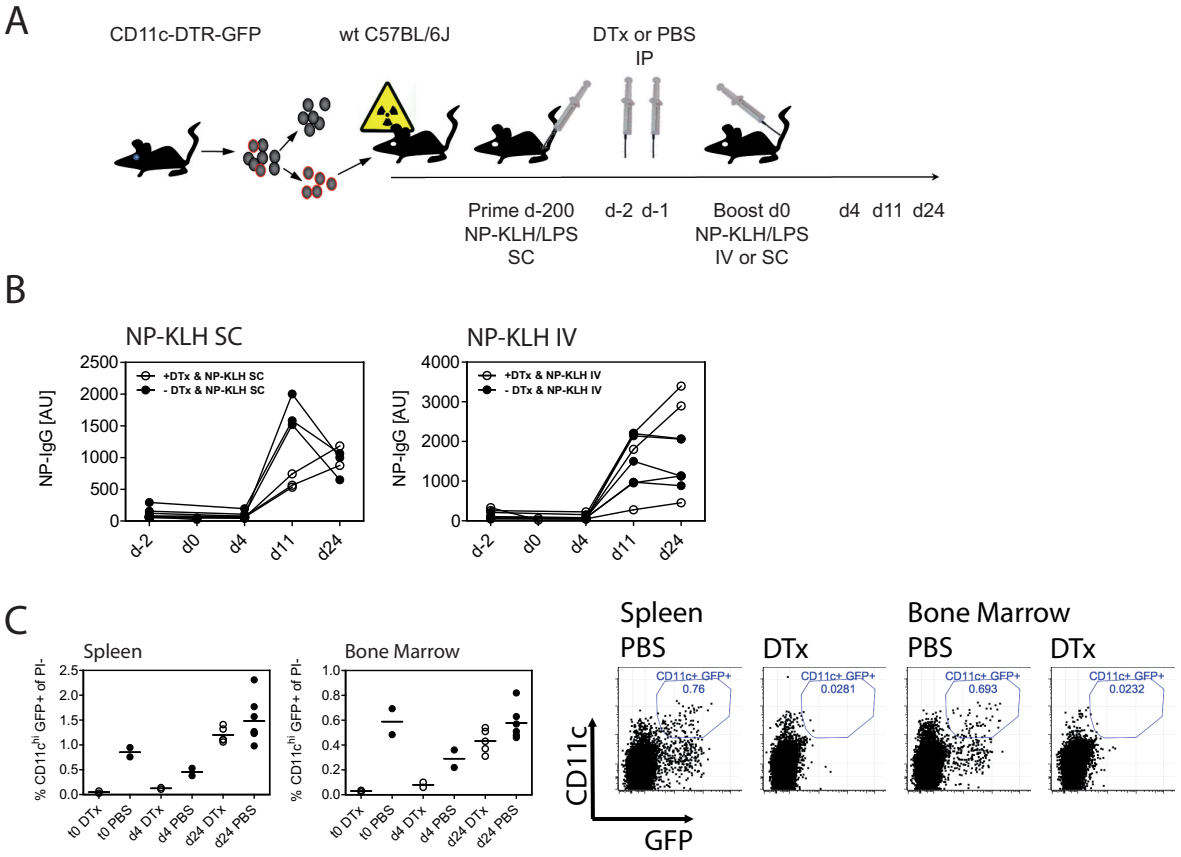

Figure 3.16: Dendritic cells are dispensable for efficient secondary humoral response after systemic boost immunization with NP-KLH.
A: Experimental setup for depletion of CD11c-expressing dendritic cells in secondary immune response. Bone marronw of CD11c-DTR-GFP-transgenic mice was transferred into lethally irradiated wildtype mice which then were after 6 weeks immunized with NP-KLH/LPS SC. 200 days after primary immunization mice were treated with Diphteria toxin or PBS for 2 days before boost with NP-KLH IV or SC. Serum was taken for NP-specific ELISA before boost and on days 4, 11, and 24 after boost. **B:** ELISA for NP-specific IgG before boost and on days 4, 11, and 24 after boost SC (left) or IV (right) with NP-KLH in CD11c-depleted (open symbols) and PBS-treated controls (closed symbols). **C:** Flow cytometric quantification of spleen and bone marrow dendritic cells after depletion of CD11c-expressing cells by Diphteria toxin (DTx) or PBS controls (PBS). Shown are frequencies of $CD11c^+GFP^+$ cells (left) and dotplots of example original flow cytometry data (right).

4 Discussion

4.1 Memory B Cells Localize to Spleen and Bone Marrow

In the mouse, spleen and bone marrow are two organs which harbor great numbers of immune cells. The spleen has long been in the focus of immunologists studying the immune system and in particular the generation and maintenance of immunological memory. The bone marrow was first recognized to be the origin of lymphocytes in mammals [Osmond and Nossal, 1974, Ryser and Vassalli, 1974]. Later it was found to be the place of residence in which survival niches for memory immune cells are provided to long-lived plasma cells [Manz et al., 1997], $CD4^+$ memory T cells [Tokoyoda et al., 2009] and, most recently, $CD8^+$ memory T cells [Sercan Alp et al., 2015].

Bone marrow and spleen are also the organs in mice in which the highest numbers of memory immune cells appear. The most isotype-switched memory B cells can be found in the spleen. But even though memory B cell frequency is lower compared to lymph nodes or spleen, the bone marrow is a large organ and can be host to many memory B cells.

4.1.1 The Bone Marrow is a Residence for Memory B Cells

The bone marrow is not only the site of B cell development and provides survival niches to memory plasma cells, it is also a site in which memory B cells reside. Although in most individual mice isotype-switched memory B cells were found in the spleen in higher numbers, there is strong evidence that the bone marrow population of memory cells is indeed relevant. For example, memory B cells specific for the experimental antigen NP were found in the bone marrow as well as in the spleen after immunization with NP-KLH. Memory B cells generated in a mucosal immune response, as identified by EdU incorporation, could be detected in the bone marrow but not in the spleen nine months after immunization[1]. Bone marrow memory B cells can also be found in humans. The migration of human memory B cells to the bone marrow was first described by Paramithiotis and Cooper [1997] and recently the presence of memory B cell in human bone marrow together with spleen, tonsils, and the blood was quantified [Giesecke et al., 2014].

Another hint to the importance bone marrow B cell memory may have, comes from wild mice. Their spleens were only about one fifth of the spleen size of non-SPF pet mice or laboratory mice from SPF conditions and the lymph nodes contained only one third of the cell numbers found in pet or lab mouse lymph nodes. Possibly the bone marrow can make up for what spleen and lymph nodes lack

[1] The point will be further addressed in chapter 4.1.3.

in capacity for the maintenance of the B cell memory. Moreover, memory B cells seem to accumulate with age in the spleen as well as the bone marrow (see chapter 4.1.2). In the pet mice, aged about one year, which were used for sorting memory B cells for receptor sequencing the number of memory B cells isolated from only the most accessible bones[2] was about two-thirds of the number of cells isolated from the whole spleen, indicating that in these particular mice the bone marrow held more IgG_{2b} memory B cells than the spleen (see chapter 3.2.3).

IgA and IgG_{2b} Memory B Cells Are More Likely to Reside in the Bone Marrow than IgG_1 Memory B Cells

The probability of an IgA or IgG_{2b} memory B cell to be present in the bone marrow was higher than for IgG_1. In single mice, the bone marrow harbors as many IgA or IgG_{2b} memory B cells as the spleen, particularly in wild mice which generally had much smaller spleens. Isotype class-switch is regulated by signals which depend on the environemnt in which the priming of the B cell takes place. The route of antigen application as well as toll-like receptor signaling influences class-switch [Major and Cuff, 1996, Pone et al., 2012b, 2015]. However, the lack in consistency in the distribution of the different isotypes to spleen or bone marrow suggests, that the isotype itself is not the decisive factor. Since the distribution is not equal for different isotypes, it is not purely coincidental. The connection between differential switch regulation and and the site of B cell instruction, i.e. IgA is strongly connected to mucosal immune responses, suggests that also the site of B cell stimulation may play a role in directing the resulting memory B cell to spleen or bone marrow.

IgG_{2b} was in all different immunizations and for mice from different origins the most abundant memory B cell isotype. IgG_{2b} is influenced by toll-like receptor 9 (TLR9) signaling [Ehlers et al., 2006, Pone et al., 2015]. Specific functions of the isotype are mediated by Fc receptors which bind to the isotype-specific constant part of the antibodies [Vidarsson et al., 2014]. Mouse IgG_{2b} is bound by the activating Fcγ receptors III, expressed by all myeloid cells, and IV which is expressed by dendritic cells and macrophages [Bruhns, 2012].

4.1.2 Memory B Cells Accumulate with Age

Using weight as a proxy for mouse age, an association between mouse age and the number of isotype-switched memory B cells was found. However, age alone cannot account for the accumulation of memory B cells in any compartment. Because age is itself only correlated with the most relevant factor, the number and extent of previous immune responses and their nature both of which are unknown. Therefore age, or even weight instead, had to substitute in the analysis. Even so, the data suggest positive relation between age and the number and frequency of memory B cells in spleen and bone marow of the mice.

Memory B cell numbers, and frequencies, can reach values which are very similar to what is seen in humans: in one particular wild mouse a frequency of 4.5% IgG_{2b} memory cells among all $CD19^+$ B cells in the spleen was measured, a value that is well in the range of what is seen for total IgG memory B cells in humans. The same is true for the bone marrow, where in the same animal a frequency of 1.9% IgG_{2b} memory among all B cells was recorded. Again, the example of the pet mice used in sorting memory B cells for receptor sequencing (see chapter 3.2.3) provides a further argument. The mice were at least 11 months old and the bone marrow had accumulated substantial numbers of memory B cells rivaling those in the spleen.

This suggests, for the B cell memory that there is not a low and fixed number of cellularly defined niches unlike as for the plasma cell survival niche in the bone marrow [Höfer et al., 2006, Radbruch

[2]The bones do not contain more than two thirds of a mouse's total bone marrow, see Benner et al. [1981].

et al., 2006]. And strikingly, no stringent assocciation between mouse weight and plasma cell numbers were observed in the same experiments – plasma cell numbers were more or less stable at low frequencies of less than 1% with maximum numbers much lower than those of memory B cells. If there is a requirement for a dedicated survival niche for memory B cells, it is likely to be less retrictive than for plasma cells.

4.1.3 Spleen and Bone Marrow Harbor Different Memory B Cell Subsets

The Signature of Memory B Cells in the Spleen

In the comparison of switched memory B cells and naïve follicular B cells from the spleen distinct differences were found on the transcriptional level as well as in the expression of surface markers detected by flow cytometry. Considering differentially transcribed genes, it could be suspected, that some of them are expressed by contamining activated B cells, e.g. *Aicda, Bid, Itgax*, and *Mki67* expression in the memory cells. However, activated cells were carefully excluded in the cell sort and published data support the finding that (spleen) memory B cells resemble a somewhat activated phenotype. In humans as well as in mice, memory B cells upregulate the expression of activation-associated genes [Bhattacharya et al., 2007, Good et al., 2009, Tomayko et al., 2008], which is interpreted as combining a sustained ability for quick response with long-term persistence of a memory B cell pool in the host [Good et al., 2009]. The pre-activated state may need to be counterbalanced, e.g. through the up-regulation of *Ahr*, encoding for the aryl hydrocarbon receptor with a role in the suppression of Bach2[3] [De Abrew et al., 2011] which is involved in the regulation of plasma cell development. This points to a delicate balance in maintaing a pre-activated state while at the same time inhibiting activation-induced pathways – like the differentiation into antibody secreting cells of pre-activated memory B cells.

In terms of migration-related markers, memory B cells upregulate CD43 which has multiple roles, e.g. the engagement of E-selectin and VCAM-1, as well as acCD44 which is also a ligand of E-selectin [Alcaide et al., 2007] and CXCR3, CD183, which can recruit B cells to inflamed tissues [Muehlinghaus et al., 2005]. Memory B cells express several integrins and down-regulate CD62L compared to naïve B cells. This suggests a role in which memory B cells in the spleen not only are pre-activated, but also have a migrational capacity outside of the spleen and could recirculate in the body.

Spleen And Bone Marrow Memory B Cells Share Phenotypic Signatures

Even though much of the attention of the work was focused on the identification of differences, for the most part memory B cells isolated from the spleen and the bone marrow closely resemble one another. The number of differentially expressed genes between them is very limited and the same is true for the expression of surface markers. Among the markers for which no differences were found are classical B cell markers like CD19, CD20, CD45R, CD38, and CD40.

The differences between spleen and bone marrow memory B cells which were identified, seem to be limited, i.e. the number of genes differentially transcribed more than two-fold higher in either organ is less than 100 transcripts. Among those some are still likely to be from contaminating cells[4] and few were regulated much more than two-fold. For the comparison between naïve and memory B cells from the spleen, the number of differentially expressed genes was much higher and among them were many with foldchange values higher than two.

[3] *Bach2* is down-regulated in the splenic memory B cells

[4] The effect from contaminating cells is suspected to be strong, because the cells were isolated from different organs and the nature of the contaminatig cells is expected also to be very different in both organs.

Memory B Cells in the Bone Marrow Differ from Spleen Memory B Cells

Bone marrow memory B cells express both CD80 and PD-Ligand 2 at higher levels than their splenic counterparts. This profile characterizes them as highly differentiated memory B cells. In a recall repsonse they would not form germinal centers but differentiate directly into antibody-secreting cells. It is an interesting question whether memory B cells with this phenotype might also do so directly in the bone marrow. In memory B cells from the bone marrow CD21 and CD62L were even further down-regulated from the level of naïve cells than in splenic memory cells. CD21 is the receptor for complement 3d and involved in B cell receptor signaling by lowering the threshold for activation via the B cell receptor [Barrington et al., 2009]. CD62L, L-selectin, mediates lymphocyte traffic through lymphoid organs but can also lead to activating signals and increased chemokine recetor responsiveness [Ivetic, 2013]. In addition, aside from CD80 and PD-Ligand 2, a number of genes involved in the interaction with T cells were down-regulated in bone marrow compared to spleen memory B cells. Taken together, bone marrow memory B cells seem to have a decreased capacity for B cell receptor stimulation or interaction with naïve T cells, which is mediated through CD86 rather than CD80, and less inclination for migration.

Bone Marrow Memory B Cells are Resting

Memory B cells isolated from the bone marrow in contrast to those from the spleen were negative for Ki-67 which is expressed in all phases of the cell cycle except G_0. The fraction of Ki-67$^+$ cells was more than three-fold less than in naïve B cells isolated from the spleen and more than ten-fold less than that of memory B cells from the spleen (chapter 3.2.2). The proportion of cells not in G_0 of the cell cycle is as low as described for resting bone marrow memory CD4$^+$ T helper cells [Tokoyoda et al., 2009] and considerably less than described for bone marrow memory cytotoxic CD8$^+$ T cells [Sercan Alp et al., 2015].

The concept is backed by evidence from gene transcription analysis. In bone marrow memory B cells an anti-apoptotic program which arrests proliferation and differentiation is in effect. The pro-apoptotic gene *Bid* [Weber et al., 2008] was down-regulated compared to spleen memory cells. On the other hand, bone marrow memory B cells expressed a higher level of *Foxp1*, atranscription factor which is involved in the suppresion of pro-apoptotic genes [van Keimpema et al., 2014]. The transcription factors c-Myb, involved in the maintenance of mature B cells [Thomas et al., 2005], and *Sox5* which decreases the proliferative capacity of B cells [Rakhmanov et al., 2014], were higher expressed in bone marrow memory B cells. The same was true for the transcriptional repressor *Bach2* which has been described to inhibit plasma cell differentiation in memory B cells [Kometani et al., 2013, Porstner et al., 2015].

Proliferation in the splenic memory compartment could also account for the failure to detect EdU$^+$ memory B cells anywhere but in the bone marrow 9 months after immunization (chapter 3.1.3). Antigen-specific memory might still be present, but was not detected due to loss of the EdU by proliferation. That is not to say, that memory B cells in the spleen are necessarily highly proliferative. Given that their transcriptional profile resembles a somewhat more activated phenotype but is more similar to naïve than to fully activated germinal center B cells [Bhattacharya et al., 2007, Tomayko et al., 2008], the Ki-67$^+$ cells could be in G_1 of the cell cycle, representing a pre-activated more alert status than the fully resting cells in the bone marrow. Another possibility would be constant proliferation of memory B cells to ensure the homeostasis of the memory compartment. Homeostatic proliferation would have to be mediated differently than in naïve B cells where it depends on BAFF [Crowley et al., 2008, Gardam et al., 2008, Pillai and Cariappa, 2009], because the maintenance of splenic memory B cells is not impaired by lack of BAFF or APRIL [Benson et al., 2008a].

Different B Cell Receptor Repertoires in Spleen and Bone Marrow

The B cell receptor sequences of memory B cells from spleen and bone marrow have accumulated equal numbers of somatic hypermutations in the heavy chain genes. In that sense, no qualitative difference was found between bone marrow and spleen memory B cells. However, the receptor repertoire, as evaluated by the heavy chain CDR3 sequences, was different. CDR3s are involved in antigen-binding of the receptor. Identical CDR3s or clonally closely related sequences are highly likely to originate from sequences which encode for receptors raised to the same antigenic epitopes. Nevertheless, the extent of clonal overlap – the proportion of CDR3s with completely identical sequences – was very limited. Memory B cells in the spleen and in the bone marrow therefore harbored, very likely, memory B cells of different specificities.

There was still some extent of sequence overlap between spleen and bone marrow of the same mouse. Especially among clones with the highest numbers of sequencing reads per sample, some but not all were found in both spleen and bone marrow. But the similarity in clonal distribution between both samples of the same mouse was still low, unlike it would be expected if memory B cell clones were evenly distributed to spleen and bone marrow or be made up of one constantly recirculating B cell memory pool. The overlap between spleen and bone marrow was limited which demonstrates that the distribution of the memory pool to the different compartments is also not completely separated.

By including information on the degree of relatedness between sequences by the calculation of phylogenetic trees, the picture will become clearer. The analysis still requires much computational power [Barak et al., 2008, von Büdingen et al., 2012] and could not be performed yet on the available data. Also, it would be very appealing to be able to deduce information about specificity from the CDR3 sequences. So far, the determinants of antibody-epitope binding have not been sufficiently understood to reliably relate from receptor sequence to antigen structure [Sela-Culang et al., 2013].

In humans as well as in mice, memory B cells recirculating in the periphery can be found [Aiba et al., 2010, Giesecke et al., 2014] and they were also detected in murine peripheral blood (chapter 3.1.1). The absolute numbers of memory B cells detectable in the peripheral blood at any one time are very low, compared to the total memory compartment. In humans, the number of memory IgG B cells in the spleen alone is 100 times higher than that in the blood. It is therefore probable that the memory B cells in the circulation do not constitute a separate population but are the momentarily recirculating proportion of a bigger memory cell pool. In humans, the frequencies of tetanus toxoid-specific B cells among $CD27^+$ memory B cells in spleen, tonsils, and peripheral blood were, in fact, found to be strikingly similar whereas none were present in the bone marrow [Giesecke et al., 2014]. This is compatible with the concept of the existence of a single recirculating pool in the periphery and secondary lymphoid organs. The bone marrow, given all the differences to memory B cells isolated from the spleen, would constitute a separate memory compartment.

Memory B cells from the spleen display a transcriptional profile which resembles a somewhat more activated phenotype than naïve B cells. By virtue of their intrinsic anti-apoptotic program recirculating cells could be less dependent on external survival signals than the more resting bone marrow memory or follicular naïve B cells. This would be an essential feature for recirculating memory B cells which are not at all times situated in the context of a putative survival niche like it has been described for other memory cells as CD4 memory [Tokoyoda et al., 2009], CD8 memory [Sercan Alp et al., 2015] or plasma cells [Cassese et al., 2003]. Naïve B cells in the spleen require BAFF receptor signals for survival, whereas splenic memory B cells do not depend on BAFF or APRIL.

Another possibility that might be considered to explain the difference between bone marrow and the canonical memory B cells in the spleen, is the concept of tissue-resident memory as described for memory T cells in mucosa, airways and the skin [Schenkel and Masopust, 2014, Shin and Iwasaki,

2013]. These cells are thought to provide a specialized first line of defense to the particular organs. There they can confront highly specialized pathogens which utilize specific attack vectors. The notion, however, is in some contrast with the resting phenotype of bone marrow memory B cells. If they were sent to the bone marrow as a specialized memory population for the defense against specialized pathogenic threats, they might be expected to be in a heightened state of alert rather than in rest. Additionally, the bone marrow is, although well-connected to the circulation, not an organ through which pathogens are likely to enter an organism. Tissue-resident memory T cells have been described for organs with epithelial layers in contact with the external environment. The bone marrow, in contrast, is a resting place for other memory cell populations as well.

4.2 Memory B Cell Response to Systemic Antigen Does not Dependent on CD11c^{hi} Dendritic Cells

If the antigen in a recall response is applied systemically, via the blood stream, CD11c^{hi} dendritic cells are dispensable for the establishment of secondary antibody response against NP-KLH (chapter 3.3). The experiment was intended as an exploratory approach to the question whether memory B cells which possess high avidity antigen surface receptors serve in antigen-presentation in the re-activation of memory T helper cells. These are resting in the bone marrow [Tokoyoda et al., 2009] and could, therefore, not be scanning the periphery for their cognate antigen. Hence, if they are to be reactivated by antigen, it must be delivered to them by suitable antigen-presenting cells.

T cell help was measured as the effect on the production of high-affinity antibodies by ELISA for antigen-specific IgG. The underlying assumptions were that (1) T cell help is necessary for the induction of an efficient secondary B cell response to the monomeric antigen NP-KLH and that (2) in a memory response it would need to be provided by memory T helper cells.

The route of antigen-application via the blood stream is unique. Antigen is delivered quickly and passively, without uptake by specialized cells, through the well-perfused organs spleen and bone marrow where it can be expected to arrive within seconds after IV application. Memory B cells are equipped with high avidity receptors and can sense minute concentrations of their cognate antigen, ingest it and present it on MHCII to (memory) T helper cells[5].

Yet, the results must be carefully evaluated, because the setting is complicated and could include more than one unknown variable. Antigen-presentation by memory B cells to memory T cells would create a situation in which a very infrequent cell type, the antigen-specific memory B cell, would have to actively engage the also highly infrequent resting sessile memory T cells in their survival niches to present antigen in search for corresponding T helper cells. Possibly, the memory B cells do not need T cell help for restimulation and differentiation into antibody-secreting cells at all. For viral proteins it has been shown that the memory B cell response is not dependent on T cell help or the presence of T cells at all and is indepent of CD11c^{+} cells or macrophages as antigen-presenting cells [Hebeis et al., 2004a, Weisel et al., 2010]. Monomeric proteins have been reported to require T cell help for re-stimulation [Aiba et al., 2010, Vieira and Rajewsky, 1990]. The differential requirement lies in the engagement of many individual receptors by comparably large virus particles composed of repetitive protein subunits which can engage multiple B cell receptors on the same B cell and thereby overrule the requirement of co-stimulatory signals from accessory cells.

Re-activation may not so much depend on antigen-presentation by specialized cells, but on antigen-delivery to the resting places of the memory cells. This is demonstrated by the delay in the immune response after SC delivery of antigen in the absence of CD11c^{hi} cells. In principle, involvement

[5] Or, by cross-presentation on MHCI, to cytotoxic T cells.

of other antigen-presenting cells besides dendritic cells, such as macrophages [Hume, 2008] or even stromal cells [Barrington et al., 2002] is conceivable here, as there may be differential requirements between secondary lymphoid organs, as in the experiment by Weisel et al. [2010], and the delivery of blood-borne antigen to memory B cells within the bone marrow. A role for B cells is not unlikely. On the other hand, antigen presentation by naïve B cells is known *in vivo* and also antigen-presentation after uptake from the circulation has been described [Rodríguez-Pinto, 2005, Xu et al., 2015].

No effect of dendritic cell depletion could be observed when the antigen was delivered by the blood stream. That an effect of the absence of dendritic cells generally would be observable at all is demonstrated by the fact that the secondary antibody response to NP was impaired in the SC boost. Whether the noted absence of the same deleterious effect in the case of IV immunization and delivery of the antigen via the blood is actually dependent on the reactivation of memory T cells by presentation of antigen through memory B cells, remains an open question. It could be elucidated by a situation in which antigen presentation by memory B cells is made impossible, i.e. by conditionally deleting MHCII in B cells in the recall situation, or by use of the same hapten with a different carrier protein in the boost than in the primary response. Cognate memory T cells would not exist, while naïve T cell help was still possible and memory B cells to the hapten were present.

4.3 Conclusions and Open Questions

The work described here, revealed many interesting and so far unknown details about the lifestyle and molecular characteristics of memory B cells. Memory B cells were quantified in mice with different immunological backgrounds. The distribution of memory B cells to the secondary lymphoid organs and the bone marrow reveals a role of the bone marrow as a qualified residence for memory B cells. The bone marrow had previously not been regarded as an important site in the maintenance of memory B cells. Here, for the first time, the accumulation of memory B cells in the murine bone marrow was quantified and the cells characterized in molecular detail and compared to memory B cells from the spleen. The insight gained from the analyses tempts some highly interesting conceptual considerations and suggests the formulation of hypotheses on which further research on B cell memory may be based.

4.3.1 B Cell Memory – Differences in Phenotype, Differences in Lifestyle

Memory B cells from the bone marrow share much of their molecular configuration with their splenic counterparts. However, they differ in terms of proliferation, surface marker expression, gene transcription, and receptor repertoire to an extent which forbids the conclusion that memory B cells isolated from the bone marrow merely were recirculating B cells which happened to be present at the time of the analysis. Thus, constant and high-rate exchange of memory B cells in the bone marrow by those from the spleen can be ruled out. Qualitative differences between isotype-switched memory B cells from spleen and bone marrow have been defined above. Human data suggest, that the differences between memory B cells in the secondary lymphoid organs and the blood are not as great as those described here for murine spleen and bone marrow memory B cells[6].

Resting B Cell Memory in the Bone Marrow – Recirculating Memory in the Spleen?

Considering the differences between spleen and bone marrow memory B cells and the indications for differential survival mechanisms of memory B cells from spleen and bone marrow, it is conceivable that they also differ in lifestyle. It can be hypothesized that memory B cells from the spleen, or the

[6]Unless, of course, both sets are constantly migrating but are recirculating through different compartments with limited overlap in spleen and bone marrow.

secondary lymphoid organs in general, constitute the pool that supplies memory B cells recirculating throughout the periphery. The bone marrow, in contrast, appears to be a place of rest for a different subset of memory B cells.

This would require that memory B cells in recirculation and possibly also in the spleen, have an intrinsic capacity for survival and do not rely on constant cell contact-dependent or locally available soluble factors in a cellular survival niche as it has been described for other memory cell types. Survival signals could be derived from soluble factors present in lymph or blood or be periodically refreshed when passing through suitable environments, e.g. lymphatic tissues. Molecular survival requirements for bone marrow memory B cells might be different and could be provided in a local niche environment. Histological investigation of the distribution of memory B cells within the tissues would be a sensible approach to uncover whether they preferentially reside next to cells that could provide a survival niche, and if so, which.

Moreover, the difference in proliferation could point to the fact that, while the memory B cells in the bone marrow do not proliferate at all, memory B cells in the spleen undergo replenishment by low but constant proliferation much like it has been described for mature naïve B cells in secondary lymphoid organs by homeostatic proliferation.

A division of localization between bone marrow and spleen, or secondary lymphoid organs and the periphery, might indicate a division of labor in recall confrontation with pathogens. This would very likely be reflected by differences in the nature of the antigens, in magnitude or kinetics of spleen and bone marrow memory B cell responses or differential requirements for antigenic-restimulation, e.g. means of antigen-delivery and co-stimulatory signals.

Differential Triggers for Spleen and Bone Marrow Homing

Spleen and bone marrow memory B cells differ in the expression of migration-related molecules such as integrins and other adhesion molecules. The differential homing signature of memory B cells in the spleen and bone marrow could result from the induction of memory formation in different tissue environments or differential capability for relocalization after re-activation from spleen or bone marrow or from differences in the migrational activity at the time of analysis as well as from a combination of these. Blocking experiments during antigen-rechallenge could be one useful approach to clarifying the functional effects of the homing molecules in memory B cells.

Another hint to the background of the distribution of memory B cells to different organs is the differential distribution by isotype. Isotype class-switch is regulated by cytokine milieu and toll-like receptor signaling. Milieu-dependent factors which influence chemokine expression by accessory cells and the toll-like receptor stimuli during the B cell response are likely to have an impact on the integrin-configuration of the cells generated in a particular environment such as mucosa-associated tissues. This is one possible reason for higher representation of IgA and IgG_{2b} memory B cells in the bone marrow than IgG_1.

Not only the isotype distribution to spleen and bone marrow memory B cells differs. In addition, the receptor repertoire is not evenly distributed to spleen and bone marrow. This suggests a preferential distribution of memory B cells which were generated in different immune responses to different compartments with a preferential localization in either organ. Antigen-specificity and with it receptor sequences are directly dependent on the antigens to which a B cell clone is reactive. Antigenic challenges are likely to differ in different sites of the induction of B cell-activation as each pathogen follows a particular and specialized strategy of entering the host organism.

4.3.2 The Role of Memory B Cells in Recall Immune Responses

Ultimately, if one accepts the concept of cellular memory in the immune system and a role for memory B cells in the immunity to pathogenic agents[7], memory B cells must be re-activated quickly in the case of recurring presence of their cognate antigen to be able to fulfill their role in ensuring protective antibody titers.

By the concept of a compartmentalized B cell memory, IgG_{2b} memory B cells in the bone marrow display a phenotype of memory cells which would not form germinal centers but quickly differentiate into antibody-secreting cells upon restimulation. The concept however, was based on observations of memory B cells from the spleen. It remains to be clarified whether it applies to bone marrow memory B cells which express CD80 and PD-ligand 2 but may differ in respect to other molecular features. If the concept holds true for bone marrow memory cells, they could possibly, given they receive all necessary co-stimulatory signals, differentiate into plasma cells directly in the bone marrow.

Considering the overall amount of differences between them, a differential behavior of isotype-switched memory B cells from the spleen and from the bone marrow is to be expected. The differences in migration-related molecules may result in a differential ability or necessity for migration upon reactivation. The expression pattern of co-stimulatory molecules involved in the interaction with T cells suggests for the bone marrow memory B cells a more pronounced capacity or requirement to interact with T helper cells or to present antigen to them for memory T cell reactivation.

It also is an open question, whether bone marrow isotype-switched memory constitutes a more potent or a more restricted subset in the capacity for recall response. Reduced CD21 expression could mean the latter, as this may raise the threshold for activation through B cell receptor signaling or lower the capacity for sensing complement-bound antigen. Taken together with the arrest in the G_0 phase of the cell cycle, it can be speculated that the resting memory B cells of the bone marrow constitute a back-up memory compartment which is not to be re-activated as long as there is still a sufficient specific antibody concentration to ensure the formation of complement-antigen complexes. Lower CD21 expression and higher activation-threshold might then be overcome by T cell help.

[7] It has been argued by Zinkernagel [2012] and, with respect to T cells, by Bell and Westermann [2008], that the protection from pathogens is mainly mediated by other mechanisms and does not actually depend on the presence of memory cells.

Summary

Memory B Lymphocytes are cells of the immune system which propagate one of its key features: Immunological memory, that is an organisms' ability to respond in a more rapid and enhanced manner to an antigenic stimulus it has encountered before. Memory B cells have expanded in response to antigen and remain stably present in an organism over long periods. It is the aim of this thesis to elucidate the validity of the general concept of memory immune cells – like plasma cells or CD4 and CD8 memory T cells – resting in specialized cellular niches for the maintenance of memory B cells.

Murine Memory B cells in the spleen, bone marrow, lymph nodes and peripheral blood were quantified in laboratory mice which had received different imunizations and in wild and pet mice which did not live under pathogen-reduced SPF conditions. As expected, large numbers of memory B cells were localized in the spleen, but a prominent population was found in the bone marrow. To understand and compare the conditions of their survival in both organs, memory B cells from spleen and bone marrow were isolated and profiled for gene transcription, expression of surface markers as well as their B cell receptor repertoire.

In terms of gene transcription and surface marker expression, memory B cells from spleen and bone marrow were very similar. However, memory B cells from the bone marrow had a more differentiated memory phenotype and express higher levels of CD80 and PD-L2, but less CD62L and CD21. Memory B cells of the spleen had a more activated phenotype and whereas bone marrow memory B cells expressed the transcription factors Foxp1 and Myb for maintenance and for suppression of apoptosis. Bone marrow memory B cells were resting in the G_0 phase of the cell cycle, whereas memory B cells in the spleen did not, as shown by expression of the cell cycle marker Ki-67. For the first time this provides evidence for the existence of a resting population of memory B cells in the murine bone marrow.

To address the hypothesis that there may be a role for memory B cells in antigen presentation to memory T cells, dendritic cells were depleted in a recall response to NP-KLH. If the antigen is applied IV through the blood stream, dendritic cells are dispensible for efficient secondary antibody response.

No difference was found in the extent of somatic hypermutations per V gene. However, the comparison of the B cell receptor repertoire from spleen and bone marrow memory cells revealed limited overlap in CDR3 sequences. This demontrates that both compartments harbor memory cells with different specificities which rules out extensive exchange of cells between the memory populations of both organs.

In contrast to the notion of recirculating B cell memory, it could be shown that a separate resting population within the B cell memory compartment exists in the bone marrow. Memory B cells in

spleen and bone marrow differ in a number of features leading to the concept that the bone marrow is resting and spleen memory B cells are in a pre-activated state and have higher migratory capacity and are probably the origin of the recirculating memory cells in the periphery.

Zusammenfassung

Gedächtnis-B-Zellen sind Immunzellen, die an der Aufrechterhaltung des immunologischen Gedächtnis beteiligt sind. Darunter versteht man die Fähigkeit eines Organismus einem berits bekannten Antigen mit einer schnelleren und stärkeren Immunreaktion zu begegnen.

Gedächtnis-B-Zellen verbleiben nach ihrer Expansion in einer Immunantwort für lange Zeit im Organismus. Ziel dieser Arbeit ist es, der Frage nachzugehen, ob auch für Gedächtnis-B-Zellen das allgemeine Konzept gilt, nach dem die Gedächtniszellen des Immunsystems, Plasmazellen oder CD4- und CD8-Gedächtniszellen, in spezialisierten zellulären Nischen überleben.

Dazu wurden Gedächtnis-B-Zellen aus Milz, Knochenmark, Lymphknoten und Blut von Mäusen isoliert, die zuvor nach verschiedenen Protokollen immunisiert worden waren und aus wilden sowie aus einer Tierhandlung stammenden Mäusen, die nicht unter SPF-Bedingungen gehalten wurden. Wie erwartet konnten Gedächtnis-B-Zellen in großer Zahl in der Milz aufgefunden werden. Aber daneben existiert eine große Population im Knochenmark. Zur Aufklärung der Unterschiede wurde Gedächtnis-B-Zellen beider Populationen isoliert und Transkriptionsprofile, eine Analyse der Expression von Oberflächenmarkern und die Sequenzierung des B-Zell-Rezeptor-Repertoires durchgeführt. Hinsichtlich der Gentranskription und der Oberflächenmarker sind die Gedächtnis-B-Zellen der Milz und des Knochenmarks einander sehr ähnlich. Allerdings zeigten die Gedächtnis-B-Zellen des Knochenmarks einen differenzierteren Gedächtnisphänotyp und exprimierten mehr CD80 und PD-L2 aber weniger CD62L und CD21. Die Gedächtnis-B-Zellen der Milz wiesen einen aktivierteren Phänotyp auf, während Gedächtnis-B-Zellen des Knochenmarks die Transkriptinsfaktoren Foxp1 und Myb zur Unterdrückung der Expression apoptotischer Gene und Homöostase exprimieren. Knochenmarks-Gedächtnis-B-Zellen ruhen in der G_0-Phase des Zellzyklus, das gilt für Gedächtnis-B-Zellen aus der Milz nicht, wie die Expression des Proliferatinsmarkers Ki-67 zeigt.

Um die Hypothese zu prüfen, dass Gedächtnis-B-Zellen eine Rolle in der Antigenpräsentation spielen könnten, wurden im Kontext einer Gedächtnisatwort gegen das Antigen NP-KLH die dendritischen Zellen depletiert. Wird das Antigen i.v. in die Blutzirkulation appliziert, sind dendritische Zellen für eine effektive Sekundärantwort nicht nötig.

Hinsichtlich der Anzahl der Mutationen in den V-Regionen der schweren Immunglobulin-Ketten bestanden keine Unterschiede zwischen Milz- und Knochenmarks-Gedächtnis-B-Zellen. Der Vergleich der CDR3-Verteilung zeigte eine sehr begrenzte Zahl an Sequenzen, die in beiden Populationen aufgefunden wurden und große Unterschiede in der Verteilung auf beide Organe. Das zeigt, dass ein Austausch zwischen den beiden untersuchten Kompartimenten des B-Zell-Gedächtnis nicht in großem Ausmaß stattfindet.

Im Gegensatz zum gegenwärtigen Verständnis eines rezirkulierenden B-Zell-Gedächtnis, konnte in dieser Arbeit die Existenz eines eigenständig neben dem rezirkulierenden Anteil bestehenden ruhenden Bestandteil des B-Zell-Gedächtnisses im Knochenmark gezeigt werden. Gedächtnis-B-Zellen in Milz und Knochenmark unerscheiden sich in etlichen Eigenschaften. Daraus lässt sich das Konzept einer Aufteilung des B-Zell-Gedchtnisses ableiten, in dem das Knochenmark den ruhenden, nicht proliferierenden Teil beherbergt und der prä-aktivierte Anteil sich in der Milz aufhlt, über eine höhere Migrationsneigung verfügt und wahrscheinlich den durch die Peripherie rezirkulierenden Teil des B-Zell-Geächtnisses auffüllt.

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Acronyms

APC	allophycocyanin
APRIL	a proliferation-inducing ligand, Tnfsf13
BAFF	B cell activating factor, TNFSF13b, BLyS, TALL-1, THANK, TNFSF20, zTNF4
BID	BH3 interacting domain death agonist
BSA	bovine serum albumin
bp	base pairs
CD	cluster of differentiation
CD1d	CD1d antigen
CD3	T cell receptor
CD4	Ly-4, L3T4
CD5	Ly-1, Ly-12, Ly-A, Lyt-1
CD8	CD8 antigen
CD9	Tspan29
CD11c	integrin-αX, CR4, N418
CD18	CD18 antigen, LAD, LCAMB, LFA1, MF17
CD19	CD19 antigen
CD20	Ly-44, MS4A2
CD21	complement receptor 2 (CR2), C3dR
CD22	CD22 antigen, Lyb-8
CD23	Fcϵ receptor
CD24	nectadrin, HSA, Ly-52,
CD26	Dpp-4, THAM
CD27	TNFRSF17, S152, TP55
CD29	integrin β1, Fnrb, GPIIa
CD34	CD34 antigen
CD38	CD38 antigen, ADPRC 1
CD40	TRAP, HIGM1, IGM, IMD3, T-BAM, TNFRSF5
CD43	sialoporin, Galgp, Ly-48
CD44	HERMES, Ly-24, Pgp-1
CD45	PTPRC, LCA
CD47	CD47 antigen, integrin-associated signal transducer, Rh-related antigen
CD48	SLAMF-2, BCM1, BLAST-1 MEM-102, Sgp-60
CD49a	integrin α 1, VLA-1
CD49b	integrin α 2, DX5
CD49c	integrin α 3, GAPB3
CD49d	integrin α 4, VLA-4
CD49e	integrin α5, Fnra, VLA-5
CD49e	integrin α6, VLA-6
CD45R	B-220: B cell-specific CD45 isoform of 220 kD

CD51 integrin α V
CD54 ICAM-1, Ly-47, MALA-2
CD55 GPI-DAF
CD62L L-selectin, LECAM-1, LNHR, Ly-m22, Lyam-1
CD64 Fcγ receptor I
CD66a CAECAM-1, Bgp1,Cea-7, Cea1, Hv-2, MHVR1, BB-1
CD73 5′ ectonucleotidase
CD80 B7-1, CD28 Ligand, Ly-53, MIC17, TSA1
CD86 B7-2, B70, CLS1, CD28 Ligand 2, ETC-1, Ly-58, MB7-2, TS/A-2
CD93 AA4.1 C1qR1, Ly68
CD95 Fas, TNFRSF6
CD98 4F2, LY-10, MDU1, Mgp-2hc, NACAE
CD127 IL-7 receptor α chain
CD132 IL-2 receptor γ chain
CD133 CD133 antigen, Prom-1
CD138 Syndecan 1
CD183 CXCR3
CD197 CCR7 (chemokine (C-C motif) receptor 7), Cmkbr7, EBI1
CD200R OX2R
CD267 TACI, TNFRSF13B, CVID, CVID2, IGAD2, RYZN
CD268 BAFF-receptor, TNFRSF13C, BROMIX, CVID4, prolixin
CD270 HVEM, Atar, HVE-A, TNFRSF14
CD273 PD-Ligand 2, B7-DC
CD274 PD-Ligand 1, B7-H1
CD278 ICOS (inducible co-stimulator)
CDR3 3rd complementarity-determing region of immunoglobulin heavy chain
CFU colony-forming units
cDNA copy DNA
CGG chicken γ globulin
CLEC12A c-type lectin 12a
CXCR5 chemokine (C-X-C motif) receptor 5
DNA deoxyribonucleic acid
DRFZ Deutsches Rheuma-Forschungszentrum, Berlin
DTR diphteria toxin receptor
EdU 5-Ethynyl-2′-deoxyuridine
ELISA enzyme-linked immunosorbent assay
FACS fluorescence-activated cell sorting
FITC fluorescein
FMO fluorescence minus one
GC germinal center
GFP green fluorescent protein
IgA immunoglobulin A
IgD immunoglobulin D
IgG_1 immunoglobulin G subclass 1
IgG_{2a} immunoglobulin G subclass 2a
IgG_{2b} immunoglobulin G subclass 2b
IgM immunoglobulin M
IL-5 interleukin 5
IL-6 interleukin 6
IL-7 interleukin 7
IP intraperitoneal
IV intravenous
KLH Keyhole Limpet hemocyanin
LCMV lymphocytic choriomeningitis virus
LIMMA linear models for microarray data
LPS lipopolysaccharide
LPAM-1 α4β7-integrin
MACS magnetic cell sorting

MHCI	major histocompatibility complex class I
MHCII	major histocompatibility complex class II
mRNA	messenger RNA
NGS	next generation sequencing
NK cell	natural killer cell
NiP	4-hydroxy-3-iodo-5-nitrophenyl
NP	4-hydroxy-3-nitrophenylacetyl
NP-KLH	4-hydroxy-3-nitrophenylacetyl-Keyhole Limpet Hemocyanin
OVA	ovalbumin
PCR	ploymerase chain reaction
PBS	phosphate-buffered saline
PE	phycoerythrin
PFU	plaque-forming units
PI	propidium iodide
PNA	peanut agglutinin
PO	per os
RMA	robust multi-array average
RNA	ribonucleic acid
S_1PR1	sphingosine-1-phosphate receptor 1, Edg1
SC	subcutaneous
SDF-1α	stromal cell-derived factor-1α, CXCL12
SPF	specific pathogen-free
TLR9	toll-like receptor 9
TNFα	tumor necrosis factor α
YFP	yellow fluorescent protein

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Publikationen

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Lebenslauf

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

Supplementary Materials

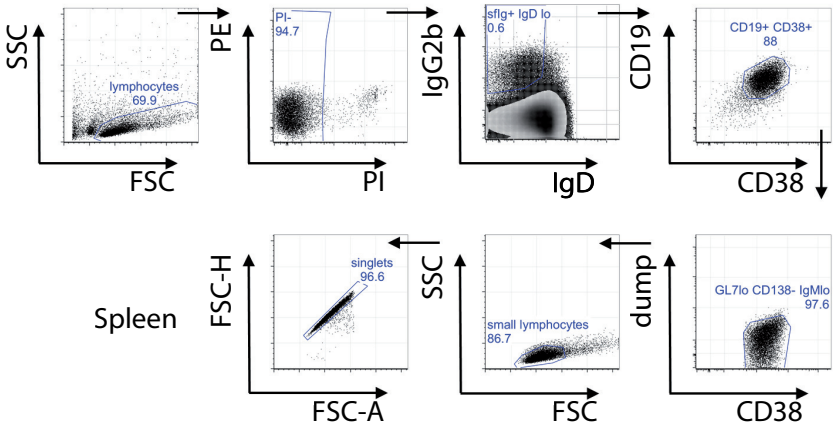

Figure 1: Gating strategy for analysis of flow cytometric quantification of murine IgG_{2b}-switched memory B lymphocytes. Shown here is an example of spleen cells which were sorted by magnetic labeling for expression of CD19 and then analysed by flow cytometry. Live cell measurements excluded dead cells by PI incorporation, cell doublets by scatter signal height and included $IgG2b^{+}IgM^{-}IgD^{-}CD19^{+}CD38^{+}CD138^{-}$ small lymphocytes, the dump channel contained antibodies for GL7 and CD11c markers.

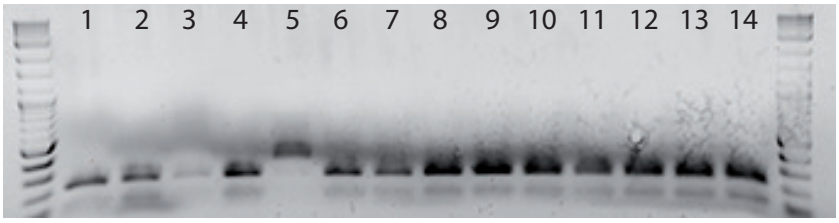

Figure 2: Classification of wild mice by PCR amplification of the Gpr33 gene.
Agarose gel separation for amplification products of Gpr33-specific PCR after Tat1 restriction for wild mouse classification. For *Mus* and *Apodemus* genera the PCR product size is 317 bp. For Tat1 restriction, expected fragment sizes for *Mus musculus* are 103 bp and 214 bp. The *Apodemus* Gpr33 gene does not contain a Tat1 restriction site. Mouse no 5 was not included in the analysis.

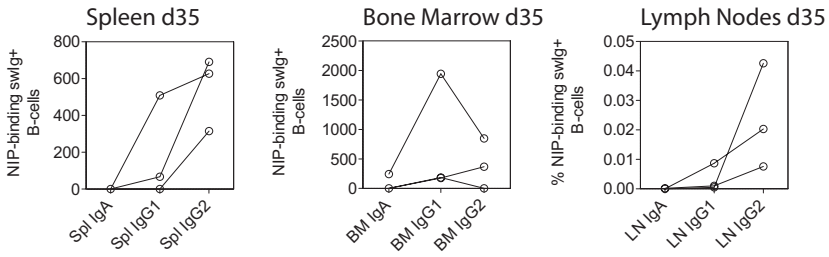

Figure 3: Switched NP-binding B cells generated after immunization with NP-KLH/LPS are $IgG_{2a/b}$.
Quantification of NP-binding isotype-switched B cells in spleen, bone marrow and peripheral lymph nodes on day 35 after immunization with NP-KLH/LPS SC. Shown are absolute numbers for spleen and bone marrow and frequencies among $CD19^{+}$ cells for lymph nodes.

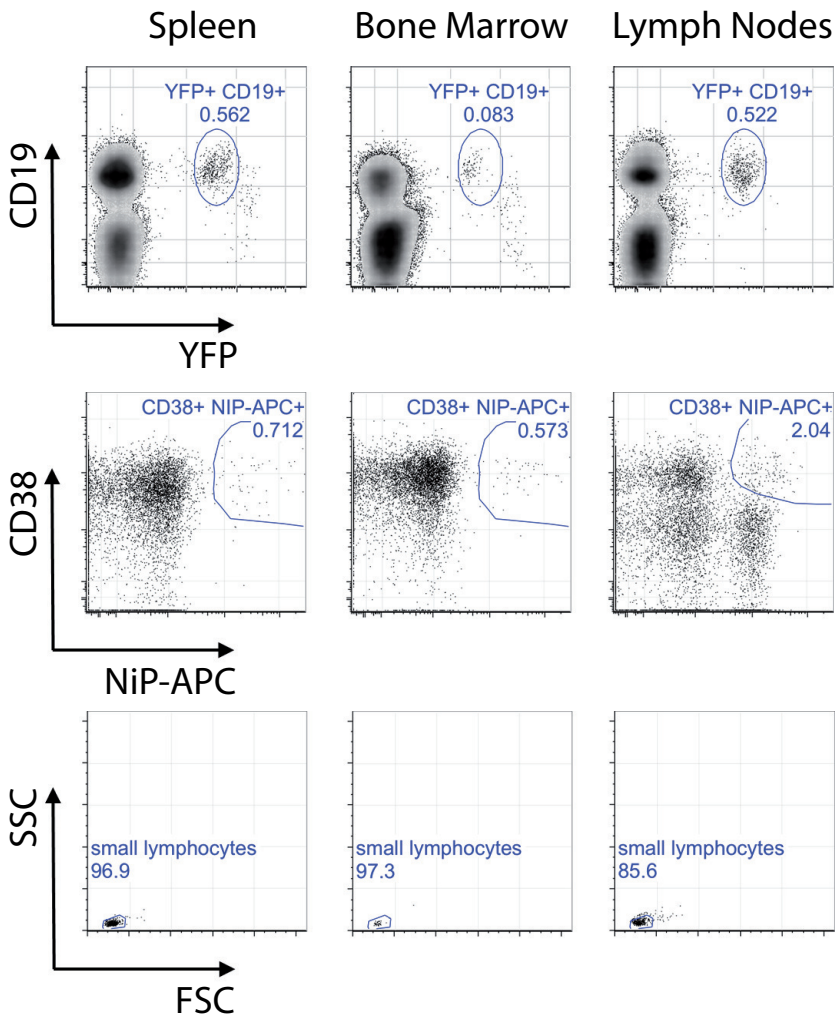

Figure 4: Spleen and bone marrow NiP-binding B cells in switch reporter mice are $CD38^+$ small lymphocytes.
YFP^+CD19^+ cells from spleen, bone marrow and peripheral lymph nodes of Cγ1-Cre×R26-stop-eYFP switch reporter mice on day 35 after immunization with NP-KLH/alum SC (top row). YFP^+ cells include NiP-binding cells (middle row). $NiP\text{-}APC^+CD38^+$ cells are small lymphocytes (lower row).

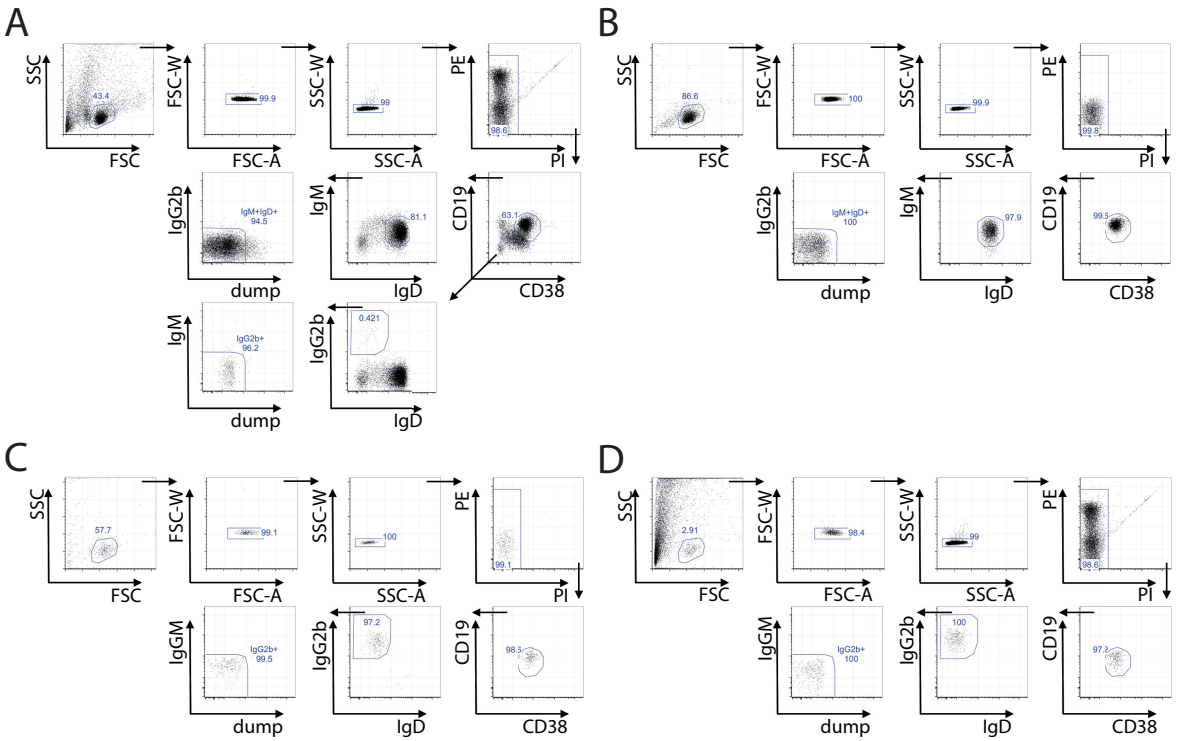

Figure 5: Gating strategy for naive follicular and IgG_{2b} memory B cells and post-sort purity checks. **A:** Gating layout for purification of spleen naïve follicular B cells and spleen and bone marrow IgG_{2b} memory B cells by FACS. Naïve cells were selected for $PI^{-}CD19^{+}CD38^{+}IgG_{2b}{}^{-}IgM^{hi}IgD^{hi}$ single small lymphocytes, memory B cells $PI^{-}CD19^{+}CD38^{+}IgG_{2b}{}^{+}IgM^{lo}IgD^{lo}$. Dump-channel contains antibodies for GL-7, CD11c and CD93. **B:** Purity control sorted for spleen naïve B cells. **C:** Purity control sorted for spleen IgG_{2b} B cells. **D:** Purity control sorted for bone marrow IgG_{2b} B cells.

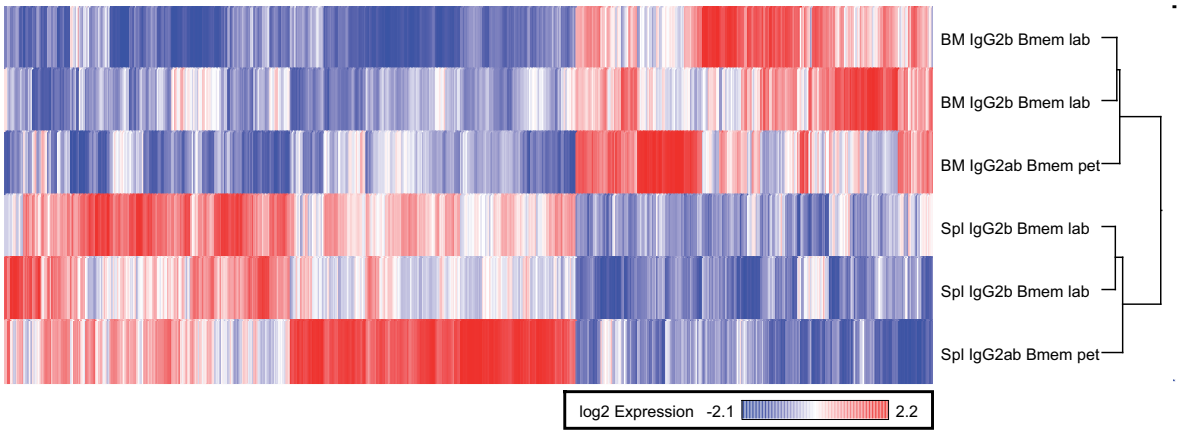

Figure 6: Relative differences in gene expression of differentially expressed genes in spleen and bone marrow memory IgG_2 B cells.
Heatmap of 1438 significantly differentially expressed genes in spleen (Spl) and bone marrow (BM) memory B cells (Bmem). Red denotes relative up-, blue down-regulation of gene expression. Cells isolated from pooled C57BL/6 SPF (lab) or pet mouse splenocytes or bone marrow.

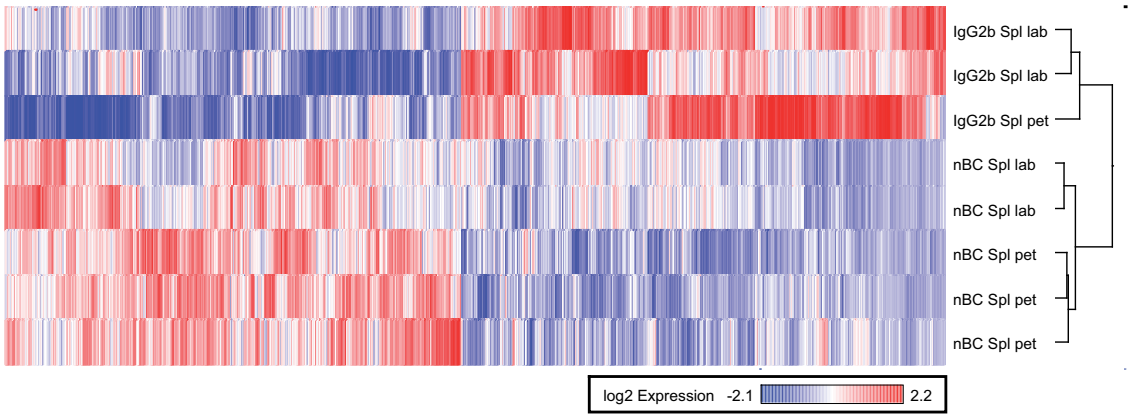

Figure 7: Relative differences in gene expression of differentially expressed genes in spleen naïve and memory IgG_2 B cells.
Heatmap of 3219 significantly differentially expressed genes in spleen (Spl) naïve (nBC) and memory B cells (Bmem). Red denotes relative up-, blue down-regulation of gene expression. Cells isolated from pooled C57BL/6 SPF (lab) or pet mouse splenocytess.

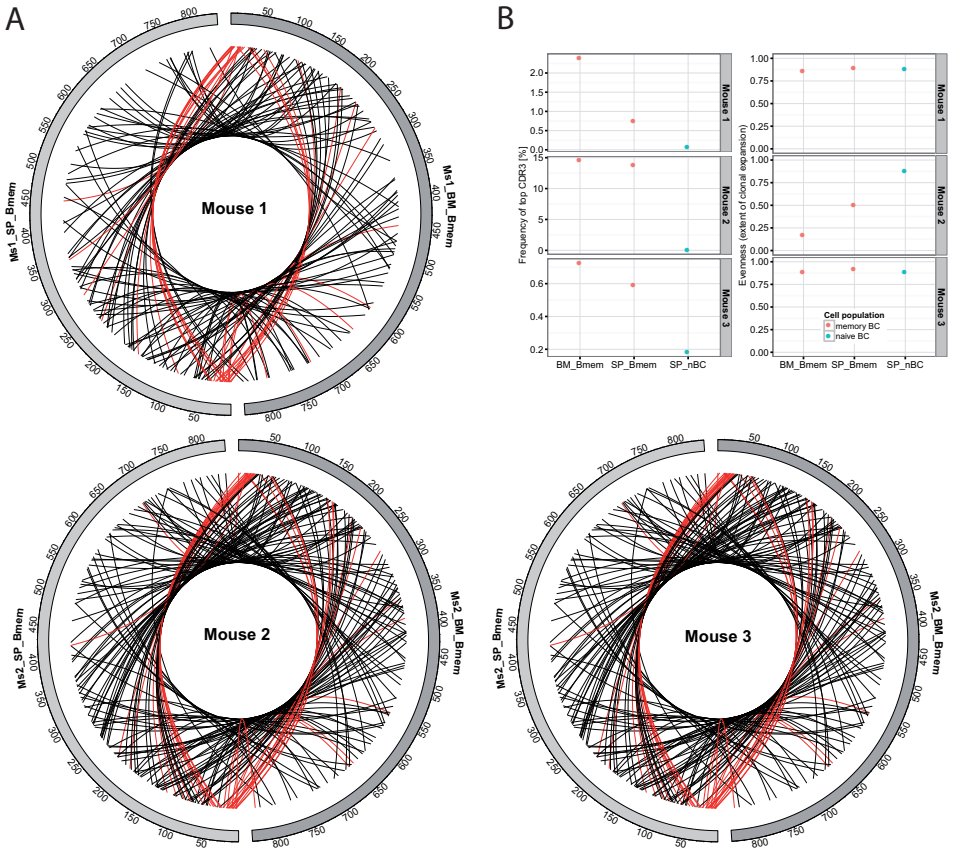

Figure 8: Distribution of most abundant B cell receptor heavy chain CDR3 sequences to spleen and bone marrow.
A: Analysis of CDR3 amino acid sequence overlap as a function of CDR3 sequence abundance ins spleen and bone marrow. Each arc represents one organ of one individual mouse. Within each arc, unique CDR3 sequences were ordered by frequency. Numbers on each arc indicate the total number of unique CDR3 amino acid sequences. Black and red lines connect rank by sequencing reads for a particular sequence in spleen and bone marrow. For improved readibility, red lines were drawn for those CDR3 sequences which were among the top 30 most abundant in at least one of the two samples.
B: Frequency of the single most abundant CDR3 amino acid sequence per organ and mouse (left) and the Shannon Evenness (right), where 0 denotes a highly clonally expanded repertoire and 1 low clonal expansion.

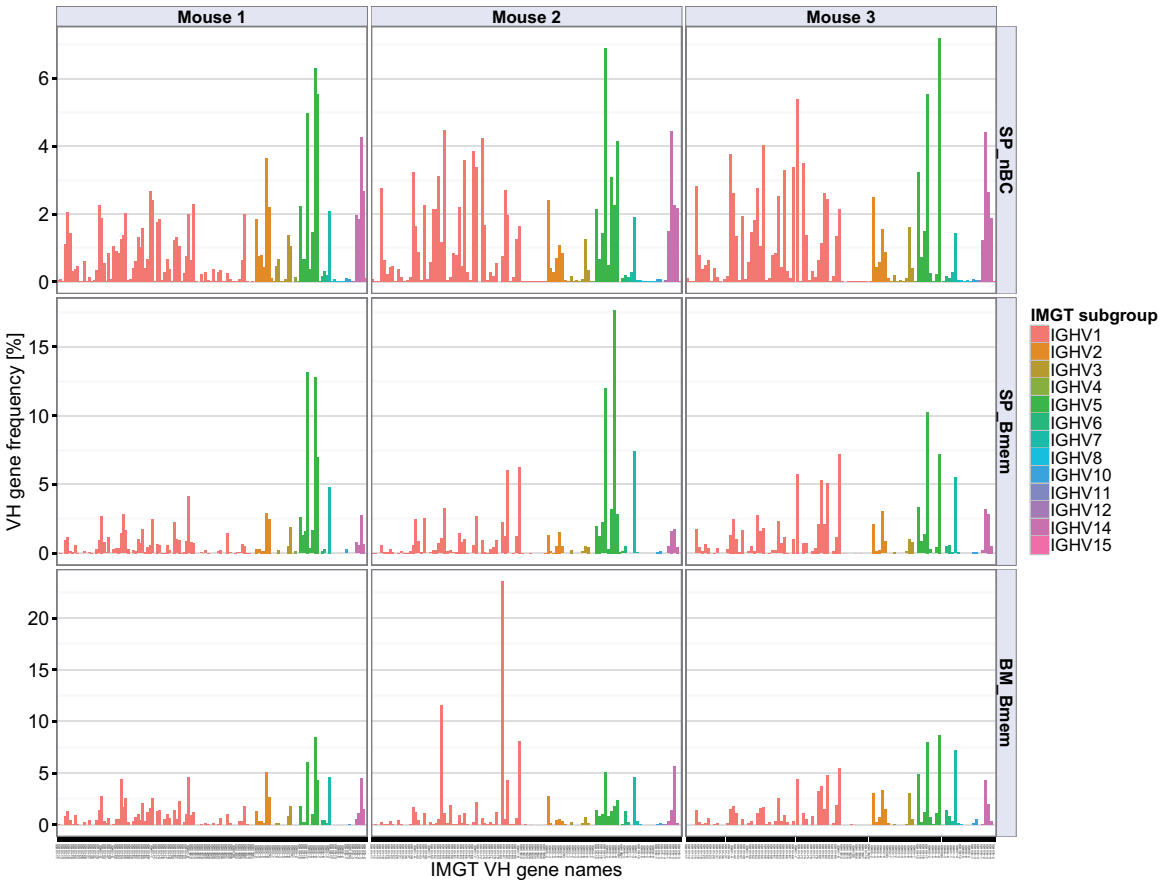

Figure 9: VH gene frequency distribution in spleen naïve (SP_nBC), spleen IgG_{2b} memory (SP_Bmem), and bone marrow IgG_{2b} memory (BM_Bmem) of individual mouse.

Table 1: Genes up-regulated in bone marrow as compared to spleen memory IgG_2 cells.
Shown are all genes significantly up-regulated (limma $p < 0.05$) with a foldchange ≥ 2. Genes for which more than one probe yielded results with more than two-fold regulation were included once, values for the probe with highest mean.

Symbol	limma p	Spleen mean	Bone Marrow mean	foldchange (BM vs Spleen)
S100a9	1.35e-04	23.28	379.83	16.31
Sox4	7.34e-06	5.92	89.11	15.05
S100a8	2.68e-04	37.22	519.01	13.94
Rag1	3.01e-06	4.83	51.21	10.60
Chi3l3	1.34e-03	4.78	45.69	9.55
Lyz2	2.32e-03	42.80	380.19	8.88
Vpreb2	3.06e-05	9.31	74.40	7.99
Ngp	1.38e-04	30.60	180.98	5.92
Cd93	8.70e-04	10.14	52.99	5.23
Camp	2.01e-04	22.94	114.32	4.98
Gm6166	1.17e-04	22.13	84.24	3.81
Myb	3.64e-03	29.17	109.03	3.74
Il7r	1.06e-06	10.64	39.51	3.71
Lcn2	1.58e-02	7.57	26.41	3.49
Dntt	1.82e-03	11.03	38.07	3.45
Igj	3.61e-03	206.94	691.30	3.34
Ltf	2.62e-03	22.07	72.09	3.27
Fabp4	2.02e-04	4.76	13.17	2.76
Lpl	2.22e-03	9.73	26.80	2.75
Ifitm6	5.59e-03	8.48	23.22	2.74
Serinc5	2.91e-04	29.56	80.24	2.71
Sspn	1.33e-02	22.39	60.53	2.70
Clec7a	2.97e-04	11.79	31.82	2.70
Sepp1	2.38e-02	216.98	567.30	2.61
Prkar2b	5.11e-03	17.67	44.86	2.54
Syk	4.52e-02	65.84	165.92	2.52
Igll1	2.19e-03	19.19	48.21	2.51
Maf	1.12e-03	6.90	17.06	2.47
C1qb	2.91e-04	8.87	21.16	2.38
Akap12	1.22e-03	25.01	57.91	2.32
Rrm2	7.98e-03	32.85	75.77	2.31
Gm20425	5.52e-04	14.35	32.61	2.27
C1qa	3.36e-03	14.09	31.63	2.24
Ccl6	6.78e-03	12.09	26.81	2.22
Slc40a1	4.35e-03	38.05	83.47	2.19
Atp1b1	1.05e-02	86.44	180.45	2.09
Fcgr3	2.99e-02	11.96	24.65	2.06
Slc4a1	3.41e-02	14.30	29.17	2.04
Pbk	1.57e-02	14.49	29.51	2.04
Nufip2	6.70e-03	120.80	244.99	2.03

Table 2: Genes down-regulated in bone marrow as compared to spleen memory IgG_2 cells.
Shown are all genes significantly up-regulated (limma $p < 0.05$) with a foldchange ≤ 0.5. Genes for which more than one probe yielded results with more than two-fold regulation were included once, values for the probe with highest mean.

Symbol	limma p	Spleen mean	Bone Marrow mean	foldchange (BM vs Spleen)
Cd37	4.69e-02	1496.45	738.47	0.49
Emid1	3.06e-03	162.54	79.80	0.49
Nfkbid	1.51e-02	303.75	147.26	0.48
Pglyrp2	3.73e-03	26.32	12.72	0.48
Tox	2.86e-02	28.87	13.94	0.48
Smyd2	1.88e-02	47.74	22.97	0.48
Dph5	2.23e-02	95.72	45.58	0.48
Cxcl10	1.79e-03	14.56	6.86	0.47
Tnf	3.57e-03	66.23	31.19	0.47
Igkv12-46	1.63e-02	252.04	117.78	0.47
Dtx1	1.97e-04	749.50	345.08	0.46
Ada	3.47e-02	58.74	26.82	0.46
Hes1	1.39e-03	121.60	54.57	0.45
Rundc3b	3.16e-04	30.16	13.52	0.45
Rasgef1b	1.72e-03	87.28	38.60	0.44
Gpr34	5.81e-05	86.44	38.16	0.44
Il4i1	1.79e-03	292.87	124.00	0.42
Mybl1	3.37e-02	40.15	16.77	0.42
Hs3st1	3.11e-03	238.99	98.24	0.41
Serpinb6b	7.74e-03	32.15	13.20	0.41
Sema7a	3.30e-03	353.87	136.19	0.38
Fam167a	4.63e-03	59.85	22.27	0.37
Zc3h12c	1.53e-02	104.90	38.82	0.37
Klrb1f	2.50e-04	59.87	22.03	0.37
Serpine2	2.26e-02	49.73	18.27	0.37
Ccdc79	1.87e-04	39.40	13.84	0.35
Myc	3.09e-02	399.20	138.61	0.35
Cfp	1.54e-02	108.76	36.74	0.34
Nebl	6.37e-03	91.29	28.99	0.32
Cdk5r1	2.99e-02	108.33	34.39	0.32
Ccbp2	2.05e-02	611.64	186.38	0.30
Gp49a	5.64e-04	528.30	154.61	0.29
Asb2	1.02e-02	116.18	31.56	0.27
Cxcr7	1.69e-02	85.47	22.77	0.27
Pde4d	4.88e-05	52.92	14.08	0.27
Pla2g7	1.67e-03	94.98	22.64	0.24
Tlr3	3.01e-03	84.61	19.34	0.23
Cr2	4.09e-07	1236.62	235.57	0.19
Ffar2	3.27e-04	426.00	64.42	0.15
S1pr3	7.42e-03	790.72	118.07	0.15

Table 3: Genes up-regulated in spleen memory IgG2_2 (Bmem) as compared to naïve B cells (nBC). Shown are all genes significantly up-regulated (limma $p < 0.05$) with a foldchange ≥ 2. Genes for which more than one probe yielded results with more than two-fold regulation were included once, values for the probe with highest mean.

Symbol	limma p	naïve (mean)	memory (mean)	foldchange (memory vs naive)
Ighg2c	2.18e-06	48.96	1916.34	39.14
Gatm	3.11e-03	16.62	150.45	9.05
Cpd	6.61e-04	13.18	117.08	8.88
Fgl2	1.96e-04	56.66	466.76	8.24
Ighg2b	3.05e-03	927.69	6342.13	6.84
Igha	1.39e-03	181.89	1157.89	6.37
Gp49a	7.32e-05	126.83	771.25	6.08
Etl4	1.51e-04	13.45	76.97	5.72
Csf2rb	1.38e-04	119.56	640.70	5.36
Nt5e	2.45e-03	94.43	505.46	5.35
Tmem51	9.71e-06	52.38	275.87	5.27
Lmo7	2.04e-03	26.94	135.91	5.05
B4galt6	7.18e-05	29.54	147.57	4.99
Sspn	4.24e-03	218.79	1065.14	4.87
Lipc	9.28e-05	54.70	262.43	4.80
Itgax	1.48e-02	37.42	176.93	4.73
Runx2	2.84e-03	24.18	112.28	4.64
Tmem180	2.54e-03	7.28	33.53	4.60
Hba-a1	1.48e-03	1045.18	4792.54	4.59
Ahr	2.51e-02	49.91	228.26	4.57
S1pr5	3.28e-02	23.92	103.24	4.32
Cxcr3	5.19e-03	382.88	1649.09	4.31
Gpr34	3.31e-03	32.64	140.50	4.30
Gbp8	3.74e-02	52.12	217.71	4.18
S100a6	1.02e-02	112.43	468.52	4.17
Itgb1	1.48e-06	137.51	571.28	4.15
Spn	1.91e-03	84.65	347.00	4.10
Dmxl2	2.56e-03	15.55	62.69	4.03
Atp11a	7.78e-03	35.13	140.49	4.00
Sirpa	5.07e-06	60.79	242.19	3.98
Pla2g7	3.63e-03	47.42	188.54	3.98
App	2.16e-04	87.04	343.63	3.95
Cd9	1.45e-02	63.48	244.99	3.86
Serpine2	2.94e-03	25.27	97.14	3.84
Nid1	1.76e-03	154.37	592.09	3.84
Cdk14	7.63e-04	6.79	26.00	3.83
Axl	2.45e-03	17.43	65.77	3.77
Cxcr7	1.16e-02	36.41	137.26	3.77
Dnaja4	8.78e-04	17.56	65.23	3.71
Cadm1	1.64e-06	22.43	82.68	3.69
Ccbp2	1.62e-02	302.58	1107.77	3.66
Fam46c	4.94e-03	80.76	293.33	3.63
Mki67	8.59e-03	51.89	188.11	3.63
Emr1	2.99e-05	77.61	279.10	3.60
Hbb-b2	1.84e-03	669.19	2385.01	3.56
Osbpl3	3.53e-03	19.74	70.33	3.56
Aicda	1.55e-02	73.12	259.43	3.55
Fzd6	4.96e-02	26.09	91.85	3.52
Naip5	2.35e-03	92.06	322.84	3.51

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Table 3 – continued from previous page

Symbol	limma p	nBC mean	Bmem mean	foldchange
Nebl	2.53e-04	48.59	169.69	3.49
Fcer1g	1.02e-04	51.06	177.43	3.47
Zbtb32	3.86e-03	75.20	261.21	3.47
Oosp1	6.52e-03	20.98	72.65	3.46
Lcp2	6.93e-03	65.01	221.20	3.40
Ffar2	5.13e-03	175.15	595.40	3.40
Fah	1.05e-03	81.22	274.17	3.38
Mllt4	2.50e-03	43.44	145.39	3.35
Rhobtb1	5.08e-04	18.64	62.02	3.33
Camk2n1	8.94e-04	38.40	127.19	3.31
Prdm1	2.14e-05	16.60	54.10	3.26
Atxn1	1.12e-04	121.34	392.22	3.23
Lphn2	3.09e-02	10.68	34.32	3.21
Zfp612	4.18e-02	36.65	117.59	3.21
Ms4a4b	1.08e-03	51.85	165.82	3.20
Anxa2	7.67e-03	306.74	978.81	3.19
Havcr1	6.35e-03	76.41	243.31	3.18
Kit	1.87e-02	9.47	30.02	3.17
Actn1	3.44e-03	33.81	106.99	3.16
Basp1	1.18e-02	175.87	552.73	3.14
Slpi	1.61e-02	182.27	572.79	3.14
Ryk	8.47e-04	43.09	135.06	3.13
Morn1	1.59e-03	36.63	114.63	3.13
Rbm47	4.93e-03	15.04	46.61	3.10
Itm2c	1.69e-03	192.14	590.52	3.07
Lgals1	3.80e-03	330.74	1012.94	3.06
Tjp1	1.48e-03	15.67	47.81	3.05
Tbc1d9	5.94e-05	19.36	58.64	3.03
Ccr1	2.76e-03	74.11	223.30	3.01
St8sia6	6.09e-04	393.77	1182.22	3.00
Tbx21	8.70e-03	189.17	565.57	2.99
Cd44	3.30e-03	598.47	1774.44	2.96
Grk5	2.44e-04	94.38	277.80	2.94
Adm	4.38e-05	24.21	70.91	2.93
Mpeg1	3.10e-03	496.59	1454.44	2.93
Abcb1b	1.20e-03	30.97	90.45	2.92
Sema7a	1.76e-02	209.28	606.07	2.90
Rilpl1	3.08e-05	20.48	59.19	2.89
Ccr9	4.73e-03	17.01	48.66	2.86
Tjp2	5.84e-03	41.71	118.89	2.85
Hmgn3	1.82e-03	283.65	805.38	2.84
S100a4	4.58e-04	27.14	76.91	2.83
Sema4f	1.32e-02	16.13	45.33	2.81
Cbfa2t3	5.99e-04	274.93	772.45	2.81
Tnfsf13b	1.68e-03	22.41	62.89	2.81
Hoxa5	3.33e-02	29.76	83.24	2.80
Atxn7l1	3.44e-03	25.90	71.87	2.78
Krt222	3.20e-03	52.43	144.54	2.76
Myo1f	1.66e-02	136.60	375.61	2.75
Mybl1	1.37e-03	23.05	63.22	2.74
Wipf3	2.18e-02	33.60	91.82	2.73
Dapk2	1.13e-02	30.92	83.85	2.71
Il2rb	1.68e-04	40.85	110.76	2.71
Ly96	2.89e-04	68.07	182.66	2.68

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Symbol	limma p	nBC mean	Bmem mean	foldchange
Ak8	2.73e-03	16.91	45.29	2.68
Lacc1	2.82e-03	62.62	167.54	2.68
Atxn1	1.78e-02	96.58	257.90	2.67
Zbtb20	1.31e-02	529.55	1408.81	2.66
Rcn3	6.57e-04	79.37	210.65	2.65
Diras2	2.17e-03	101.42	266.49	2.63
Gpr137b	8.49e-03	299.56	786.45	2.63
Trabd2b	1.21e-03	49.24	129.12	2.62
Apoe	6.45e-03	384.96	1003.67	2.61
Cdh17	1.17e-02	120.75	312.32	2.59
Ccdc122	1.84e-03	64.72	167.21	2.58
Hs3st1	8.40e-03	161.51	416.93	2.58
Dnm3	3.70e-05	22.53	58.14	2.58
Fam135a	1.29e-03	10.92	28.09	2.57
Akap5	2.33e-03	17.05	43.68	2.56
Neil1	1.41e-02	287.20	732.93	2.55
Cdc14b	1.16e-02	96.89	246.81	2.55
Hspa12a	2.67e-04	31.46	79.71	2.53
Slc39a4	3.99e-03	15.04	38.08	2.53
Scamp1	5.85e-06	13.34	33.75	2.53
Sowahc	5.23e-03	76.93	194.41	2.53
Plcd3	1.89e-02	33.51	83.97	2.51
Specc1	2.92e-03	9.99	24.92	2.49
Tmbim1	1.25e-03	36.14	89.77	2.48
S1pr3	2.88e-02	541.41	1344.53	2.48
Upb1	1.11e-04	40.02	98.90	2.47
S100a8	2.74e-02	13.83	34.16	2.47
Gm10451	1.19e-03	31.86	78.53	2.47
Lipa	2.54e-04	77.17	190.01	2.46
Clec2g	7.18e-05	7.60	18.70	2.46
Itgae	4.93e-03	29.06	71.52	2.46
Nostrin	1.55e-05	16.20	39.68	2.45
Anxa6	4.36e-03	149.44	365.98	2.45
Kcnn4	3.48e-02	168.72	412.49	2.44
Sorbs2	6.77e-03	46.28	113.11	2.44
Ccdc28b	1.50e-02	96.25	234.27	2.43
Bhlhe41	2.97e-03	31.62	76.53	2.42
Rhbdf1	1.69e-02	214.18	517.92	2.42
Gm3715	3.64e-02	82.50	199.14	2.41
Rgs13	3.62e-02	20.03	48.10	2.40
Fas	1.33e-02	105.11	252.28	2.40
Tmcc3	3.90e-05	109.25	262.17	2.40
Igj	2.70e-02	119.05	285.21	2.40
Sh3bgrl2	3.78e-05	8.83	20.94	2.37
Slc7a7	5.39e-03	59.91	142.08	2.37
Asb2	4.13e-02	84.30	199.77	2.37
Rap1gap2	2.15e-02	75.41	178.53	2.37
Ahnak	7.05e-03	632.26	1493.56	2.36
Neo1	3.03e-02	12.26	28.96	2.36
Ppfibp2	1.85e-04	81.82	193.05	2.36
Tcf7	7.94e-03	30.18	70.98	2.35
Ass1	2.50e-04	36.69	86.24	2.35
Nrp2	7.72e-04	54.35	127.61	2.35
Cd80	1.69e-02	23.12	53.99	2.33

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Table 3 – continued from previous page

Symbol	limma p	nBC mean	Bmem mean	foldchange
Ehf	3.36e-03	6.32	14.74	2.33
Emb	1.21e-02	53.28	123.88	2.33
Pld4	2.17e-03	597.77	1387.42	2.32
Tmprss13	3.84e-03	29.61	68.48	2.31
Klrb1f	1.40e-02	43.07	99.54	2.31
Kcnk6	2.17e-02	84.37	194.30	2.30
Zfp365	9.68e-04	9.93	22.76	2.29
Jdp2	2.19e-02	46.57	105.57	2.27
Tmcc2	4.57e-02	6.37	14.45	2.27
Nipa1	1.15e-02	10.13	22.93	2.26
Ighg1	2.08e-02	68.50	154.92	2.26
Tenm4	4.99e-03	17.52	39.56	2.26
P4htm	5.41e-03	31.22	70.49	2.26
Ipcef1	5.32e-03	126.92	286.37	2.26
Hpse	7.94e-05	180.24	406.57	2.26
Hspb3	2.75e-02	16.12	36.34	2.25
Cobl	8.19e-03	14.70	33.11	2.25
Nek6	2.27e-03	41.43	93.31	2.25
Vps26b	1.59e-02	18.09	40.70	2.25
Pls3	3.71e-03	17.25	38.77	2.25
Laptm4b	2.90e-02	59.23	133.15	2.25
S100a9	2.75e-02	17.23	38.62	2.24
Camkmt	1.05e-02	38.20	85.56	2.24
Ptpn22	8.52e-03	526.83	1178.67	2.24
Optn	1.17e-03	66.33	148.28	2.24
Pde8a	1.17e-05	158.59	354.36	2.23
Zdhhc2	1.03e-02	24.58	54.85	2.23
Rassf4	3.36e-03	263.92	587.70	2.23
Nek2	2.02e-03	44.77	99.35	2.22
Tmem154	9.52e-04	152.10	337.06	2.22
Parm1	4.11e-02	71.28	157.71	2.21
Mreg	4.16e-04	197.36	436.26	2.21
Pon3	1.36e-02	46.41	102.27	2.20
Slc36a4	2.15e-02	74.29	163.53	2.20
Usp11	2.22e-02	89.26	196.13	2.20
Cd59a	5.48e-03	25.22	55.29	2.19
Celsr1	1.57e-02	7.15	15.61	2.18
Ryr1	2.38e-03	165.01	358.76	2.17
Fscn1	1.63e-02	432.70	940.13	2.17
Eid2	5.65e-03	59.22	128.66	2.17
Cacna1s	9.28e-03	61.98	134.25	2.17
Ighg3	1.30e-02	114.49	246.41	2.15
Lrig1	7.54e-03	9.38	20.07	2.14
Lysmd2	3.47e-06	55.13	117.90	2.14
Clic4	6.58e-03	876.94	1866.05	2.13
Zc3h12c	1.23e-02	85.05	180.64	2.12
Gstt1	8.16e-04	32.08	68.02	2.12
Adssl1	1.05e-04	73.48	155.75	2.12
Eml6	3.84e-03	11.07	23.42	2.11
Tnfrsf1b	8.60e-04	132.57	279.61	2.11
Pdcd1lg2	4.80e-06	135.13	284.90	2.11
Pvt1	1.46e-02	141.19	297.63	2.11
Nusap1	7.10e-03	54.41	114.50	2.10
Parp3	1.88e-03	241.92	508.76	2.10

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Table 3 – continued from previous page

Symbol	limma p	nBC mean	Bmem mean	foldchange
Cxcl10	1.41e-03	12.18	25.59	2.10
Traf1	6.32e-03	250.73	525.46	2.10
Kcnt2	2.48e-03	5.16	10.80	2.09
Gramd1b	8.88e-04	46.72	97.72	2.09
Bmpr1a	2.08e-02	90.04	187.81	2.09
Inf2	1.85e-03	148.89	310.52	2.09
BC064078	2.90e-03	183.08	381.54	2.08
Pogk	8.46e-05	31.14	64.70	2.08
Cln8	7.64e-03	47.08	97.53	2.07
Tmtc2	7.73e-04	9.56	19.80	2.07
Mical3	4.44e-03	61.58	127.30	2.07
Plcxd1	5.59e-04	13.10	26.95	2.06
Mll1	1.55e-02	515.70	1060.89	2.06
Smpdl3a	1.00e-02	473.76	973.92	2.06
Anks1b	2.06e-02	4.56	9.37	2.05
Wee1	9.31e-03	107.16	220.15	2.05
Myadm	3.08e-02	606.03	1244.37	2.05
Cd86	4.01e-02	354.40	727.53	2.05
Pawr	3.91e-02	30.86	63.31	2.05
Syt11	8.15e-03	108.01	221.52	2.05
Zeb2	3.61e-03	45.95	94.15	2.05
Rdh12	4.39e-03	176.31	360.78	2.05
Pik3r6	4.04e-02	208.18	425.22	2.04
Dnase1l3	6.66e-04	33.96	69.33	2.04
Pbx3	3.35e-03	104.27	212.78	2.04
Cdon	1.54e-02	49.55	100.97	2.04
Lair1	2.02e-04	19.59	39.80	2.03
Slc43a3	1.74e-02	25.28	51.17	2.02
Slc20a2	1.33e-03	146.44	296.20	2.02
Chka	1.19e-04	136.36	275.42	2.02
Racgap1	4.34e-03	56.91	114.49	2.01
F9	1.16e-02	22.66	45.42	2.00
Vwa5a	2.41e-02	112.09	224.31	2.00

Table 4: Genes down-regulated in spleen memory IgG_2 (Bmem) as compared to naïve B cells (nBC). Shown are all genes significantly up-regulated (limma $p < 0.05$) with a foldchange ≤ 0.5. Genes for which more than one probe yielded results with more than two-fold regulation were included once, values for the probe with highest mean.

Symbol	limma p	naïve (mean)	memory (mean)	foldchange (memory vs naive)
Slfn2	3.05e-02	1751.106	871.7285	0.50
Trpm7	1.91e-02	291.9901	145.0072	0.50
Zfp36l1	2.36e-03	1214.7645	600.6097	0.49
Socs5	3.02e-02	219.3817	108.1347	0.49
Luc7l	1.28e-02	124.9332	61.5343	0.49
Rock1	2.87e-02	333.1156	164.0562	0.49
Frmd4a	3.38e-04	38.9544	19.1726	0.49
Pde7a	2.02e-02	232.407	114.3549	0.49
Smc1a	8.14e-03	198.1094	96.8956	0.49
Bach1	3.16e-03	58.3206	28.4022	0.49

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Table 4 – continued from previous page

Symbol	limma p	nBC mean	Bmem mean	foldchange
Hist1h2ap	1.51e-02	1201.0854	582.4962	0.48
Ctbp2	1.89e-03	44.2981	21.4815	0.48
Arid4b	1.34e-03	202.6679	98.1716	0.48
Cap1	7.34e-03	427.5306	206.7598	0.48
Rnf122	4.14e-03	257.0435	123.8894	0.48
Psmd4	2.78e-02	531.0741	255.0735	0.48
Abcc1	5.51e-03	50.3008	24.0811	0.48
Gch1	4.21e-02	265.9141	127.2428	0.48
Acaa2	2.77e-02	118.4778	56.4746	0.48
Akap12	8.22e-04	132.775	63.1393	0.48
Ddx46	2.88e-03	119.0824	56.6109	0.48
Junb	4.96e-02	297.4498	140.796	0.47
Ripk3	3.82e-02	196.1621	92.7458	0.47
Idi1	8.60e-03	110.9276	52.3743	0.47
Sptbn1	8.44e-03	424.1501	199.9973	0.47
Zfp608	1.68e-03	585.4379	275.0565	0.47
Pkn1	4.77e-02	41.5946	19.542	0.47
Treml2	2.67e-02	468.8969	220.2589	0.47
Rbm6	3.71e-03	181.4643	85.1201	0.47
Ankfy1	1.19e-02	37.4736	17.5687	0.47
Ifngr2	2.92e-02	303.9382	142.4308	0.47
Prrc2c	7.45e-03	32.2299	15.071	0.47
Ankrd11	1.08e-02	532.434	248.9218	0.47
Cpsf6	2.88e-02	65.3342	30.4701	0.47
Chd4	2.03e-03	428.4511	197.8785	0.46
Atic	2.32e-03	127.7109	58.8934	0.46
Pten	2.19e-02	120.9786	55.7311	0.46
Hnrnpr	4.22e-02	188.669	86.8209	0.46
Rfc1	2.40e-02	152.0779	69.9038	0.46
Brwd1	1.81e-02	451.8833	206.7681	0.46
Timeless	1.06e-02	66.7946	30.3803	0.45
Ets1	9.08e-03	426.7923	193.6162	0.45
Baz2b	3.69e-02	110.5577	49.7162	0.45
Sgms1	9.48e-05	536.806	241.1199	0.45
Acpp	5.84e-03	25.9676	11.6627	0.45
Utp23	7.08e-03	76.7908	34.4549	0.45
Fcer2a	5.00e-03	2313.5762	1034.9199	0.45
Rnf114	4.04e-02	320.9786	143.4925	0.45
Dgkd	1.35e-03	47.1815	21.0409	0.45
Pgm2l1	1.68e-02	69.1332	30.8274	0.45
Kpnb1	4.34e-02	18.9811	8.4578	0.45
Zmynd11	1.67e-02	175.695	78.1933	0.45
Pnn	3.39e-03	47.9022	21.2716	0.44
Eid1	2.60e-04	359.5545	158.7167	0.44
Btg2	3.49e-02	1279.9305	560.1772	0.44
Edaradd	2.96e-02	227.5042	98.5453	0.43
H2-T24	4.48e-02	144.7613	62.648	0.43
Ppargc1a	3.97e-02	30.2556	13.0134	0.43
Stat2	2.37e-02	66.4887	28.1225	0.42
Arfgef1	1.39e-02	66.2569	27.9758	0.42
Zc3h13	5.20e-03	215.8886	91.1073	0.42
Rbm25	8.57e-03	64.2527	26.9933	0.42
Smc2	5.95e-03	75.1829	31.319	0.42
Lifr	1.66e-02	74.5472	31.0034	0.42

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Table 4 – continued from previous page

Symbol	limma p	nBC mean	Bmem mean	foldchange
Jak1	2.88e-02	234.3978	97.3017	0.42
Smad1	2.12e-02	59.7424	24.5689	0.41
Zfp318	1.53e-03	427.1319	175.4947	0.41
Heg1	4.36e-02	328.1528	134.3018	0.41
Kdm6b	3.35e-02	363.3948	148.3635	0.41
Tra2b	2.16e-02	235.5423	95.4859	0.41
Rtp4	1.48e-02	393.8404	158.9805	0.40
Eif3a	1.19e-02	179.3997	70.6177	0.39
Sorl1	1.29e-03	261.1188	101.4771	0.39
Spaca1	1.15e-03	36.5889	13.9375	0.38
Csrp2	1.04e-03	50.7424	19.2638	0.38
Sf3b2	4.09e-03	619.3463	231.8472	0.37
Gpr171	4.15e-02	1416.6696	528.1106	0.37
Cd2ap	7.93e-03	230.635	84.6944	0.37
Tnik	1.18e-02	66.0408	23.6666	0.36
Pank3	4.51e-02	132.5943	46.4006	0.35
Sdc4	5.62e-03	816.6609	281.0916	0.34
Cnksr3	4.74e-03	101.2672	34.3977	0.34
Rnf145	3.06e-02	154.312	52.3408	0.34
Bptf	8.39e-03	103.0084	33.9521	0.33
Zfp53	1.11e-02	558.5267	182.5274	0.33
Fam101b	1.23e-04	704.0447	226.6349	0.32
Tle1	2.06e-04	80.0835	25.3192	0.32
Itgb3	2.33e-02	211.699	66.9156	0.32
Tmod3	7.02e-04	803.4293	252.4842	0.31
Il12a	4.76e-02	159.419	50.0012	0.31
Pde2a	4.02e-02	889.2819	270.3735	0.30
Calcrl	5.58e-03	200.3399	60.7317	0.30
Mcoln3	2.05e-02	109.9694	33.3309	0.30
Cd93	7.59e-03	71.6875	20.3144	0.28
Agpat9	1.66e-02	113.7446	32.0356	0.28
Afap1	4.64e-02	165.6359	45.9927	0.28
Satb1	4.27e-02	1293.5544	359.0945	0.28
Rgl1	2.94e-02	91.5046	25.2845	0.28
Vav3	6.06e-03	111.5444	29.712	0.27
Zfp191	2.11e-03	195.8056	51.2377	0.26
Tsc22d1	9.38e-03	229.7779	59.0539	0.26
Tsc22d1	1.59e-02	123.6942	30.4278	0.25
Ctnna1	6.22e-03	340.9226	80.0979	0.23
Ighm	1.84e-02	4642.9888	918.9008	0.20
Inpp4b	4.59e-02	148.4967	27.2579	0.18
Maf	2.12e-03	86.7912	15.2933	0.18
Ctnna1	6.60e-03	413.5661	70.3076	0.17
Sox4	3.97e-03	83.0683	12.352	0.15