Regulation of innate and adaptive B-cell responses by the proteins SLy2 and CXCR1

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Abbreviations

aa	Amino acid
ASC	Antibody-secreting cell
BCR	B-cell receptor
BM	Bone marrow
Btk	Bruton's tyrosine kinase
Ca^{2+}	Calcium
CD	Cluster of differentiation
CXCR	CXC chemokine receptor
FO	Follicular
GC	Germinal center
GPCR	G-protein coupled receptor
HDAC	Histone deacetylase
Ig	Immunglobulin
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
kDa	Kilodalton
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MZ	Marginal zone
NLS	Nuclear localization signal
P23	Pneumovax23
PCV13	Prevenar13
PI3K	Phosphatidylinositol 3-kinase
pPS	Pneumococcal polysaccharide
PRR	Pattern-recognition receptor

SAM	Sterile α motif
SH3	Src homology 3
SHM	Somatic hypermutation
SLy2	Src homology domain 3 lymphocyte protein 2
TD	Thymus/ T-cell dependent
T_{FH} cell	T follicular helper cell
TI	Thymus/ T-cell independent
TLR	Toll-like receptor

Summary

Innate and adaptive B cells are the main cellular effectors of humoral immunity as they possess the unique ability to produce life-saving antibodies. Depending on the type of antigen that is encountered, B-cell responses can be characterized into two major categories, namely thymus/ T-cell-independent (TI) and thymus/ T-cell-dependent (TD). Responses to TI antigens are mainly mediated by innate B-cell subsets that rapidly provide low-affinity antibodies as an important first line of defense. On the other hand, TD antigens trigger an adaptive germinal center reaction, resulting in generation of high-affinity plasma cells and the formation of immunological memory. Both, TI and TD antibody responses are tightly controlled by various regulatory pathways that remain incompletely understood.

In this work, we dissected the role of the immunoinhibitory adaptor protein SLy2 and the chemokine receptor CXCR1 for innate and adaptive B-cell responses. To this end, we systematically analyzed B-cell compartments and antibody titers in mice globally lacking the expression of SLy2 or CXCR1. Complementarily, we studied SLy2-overexpressing mice.

Initially, we performed *ex vivo* analyses on B cells and natural antibodies under homeostatic conditions. In addition, *in vitro* proliferation, class-switch and antibody secretion of isolated B cells were investigated. Subsequently, animals were immunized with TI and TD antigens to measure their specific B-cell response. Since infections with the human pathogen *Streptococcus pneumoniae* are responsible for a huge burden of disease worldwide despite the availability of vaccines, we set a special focus on pneumococcal antigens. For this reason, we also implemented *in vivo* survival studies in the context of acute pneumococcal lung infection.

Our findings demonstrate that SLy2 negatively controls the production of IgM and IgG antibodies to TI and TD pneumococcal vaccines, respectively. We further show a severe reduction of bone marrow-resident progenitor cells in SLy2-overexpressing mice. By contrast, SLy2-deletion causes an improvement of B-1 cell immunity. Besides, we report a previously unknown relevance of CXCR1 for adaptive B-cell responses. Specifically, CXCR1-deficient mice harbor increased populations of follicular B cells, accompanied by strengthened IgG_1 responses to TD immunization. In summary, we provide novel insights into the regulation of antibody responses which could be useful for the development of future immunotherapeutic interventions.

Zusammenfassung

Angeborene und adaptive B-Zellen sind die wichtigsten zellulären Effektoren der humoralen Immunität, da sie die einzigartige Fähigkeit besitzen, lebensrettende Antikörper zu produzieren. In Abhängigkeit des induzierenden Antigens können B-Zell-Antworten in zwei Hauptkategorien eingeteilt werden: Thymus/T-Zell-unabhängig (TU) und Thymus/T-Zell-abhängig (TA). Reaktionen auf TU-Antigene werden hauptsächlich durch angeborene B-Zellen vermittelt, indem diese niedrig-affine Antikörper als erste wichtige Verteidigungslinie bereitstellen. TA-Antigene hingegen triggern eine adaptive Keimzentrumsreaktion, die zur Ausdifferenzierung hoch-affiner Plasmazellen und der Bildung eines immunologischen Gedächtnisses führt. Diese Antikörperreaktionen unterliegen der strengen Regulation verschiedenster molekularer Kontrollmechanismen, die bis heute nicht vollständig verstanden sind.

In dieser Arbeit wurde die Rolle des Adapterproteins SLy2 und des Chemokin-Rezeptors CXCR1 für angeborene und adaptive B-Zell-Reaktionen untersucht. Hierfür analysierten wir systematisch die B-Zell-Kompartimente und Antikörpertiter in SLy2- und CXCR1-defizienten Mäusen. Ergänzend wurden SLy2-überexprimierende Mäuse für die Studie herangezogen. Zunächst führten wir *ex vivo*-Untersuchungen auf Basis homöostatischer Bedingungen durch. Außerdem wurde die *in vitro*-Proliferation und -Antikörpersekretion isolierter B-Zellen studiert. Anschließend wurden die Tiere immunisiert und ihre spezifische B-Zell-Antwort gemessen. Da Pneumokokken-Infektionen trotz verfügbarer Impfstoffe weltweit für eine enorme Krankheitslast verantwortlich sind, legten wir einen besonderen Fokus auf Pneumokokken-Antigene. Zusätzlich führten wir *in vivo*-Überlebensstudien im Kontext einer akuten bakteriellen Pneumonie durch. Unsere Ergebnisse zeigen, dass SLy2 die Produktion von IgM- bzw. IgG-Antikörpern gegen TU- bzw. TA-Pneumokokken-Impfstoffe inhibiert. Ferner verursacht die Überexpression von SLy2 eine starke Reduktion Knochenmark-residenter Vorläuferzellen. Im Gegensatz dazu zeigt sich eine verbesserte B-1-Zell-Immunität nach Deletion des Proteins. Weiterhin demonstrieren wir eine bisher unbekannte Bedeutung von CXCR1 für adaptive B-Zell-Antworten. Konkret beherbergen CXCR1-defiziente Mäuse mehr follikuläre B-Zellen, einhergehend mit verstärkten IgG_1 Reaktionen auf TA-Vakzinierung.

Zusammenfassend liefern unsere Ergebnisse neue wichtige Einblicke in die Regulation von Antikörperantworten, welche für die Entwicklung neuer immuntherapeutischer Interventionen von Relevanz sein könnten.

Publications

Accepted and published

- Jaufmann J, Tümen L and Beer-Hammer S (2021) SLy2-overexpression impairs B-cell development in the bone marrow and the IgG response towards pneumococcal conjugate-vaccine. *Immun Inflamm Dis.* 2021;1–14. *DOI: 10.1002/iid3.413.*
- Jaufmann J, Carevic M, Tümen L, Eliacik D, Schmitt F, Hartl D and Beer-Hammer S (2021) Enhanced IgG1-mediated antibody response towards thymus-dependent immunization in CXCR1-deficient mice. *Immun Inflamm Dis*. 2021;9:210–222. *DOI: 10.1002/iid3.380.*
- Jaufmann J, Tümen L, Schmitt F, Schäll D, von Holleben M and Beer-Hammer S. (2020) SLy2-Deficiency promotes B-1 Cell Immunity and triggers enhanced Production of IgM and IgG$_2$ Antibodies against Pneumococcal Vaccine. *Immun Inflamm Dis. 2020; 8: 736– 752. DOI:10.1002/iid3.365.*
- Jaufmann J, Lelis FJN, Teschner AC, Fromm K, Rieber N, Hartl D and Beer-Hammer S. (2020) Human monocytic myeloid-derived suppressor cells impair B-cell phenotype and function in vitro. *European Journal of Immunology 50(1):33-47. DOI:10.1002/eji.201948240.*
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Declaration according to § 5 Abs. 2 No. 8 of the PhD regulations of the Faculty of Science

-Collaborative Publications-

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List of publications in thesis

1. Jaufmann J, Tümen L, Schmitt F, Schäll D, von Holleben M and Beer-Hammer S. (2020) SLy2-Deficiency promotes B-1 Cell Immunity and triggers enhanced Production of IgM and IgG_2 Antibodies against Pneumococcal Vaccine. *Immun Inflamm Dis. 2020; 8: 736– 752. DOI:10.1002/iid3.365.*

2. Jaufmann J, Carevic M, Tümen L, Eliacik D, Schmitt F, Hartl D and Beer-Hammer S. (2021) Enhanced IgG_1-mediated antibody response towards thymus-dependent immunization in CXCR1-deficient mice. *Immun Inflamm Dis*. 2021;9:210–222. *DOI: 10.1002/iid3.380.*

3. Jaufmann J, Tümen L and Beer-Hammer S (2021) SLy2-overexpression impairs B-cell development in the bone marrow and the IgG response towards pneumococcal conjugate-vaccine. *Immun Inflamm Dis.* 2021;1–14. *DOI: 10.1002/iid3.413.*

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Date, Signature of the doctoral committee or at least of one of the supervisors

1. Introduction

1.1. B cells

1.1.1. B cells as antibody-secreting cells

The production of specific antibodies (or immunglobulins) is a feature being exclusive to B cells and makes them indispensable for the maintenance of humoral immunity (1). Classically, B cells develop from hematopoietic progenitors within the bone marrow (BM), thereby undergoing several stages of differentiation and selection. This process involves individual rearrangement of the immunglobulin (Ig) gene loci and finally results in an enormous diversity of specific B cells (2). Expressing a unique B-cell receptor (BCR) on their surface, mature naïve B cells then leave the BM to undergo distinct fates of differentiation in the periphery (3, 4). As a consequence of stimulation, these B cells undergo marked alterations in morphology and gene expression and eventually differentiate into antibody-secreting cells (ASCs), providing humoral immune protection (5).

1.1.2. B-cell antigen recognition

Generally, antigen-recognition of B cells is driven by the engagement of the BCR-complex upon binding to its specific epitope and subsequent induction of intracellular signaling cascades (6). The antigen-specific BCR itself is composed of two heavy and two light polypeptide chains and is associated with the heterodimeric invariant proteins Igα and Igß (CD79a and CD79b, respectively). Upon BCR-antigen ligation on the surface, the signal is transduced into the cell via the cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs) of Igα and Igß (7, 8). Initially, Src-family tyrosine kinases are recruited to phosphorylate the ITAMs, which in turn leads to an activation of Syk and the induction of several kinase cascades, including the Bruton’s tyrosine kinase (Btk), phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways (7, 9). Moreover, the B cell undergoes actin remodeling, metabolic reprogramming and calcium (Ca^{2+})-influx. Eventually, these signaling events induce alterations in gene expression and effector differentiation of the B cell (10).

In addition, the BCR-complex contains accessory, regulatory co-receptors such as CD19, CD21 and CD81. Depending on the type of B cell and the background of stimulation, these co-receptors are able to either strengthen or suppress BCR-mediated signaling (11). Figure 1 provides a simplified overview of the BCR-complex.

Important to mention, full B-cell activation normally requires a second, co-stimulatory signal derived from pattern-recognition receptor (PRR) ligands, cytokines and/ or signals provided by other immune cells (12). Sole BCR-engagement without secondary stimulation might lead to Ca^{2+}-induced B-cell apoptosis as a mechanism to maintain self-tolerance (10).

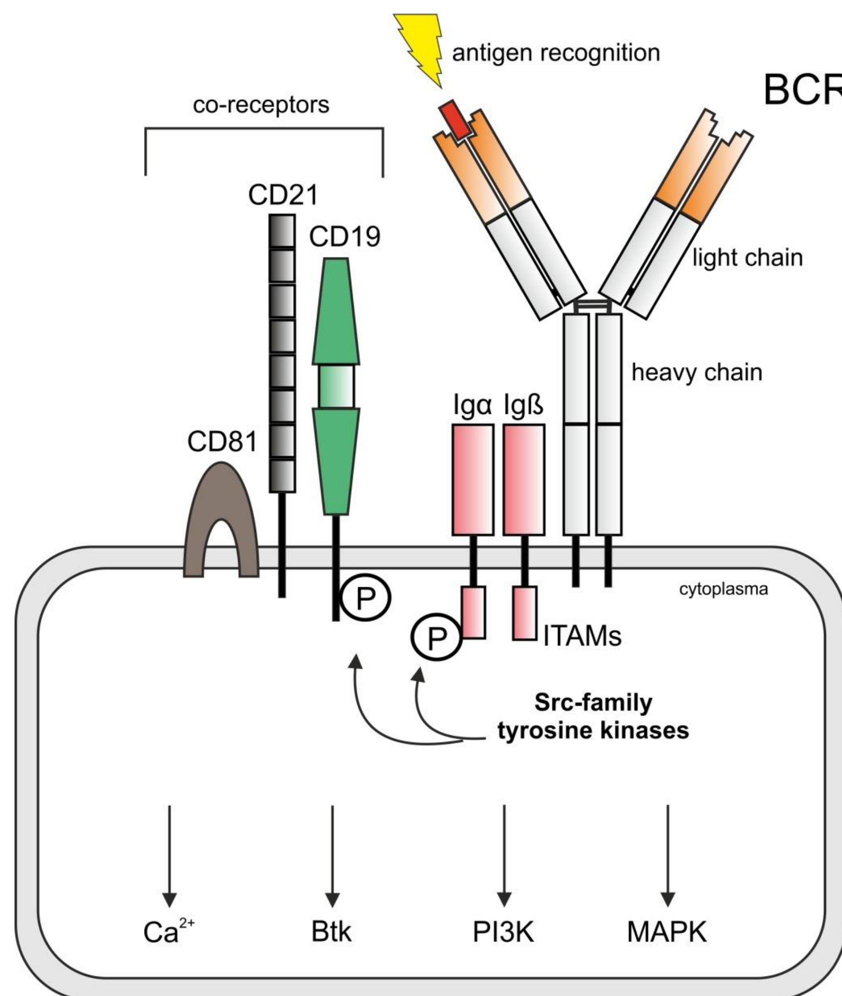

Figure 1: The B-cell receptor complex. The antigen-specific BCR is composed of two heavy and two light chains, coupled to the invariant proteins Igα and Igß. The BCR-complex further contains various regulatory co-receptors, including CD19, CD21 and CD81. Upon antigen recognition, the cytoplasmic ITAMs of Igα and Igß get phosphorylated and induce various kinase cascades as well as metabolic and cytoskeletal remodeling. Modified from "*Janeway's Immunobiology*", 9th Edition. Murphy K and Weaver C. Garland Science, Taylor & Francis Group (2017). *p. 265-282.*

1.1.3. Antibody structure and isotypes

Antibodies are the soluble version of a BCR and are secreted into the blood in large quantities. They consist of four main polypeptide chains, namely two light and two heavy chains, which are each further divided into a variable (V_L and V_H) and a constant (C_L and C_H) region. The heavy and the light chains are connected through disulfide bonds and a central hinge region defines two major Ig fragments, namely the $F_{(ab)2}$ and the F_C part. While the variable region of an antibody contains the antigen-binding site and defines its specificity, the constant region is important to its secondary effector function (13). By means of their F_C-receptors, other immune cells are able to detect antibodies bound on the surface of pathogens (14). Figure 2 provides a schematic overview of the structure of a typical antibody molecule.

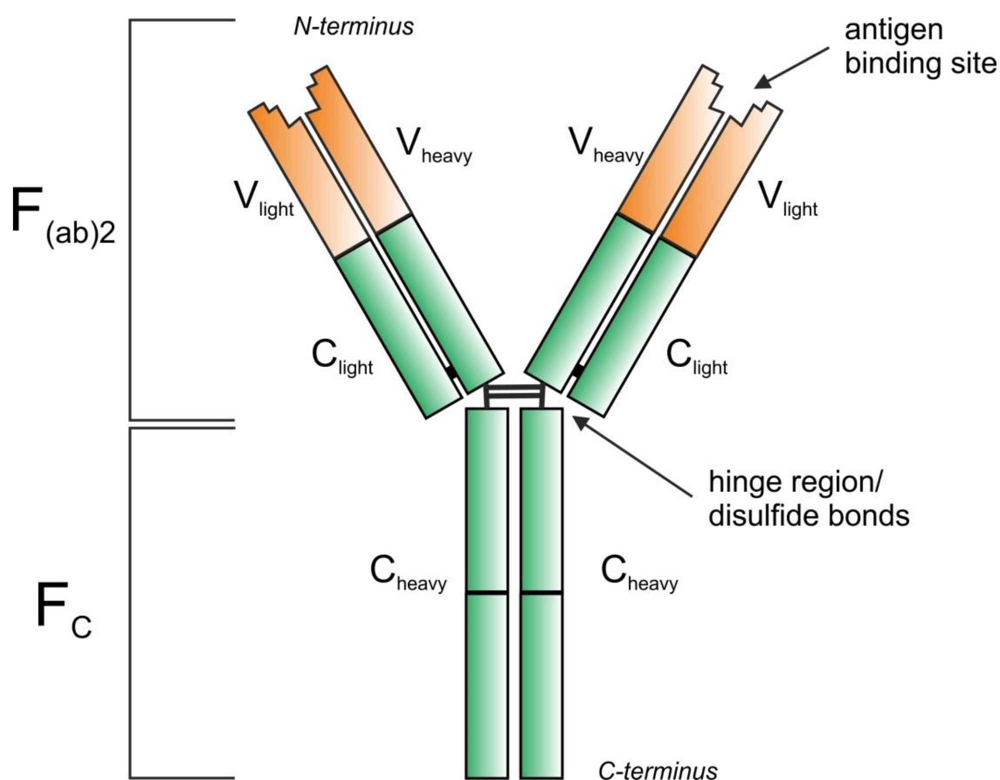

Figure 2: The basic structure of a typical antibody molecule. Antibodies are built up of two heavy and two light polypeptide chains; both of which can be further separated into a variable light (V_L), variable heavy (V_H), constant light (C_L) and constant heavy (C_H) chain. The heavy and the light chains are connected through disulfide bonds. A central hinge region separates two main antibody fragments: Two F_{ab} parts, containing the variable regions; and the F_C part made up of two constant heavy chains. The F_C-part is especially important for the functional activity of the antibody, since it can be detected by F_C-Receptors expressed on other immune cells. One antibody possesses two antigen-binding sites at its N-terminus. Modified from "*Janeway's Immunobiology*", 9th Edition. Murphy K and Weaver C. Garland Science, Taylor & Francis Group (2017). *p. 139-152; 191-198; 422-426.*

Depending on the type of antigen that is present, different classes of antibodies are induced: IgM, IgG, IgA, IgD and/ or IgE. These isotypes are distinguished according to the molecular structure of their C_H chain and greatly vary with regard to abundance, functional activity and specificity (15, 16). For instance, IgM is secreted as pentameric Ig molecule with rather low antigen-affinity, whereas monomeric IgG antibodies are usually highly specific (17). IgG is by far the most abundant subclass in mammals and is further divided into IgG_1, IgG_2, IgG_3 and IgG_4 (named by decreasing order of serum abundance) (18). The two main B-cell responses that lead to the production of these antibodies will be discussed in full detail below (please see section 1.1.4.).

Secreted antibodies are able to directly confer immunity by binding and thereby neutralizing extracellular pathogens and pathogen-derived toxins. Beyond, antibody-binding to antigenic structures is facilitating secondary defense mechanisms such as phagocytosis by innate immune cells (opsonization) and activation of the complement system (19, 20).

Table 1 summarizes the basic features of the five main classes of immunglobulins.

	Secreted structure	**Dominant distribution**	**Mean serum level (mg/mL)**	**Main effector mechanism**
IgM	Pentamer	Bloodstream	1.5	Complement activation
IgG	Monomer	Bloodstream and extracellular fluids	13.5	Neutralization and Opsonization
IgA	Monomer or Dimer	Bloodstream and secretions of the intestinal and respiratory tract	2.1	Neutralization
IgE	Monomer	Bound on mast cells and basophils	$3x10^{-5}$	Mast cell sensitization
IgD	Monomer	Mainly surface-expressed	0.004	-

Table 1: The characteristics of the antibody isotypes. Modified from "*Janeway's Immunobiology*", 9th Edition. Murphy K and Weaver C. Garland Science, Taylor & Francis Group (2017). *p. 191-198; 422-426.*

1.1.4. Classification of B cells and antigens

By means of their localization, surface phenotype and intrinsic propensity to respond towards different antigens, B cells can be divided into innate and adaptive subpopulations.

Adaptive B cells are termed follicular (FO) B cells, or alternatively B-2 cells, and are mainly located within lymphoid follicles of the spleen and the lymph nodes. These cells respond towards thymus/ T-cell dependent (TD) peptide antigens and mediate highly specific antibody-responses, accompanied by the formation of long-lived memory. As indicated by the name, TD B-cell responses require the help of cognate T follicular helper (T_{FH}) cells, sharing the same antigen specificity and delivering the second signal following antigen-BCR-ligation (5, 21).

By contrast, innate B cells encompass two different populations: Marginal zone (MZ) B cells and B-1 cells, mainly residing in the marginal sinus of the spleen and the peritoneal and pleural body cavities, respectively (22, 23). MZ B and B-1 cells are specialized to thymus/ T-cell independent (TI) antigens, thus being fully functional in the absence of T cells (24). TI antigens fall into two categories. TI type-1 antigens are microbiota-derived motifs such as lipopolysaccharide (LPS), CpG oligodeoxynucleotides (CpG-DNA) or viral RNAs and bind to Toll-like receptors (TLRs) on the B-cell surface. For optimal B-cell responses, a synergistic engagement of both TLR and BCR might be required (25, 26). TI type-2 antigens on the other hand are highly repetitive structures that are able to induce extensive cross-linking of multiple BCRs on the B-cell surface. A prototypic TI type-2 antigen is the bacterial capsular polysaccharide of *Streptococcus pneumoniae* (27).

As B-1 cells are especially relevant to this work, they are separately introduced in detail (please refer to chapter 1.1.6.).

1.1.5. T-cell dependent versus independent antibody responses

A TD antibody response is a two-step process. The first phase occurs extrafollicular and involves the BCR-dependent recognition of a protein antigen by B-2 cells. As a consequence, these cells differentiate into short-lived and dividing plasma blasts, secreting antibodies of moderate affinity to provide immediate protection (5).

In the second step, some of these activated B cells enter the B-cell follicle together with their cognate T_{FH} cells to form a highly dynamic germinal center (GC) reaction (5, 21). The GC can be divided into a light zone, containing both GC B and T_{FH} cells, and a dark zone, solely consisting of dividing B-cell clones. Within the dark zone, B cells undergo extensive proliferation and somatic hypermutation (SHM), a unique mutation mechanism targeting the variable regions of the rearranged Ig loci with the aim to increase antigen-affinity. This process is named affinity maturation. Subsequently, these B cells re-enter the light zone, where they capture antigen and process it in peptide-MHC II complexes for presentation to the T_{FH} cells. The T cells in turn provide important survival and proliferation signals to the GC B cells, including stimulation via CD40-ligand and cytokines (e.g. Interleukin-21) (28). Importantly, the greater the antigen capture and presentation rate of a B cell is, the higher its BCR-affinity and the more T-cell help it will receive, leading to so called affinity selection. As a result of several cycles of affinity maturation and affinity selection in the dark and the light zone, respectively, only those B cells with the highest antigen-affinity will successfully complete GC reaction. Eventually, these GC B cells differentiate into long-lived and class-switched plasma cells, secreting huge amounts of highly specific antibodies, mainly of the IgG_1 isotype. Besides, the GC reaction induces the formation of memory B cells, being effective protectors against future infections (29, 30).

On the contrary, TI antigens rapidly induce extrafollicular, short-lived plasma cell differentiation as an important first line defense against encapsulated bacteria (31). These antibody responses are mediated by innate immune cells and normally don’t involve GC formation (5, 32). Since they usually take place without SHM events, TI B-cell responses induce antibodies of only moderate affinity. Although class-switching to other isotypes is known to occur, MZ B and B-1 cells mainly secrete IgM and IgA (32). In addition, TI type-2 antigens trigger the production of IgG_2 (18).

Originally, it was assumed that TI antigens do not induce any immunological memory. However, recent data provides clear evidence for the existence of B-1 cell-derived memory B cells, generated in the course of GC-independent responses (33, 34).

In Figure 3 and Table 2, the most important characteristics of innate and adaptive B-cell responses are summarized.

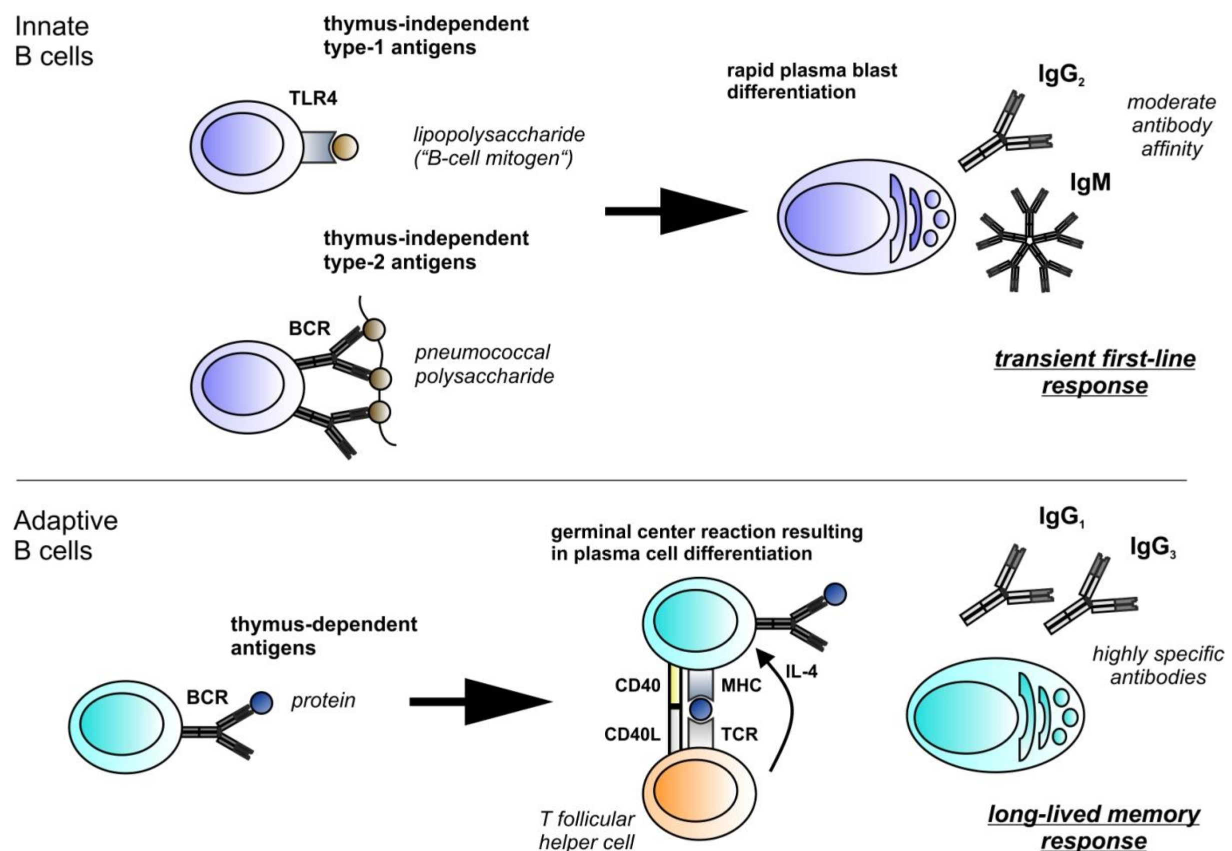

Figure 3: Innate and adaptive antibody responses. Thymus-independent type-1 and type-2 antigens such as lipopolysaccharides and pneumococcal polysaccharides, respectively, induce innate antibody responses. More precisely, they mainly activate natural B-1 or MZ B cells, which subsequently differentiate into antibody-secreting plasma blasts. These rapidly secret IgM and IgG_2 antibodies of moderate affinity. By contrast, thymus-dependent protein antigens activate the adaptive immune system, leading to the formation of a germinal center reaction. Upon affinity maturation and affinity selection driven by T follicular helper cells, adaptive B cells differentiate into long-lived plasma cells and memory cells. This response is classically inducing high affinity antibodies of the isotypes IgG_1 and IgG_3. Modified from Nutt SL, Hodgkin PD, et al. (Nature Reviews Immunology, 2015) and Vinuesa CG and Chang PP (Nature Reviews Immunology, 2013).

	B-cell population	Type of antigen	Type of response	GC	SHM	Class-Switch	Main Ig isotypes
Innate	B-1 cells MZ B cells	Non-proteins, TI type-1 and type-2	Rapid first line defense, moderate affinity	No	No/ low	Yes	IgM, IgA and IgG_2
Adaptive	FO B cells	TD protein antigens	Secondary response, highly specific	Yes	Yes	Yes	IgG_1 and IgG_3

Table 2: Features of innate versus adaptive B-cell responses. Modified from Nutt SL, Hodgkin PD, et al. (Nature Reviews Immunology, 2015) and Vinuesa CG and Chang PP (Nature Reviews Immunology, 2013).

1.1.6. B-1 cells and natural IgM

As already mentioned above, B-1 cells mainly localize to the peritoneal and pleural body cavities but can also be found within the BM and the spleen. In mice, they are clearly defined by their $B220^{low}CD19^{+}CD43^{+}IgM^{high}$ surface phenotype and are further distinguished into B-1a and B-1b cells, being $CD5^{+}$ and $CD5^{-}$, respectively (23). It is established that B-1 cells are mainly generated in the fetal liver before birth, but are only poorly reconstituted by BM precursors during adulthood (35). Instead, B-1 cells sustain their population size by self-renewal, meaning they undergo limited proliferation for replacement of dying cells (36, 37).

B-1 cells participate in both, tissue homeostasis and pathogen-specific immune responses. Under homeostatic conditions, they constitutively secret cross-reactive IgM of broad specificity and their BCR-repertoire is enriched for self-antigens (38). Accordingly, they fulfill an important housekeeping function by neutralization of dying cells and toxic metabolites (39). For instance, natural IgM antibodies have been shown to suppress the development of inflammatory arthritis by clearance of apoptotic cells (40). Further, by binding to oxidized low-density lipoproteins, B-1-derived IgM is decreasing the risk of developing atherosclerosis (41, 42).

Beyond, B-1 cells are highly responsive to TLR-mediated stimulation, thus acting as a first line defense against microbial pathogens (26, 43, 44). Upon activation, peritoneal B-1 cells rapidly egress from the body cavities and differentiate into short-lived plasma cells, producing large amounts of protective antibodies (45, 46).

Although the existence of a human equivalent of B-1 cells has been controversially discussed for many years, accumulating evidence strongly indicates the presence of a B-1 cell-like population in humans, sharing the functional and phenotypical characteristics of murine B-1 cells (35, 47, 48).

Several studies have individually highlighted the essential contribution of B-1 cells to the formation of immunity against pneumococcal infections, which will be discussed in the following section (41, 49, 50).

1.2. *Streptococcus pneumoniae* infections and vaccination

Streptococcus pneumoniae is an opportunistic pathogen in humans, asymptomatically colonizing the mucosal surface of the upper respiratory tract (51). Upon local spread, invasion of the lower airways or entry into the bloodstream, *S. pneumoniae* can cause severe diseases such as otitis media, meningitis, pneumonia or sepsis (51). The mortality of invasive pneumococcal disease is especially high within defined risk groups, including children aged <2 years, adults aged >50 years and immunocompromised people with specific comorbidities (52). Despite existing vaccines, *S. pneumoniae* remains the 4th most common microbial cause of fatal infection and the most frequent cause of community-acquired pneumonia. Thus, pneumococcal disease constitutes a major global health problem (53, 54).

The pneumococcal polysaccharide (pPS) capsule is a source of inner strain-variation and the most important determinant of pneumococcal pathogenicity. Currently, 98 different serotypes are known (55). The pPS capsule is the outer layer of *S. pneumoniae* and its thickness is directly associated with the bacterial virulence (51, 55). It conveys several strategies of immune evasion, such as avoiding the entrapment in nasal mucus of the host and inhibition of the complement system (56, 57). Regarding the serious burden of pneumococcal disease around the globe, most European countries have nowadays established guidelines for vaccination (54).

Currently, two different immunization strategies are available: pure, polysaccharide-based vaccines and protein-conjugate vaccines. Pneumovax23 (P23) is a 23-valent mixture of pPS, containing selective serotypes of *S. pneumoniae* that are frequently associated with clinical manifestations. It triggers a TI B-cell response, leading to a

transient production of serotype-specific antibodies in healthy adults. However, since P23 is not immunogenic in young children and the elderly, the *World Health Organization* and the *Robert-Koch Institute* are recommending the pneumococcal conjugate-vaccine Prevenar13 (PCV13) for the risk group candidates (58). PCV13 consists of 13 pPS-serotypes, coupled to a diphtheria (CRM_{197}) carrier-protein, thus additionally stimulating robust TD B-cell responses and memory immune cells (59). Interestingly, routine vaccination of children with pneumococcal conjugate-vaccine has been shown to successfully reduce the rate of nasopharyngeal carriage in a wide range of population groups (60).

Despite the benefits of pneumococcal immunization, its overall efficacy in preventing pneumococcal pneumonia in adults is estimated to be inadequate (58, 61, 62). Besides, immune responses to pneumococcal vaccine decline with increasing age (61, 63). Hence, there is an urgent need to expand our knowledge about underlying innate and adaptive immune responses towards *S. pneumoniae*-derived antigens.

1.3. Src homology domain 3 lymphocyte protein 2 (SLy2)

1.3.1. Structure, expression and interaction partners

Src homology domain 3 lymphocyte protein 2 (SLy2), also termed HACS1, NASH1 or SAMSN1, is the second member of the SLy/SASH1-family of three highly homologous adaptor proteins (64, 65). Mouse SLy2 is located on chromosome 16, comprises 372 amino acids (aa) and has a size of 48 kDa (65-67). In humans, SLy2 localizes to chromosome 21q11.2, a region that is frequently disrupted by translocation events in hematopoietic disorders (68). The murine and the human protein share 87% overall identity on the protein level (69).

SLy2 is particularly highly expressed in normal and malignantly transformed hematopoietic cells, but can also be found at lower expression levels in muscle tissue, brain, heart, lung and endothelial cells (64). Interestingly, SLy2 is detected in the cytoplasm as well as inside the nucleus, indicating nucleo-cytoplasmic shutting of the protein.

Adaptor proteins do not possess any intrinsic enzymatic activity, but serve as important recruiters and anchors for other proteins in intracellular signaling. By

means of highly conserved adaptor modules, they efficiently interact with other proteins (70).

Figure 4 displays the structural domains of murine SLy2 and its main corresponding functions. At the N-terminal part, SLy2 holds two nuclear localization signals (NLS1: aa 4-21, NLS2: aa 65-82), followed by a central Src homology 3 (SH3) domain (aa 166-223) and a sterile α motif (SAM) domain near the C-terminus (aa 239-305). In addition, SLy2 contains a phosphorylation site at position serine 23 (ser23) and a predicted nuclear export signal (NES) within the SAM domain (66). While the NLS and NES regulate its subcellular distribution, both SH3 and SAM domains are responsible for protein-interactions, by binding to proline-rich sequences (consensus PXXP) and forming homo- and heterodimers, respectively (71-74).

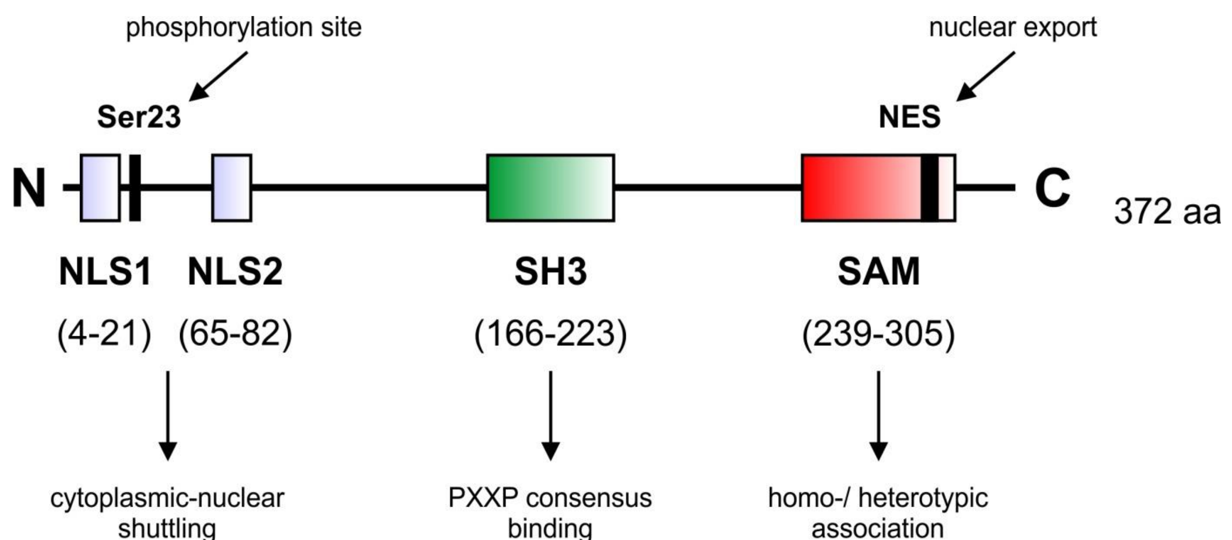

Figure 4: Structural domains of the immunoinhibitory adaptor protein SLy2. SLy2 contains a bipartite nuclear localization signal (NLS) at its N-terminal end, followed by a central Src homology 3 (SH3) domain and a sterile α motif (SAM) at its C-terminus. At position serine23 (ser23), SLy2 is phosphorylated and as a consequence interacts with 14-3-3 proteins. Moreover, sequence analyses predict the existence of a nuclear export signal (NES) within the SAM domain.

Although most of the molecular interaction partners of SLy2 remain to be identified, it is known that ser23-phosphorylated SLy2 is associating with 14-3-3 proteins in the cytoplasm. 14-3-3 proteins are adaptor molecules themselves and have been shown to prevent SLy2 from nuclear translocation, thus regulating its cellular distribution (66,

75). Interestingly, SLy2 also participates in the control of actin dynamics and cytoskeletal remodelling by direct interaction with the master regulator cortactin (67).

In the nucleus, SLy2 interacts with Sin3A-associated polypeptide 30 (SAP30) and histone deacetylase (HDAC) 1, both of which are part of the Sin3A-HDAC complex, a large multi-protein that controls gene expression (66, 76). HDACs usually suppress transcription by the removal of acetyl groups from N-terminal histone residues, thereby stabilizing the chromatin structure (77, 78). HDAC-target genes for example include the anti-proliferative factors p27 and p53, thus HDACs are involved in the regulation of cell cycle progression (79). Experiments with transfected HEK293T cells revealed a markedly increased activity of HDAC1 in the presence of SLy2, indicating a regulatory role of the adaptor protein for HDAC-enzymatic activity (66).

1.3.2. SLy2 in B-1 cell responses towards *S. pneumoniae*

SLy2 is an inhibitory adaptor protein that is upregulated in murine and human B cells as a consequence of stimulation (69). Correspondingly, overexpression of SLy2 in B cells negatively affects their proliferation rate and spreading behavior (67, 69). Investigations of SLy2-Transgenic (Tg) and SLy2-Knockout (Ko) mice further suggest a special role for SLy2 with regard to innate B-1 cells.

More precisely, while SLy2-Ko mice display normal numbers of BM progenitors and splenic mature B cells, they harbor increased percentages of innate B-1 cells. As a consequence thereof, the basal levels of natural serum IgM are increased in these mice (80). On the contrary, SLy2-Tg mice significantly lack peritoneal B-1 cells associated with decreased titers of homeostatic serum IgM (81).

As shortly mentioned above, innate B-1 cells are an important source of immunity against pneumococcal antigens. In the first place, B-1 cell-derived natural IgM targeting phosphorylcholine on apoptotic cells efficiently cross-reacts with *S. pneumoniae* and therefore inhibits early pathogen replication (23, 38, 82). Second, B-1 cells rapidly differentiate into ASCs upon recognition of pneumococcal antigens. Conveniently, the antibody response towards the polysaccharide vaccine P23 is significantly impaired in SLy2-Tg mice. Hence, SLy2 can be considered as an important regulator of B-1 cell responses towards pneumococcal infection (81). One mechanism leading to these defects is the suppression of Interleukin-5-Receptor α

(IL-5Rα) expression on the surface of SLy2-overexpressing B-1 cells (81). The IL-5/ IL-5Rα signaling pathway is essential for the proliferation and differentiation of B-1 cells (83). In summary, these results allow the hypothesis that SLy2 functions as an inhibitory adaptor in IL-5 signaling, thereby modulating B-1-cell effector function (81).

1.3.3. SLy2 in Trisomy 21

SLy2 belongs into a group of 9 genes that are additionally amplified in Down syndrome (DS) individuals and are likely to contribute to the disease phenotype associated with Trisomy 21 (84). In 2015, our group could demonstrate increased expression levels of SLy2-mRNA in peripheral blood cells derived from DS patients, accompanied by drastically reduced titers of global serum IgM as compared to healthy controls (81).

Aside from that, DS patients suffer from several immune deficiencies including abnormal lymphocyte counts and inadequate antibody responses towards vaccination (85-87). Further, DS goes ahead with an increased susceptibility to respiratory tract infections (88). Interestingly, a Swedish study on the mortality of DS patients identified infectious pneumonia as the leading cause of death (89).

The susceptibility towards pneumococcal infections in combination with the lack of natural antibodies indicates a possible defect of B-1 cells in the context of DS. Since SLy2 is a regulator of B-1 cell function, it is reasonable to speculate that its overexpression in DS patients might contribute to their increased morbidity and mortality with regard to pneumococcal diseases (81).

1.3.4. SLy2 in proliferative diseases

Differential expression of SLy2 is associated with the development of several human proliferative disorders. For instance, downregulation or loss of SLy2 significantly correlates with severity of symptoms and shorter disease-related survival of patients suffering from hepatocellular carcinoma and gastric cancer (90-92). Notably, several transcriptome studies demonstrate altered SLy2-expression levels in the context of malignant transformations such as lung cancer and ulcerative colitis-associated colorectal cancer (93, 94).

Besides, SLy2 is implicated in the development of Multiple Myeloma (MM), a proliferative disorder characterized by the expansion of malignant plasma cells (95). The disease symptoms can be highly heterogeneous and among others include bone pain and destruction, renal dysfunction and hypercalcemia. In almost all cases, MM is proceeded by a pre-malignant, asymptomatic state termed Monoclonal Gammopathy of Undetermined Significance (MGUS). There are frequent genetic abnormalities that have been associated with MM, including chromosomal losses/ gains, translocation events and point mutations (95). Interestingly, SLy2 is located within a chromosomal aberration region of 105 MM patients, investigated in the course of a Malaysian study (96). In C57BL/KaLwRij mice, a strain spontaneously developing MM-like disease, a large genomic deletion within chromosome 16 has been reported that leads to a complete knockout of SLy2 (97). In addition, the expression of SLy2 is significantly reduced in plasma cells (PCs) from MGUS-donors as compared to healthy controls, suggesting a protective function of the adaptor protein in MM progression (98). Lower PC-specific expression of SLy2 further correlates with increased burden of BM-infiltrating PCs in these patients (98).

Intriguingly, treatment with HDAC-inhibitors has been shown to be beneficial for MM patients, and HDAC-mediated regulation of CD38-expression on malignant PCs correlates with the disease outcome (99, 100). This is especially interesting, since SLy2 is known to interact with HDAC1 in the nucleus (66). We recently reported the upregulation of SLy2 in the human MM cell line RPMI 8226 after stimulation with IL-6, accompanied by alterations in HDAC-activity. Although the underlying mechanisms remain to be identified, these results strongly indicate a protective function of SLy2 in MM development by regulating HDAC-activity (101).

Summed up, accumulating evidence points to SLy2 as an important prognostic factor in human cancerous diseases with a putative function as tumorsuppressor.

1.4. Chemokines and chemokine receptors

1.4.1. Classification and structure

Chemotactic cytokines (or chemokines) are a super family of approximately 50 small secreted molecules (8-12 kDa) that regulate various immunological processes (102). These include immune cell development, survival, positioning and migration towards sites of inflammation (103, 104). Based on the sequential position of conserved cysteine residues, chemokines are classified into four subgroups, namely CXC, CC, C and CX_3C (105).

In immune cells, CC and CXC chemokine signaling generally occurs through binding of the chemokines to their respective G-protein coupled receptors (GPCR) (106). GPCRs consist of seven transmembrane (TM) α-helices, spanning the plasma membrane and being connected by six intra-/ extracellular loops. Ligand binding and receptor activation occur at the extracellular region. The cytoplasmic part of the GPCR is coupled to small heterotrimeric G-proteins that mediate downstream signal transduction. Figure 5 illustrates the simplified structure of a GPCR.

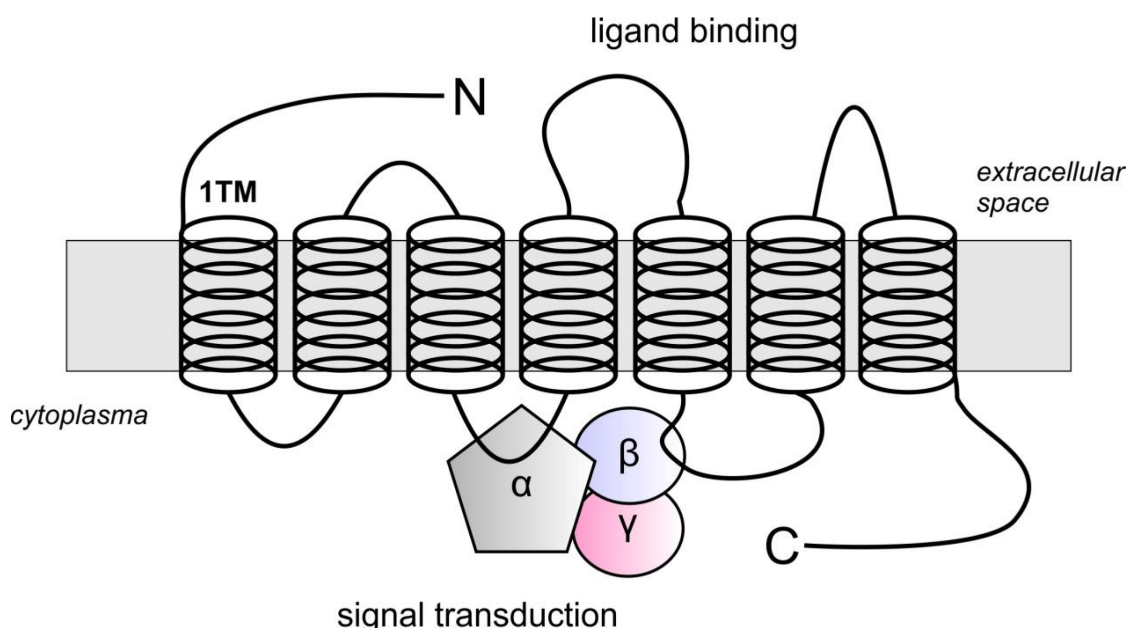

Figure 5: The basic structure of a G-protein coupled chemokine receptor. A GPCR consists of 7 transmembrane (TM) regions, spanning the lipid bilayer and being connected through six extramembraneous loops. While ligand binding is mediated by extracellular receptor parts, the signal is transduced inside the cell by trimeric G-proteins, inducing a variety of signaling cascades. Modified from Stillie RM. et al. (JLB 2009).

More precisely, the Gα-subunit of the G-protein is bound to guanosine diphosphate (GDP) in its inactive state. However, upon receptor engagement, GDP dissociates to allow binding of guanosine triphosphate (GTP). As a consequence, the Gßγ-dimer is released from the receptor and several intracellular signaling cascades are induced (107, 108).

Currently, 17 CXC chemokines and 7 corresponding chemokine receptors are known, with most of them displaying promiscuous binding behavior (103, 109). In Table 3, all of them are listed with their respective function in immunity.

Chemokine	**Receptor**	**Functions in immunity**
CXCL1/ GROα	CXCR2	Neutrophil trafficking
CXCL2/ GROß/ MIP-2α	CXCR2	Neutrophil trafficking
CXCL3/ GROy/ MIP-2ß	CXCR2	Neutrophil trafficking
CXCL4	CXCR3	Pro-coagulant
CXCL5/ ENA-78	CXCR2	Neutrophil trafficking
CXCL6/ GCP-2	CXCR1, CXCR2	Neutrophil trafficking
CXCL7/ NAP-2	CXCR2	Neutrophil trafficking
CXCL8/ IL-8	CXCR1, CXCR2	Neutrophil trafficking
CXCL9	CXCR3	Th1 response, NK cell trafficking
CXCL10/ IP-10	CXCR3	Th1 response, NK cell trafficking
CXCL11	CXCR3, CXCR7	Th1 response, NK cell trafficking
CXCL12/ SDF-1	CXCR4, CXCR7	Immune cell Bone marrow homing
CXCL13/ BCA-1	CXCR5, CXCR3	B cell and T_{FH} cell positioning in LN
CXCL14	Unknown	Macrophage skin homing
CXCL15	Unknown	Unknown
CXCL16	CXCR6	Unknown
CXCL17	Unknown	NKT and ILC migration and survival

Table 3: CXC chemokines and their receptors. Modified from Zlotnik A and Yoshie O. (Immunity, 2012) and Griffith JW. et al. (Ann. Rev. Immun. 2014).

1.4.2. CXC chemokine receptor (CXCR) 1 and 2

Human CXCR1 (IL-8Rα) and CXCR2 (IL-8Rß) are high-affinity receptors for CXCL8 (IL-8), are both encoded on human chromosome 2q35 and share 77% identity on the amino acid level (110, 111). IL-8 is an inflammatory chemokine, rapidly released by macrophages, epithelial/ endothelial cells and muscle cells at sites of inflammation in order to attract immune cells. Of note, the IL-8-signaling pathway significantly contributes to various human diseases including diabetes and cancer (112-114). Further, CXCR1 mediates the antibacterial host defense in cystic fibrosis lung disease and its loss is associated with impaired bacterial clearance and disease severity in these patients (115, 116).

Despite their pronounced structural homology, human CXCR1 and 2 can be distinguished according to their selectivity of ligand binding. While CXCR2 effectively binds many ligands (CXCL1, 2, 3, 5, and 8), CXCR1 is restricted to IL-8 and CXCL6 sensing (117). Both receptors are highly expressed on the surface of neutrophils, macrophages, T cells and also on homeostatic and malignantly transformed B cells (118-120). Interestingly, the consequences of dual inhibition of CXCR1 and 2 substantially differ from those induced by selective CXCR2 inhibition alone, indicating distinct functions of the receptors (121, 122).

In 2006, a murine CXCR1-homologue has been identified on chromosome 1, sharing 64% identical amino acids and similar genomic organization with the human CXCR1 gene. Its expression was detected in liver, kidney, spleen and neutrophils of mice and was clearly distinguishable from the cDNA of CXCR2 (118, 123). The role of CXCR1 for neutrophil biology was subsequently studied by the use of CXCR1-deficient mice (124).

While murine CXCR2 is responsible for neutrophil transmigration *in vitro* and *in vivo*, CXCR1 controls their bacterial killing activity. More precisely, CXCR1 regulates the production of reactive oxygen species (ROS) and TLR-5-expression levels and is required for functional sensing and elimination of *Pseudomonas aeruginosa* by neutrophils (124).

Taken together, human and murine CXCR1 have been extensively studied in the context of neutrophils. By contrast, their role for innate and adaptive antibody responses remains largely unknown.

2. Aim of the study

The major aim of this study was to dissect the role of the adaptor protein SLy2 and the chemokine receptor CXCR1 for innate and adaptive B-cell responses. We were especially interested in the antibody production towards TI and TD antigens in the context of SLy2- and CXCR1-deficiency. To this end, we made use of two genetically modified mouse strains, globally lacking the expression of SLy2 or CXCR1. Complementarily, we planned studies with SLy2-Transgenic (SLy2-Tg) mice that specifically overexpress the immunoinhibitory adaptor in lymphocytes. The objective of our work included *in* and *ex vivo*, as well as *in vitro* experiments for a detailed examination of cellular and humoral immunity.

Since pneumococcal infections are still responsible for a huge burden of disease worldwide despite the availability of vaccines, we aimed to set a special focus on *S. pneumoniae*-derived antigens. To this end, we intended to investigate immune responses to TI and TD pneumococcal immunization with Pneumovax23 and Prevenar13, respectively. Complementarily, we designed and planned survival analyses in a murine model of acute pneumococcal lung infection.

3. Results and Discussion

3.1. Role of SLy2 for B-cell responses

SLy2 belongs into a group of three highly homologous proteins that were classified into one family of adaptors based on their pronounced sequence similarity and conserved functional domains. The members SLy1 (SH3 protein expressed in lymphocytes 1), SLy2 and SLy3/ SASH1 (SAM and SH3 domain containing 1) display individual expression patterns and are involved in diverse biological processes. What they have in common is their frequent association with proliferative disorders (93, 98, 125-129).

Both SLy1 and SLy2 are expressed in lymphocytes, while these appear to be devoid of SLy3/ SASH1-expression (129). SLy1 is mainly implicated in the development of thymocytes, by promoting the transition of T-cell precursors from the double-negative to the double-positive state (130). Besides, it regulates peripheral T-cell responses and the function of splenic MZ B cells (131, 132). Further, SLy1-deficiency results in ribosomal instability and accumulation of the apoptotic factor p53 in natural killer (NK) cells (133).

By contrast, SLy2 is thought to be primarily relevant in the context of antibody responses. Our group previously reported impaired IgM production towards the pneumococcal polysaccharide vaccine P23 in SLy2-Tg mice by reduced expression levels of IL-5Rα on B-1 cells (81). Therefore, we next aimed to investigate whether *S. pneumoniae*-directed responses might be improved in the absence of the adaptor protein SLy2.

3.1.1. SLy2 in B-cell homeostasis and activation

Initially, we found increased ratios of B-1b cells in the BM of SLy2-deficient mice, accompanied by elevated titers of homeostatic IgM in the serum and the spleen (134). These findings conform to previous studies that have identified BM-resident B-1 cells as the main source of pre-antigenic IgM (17, 45). Based on this knowledge we can conclude that SLy2 negatively regulates BM B-1 cell-dependent production of natural antibodies. This is also in accordance with the reduced IgM titers observed in SLy2-Tg mice (81). As already introduced above, homeostatic IgM is produced

independently of antigen exposure, as it is present at normal levels also in germ-free mice. Interestingly, natural IgM specificities are highly enriched for self-antigens and B-1 cells are even positively selected for the expression of autoreactive BCRs. This skewed repertoire is essential to tissue homeostasis, as it selects for antibodies that remove apoptotic and neoplastic cells from the circulation (17). Steady state IgM enhances phagocyte clearance of damaged cells and suppresses TLR-induced inflammation, thereby attenuating disease activity in the context of autoimmune diseases (135, 136). For instance, in a murine model of collagen-induced arthritis, the administration of natural antibodies against phosphorylcholine significantly attenuated severity of symptoms (136). On the contrary, autoimmunity has been associated with an accumulation of B-1 cells, where they might even contribute to disease onset by specific antibodies and enhanced antigen presentation to autoreactive T cells (137). Thus, the modulation of pre-existing IgM levels by SLy2 is of utmost interest and should be subject to future studies. Differential expression of the adaptor protein could contribute to the progression of autoimmune diseases by regulating titers of naturally arising antibodies.

The potential of B-1a cell development is considered being restricted to neonatal resources such as the fetal liver. Subsequently, peritoneal B-1a cells maintain their population size by limited proliferation in an IL-5/ IL-5Rα-dependent manner (83). While hematopoietic stem cells (HSC) from adult BM are unable to reconstitute the B-1a cell compartment, they successfully give rise to both B-2 and B-1b cells; suggesting that B-1a and B-1b populations undergo different developmental pathways (138, 139). However, the factors that drive BM B-1b cell survival and proliferation have not yet been established. There are some studies, indicating a role of IL-9 for B-1b cell expansion, which is secreted by bone marrow stromal cells (BMSCs) (23, 140, 141). SLy2-expression is not restricted to lymphocytes but is widely expressed in many body tissues. Thus, we performed expression analyses on BMSCs from SLy2-deficient and Wt animals in order to assess IL-9 expression levels. However, these were comparable between the genotypes, indicating that the BM B-1b phenotype we observed occurred independent of IL-9 production (134). Hence, the underlying mechanisms leading to the increased percentages of B-1b cells in SLy2-deficient mice remain to be solved. To this end, both B-1 cell intrinsic expression patterns and BM niche constituents should be considered.

Aside from B-1 cells, innate MZ B cells of the spleen contribute to natural antibody levels at steady state (17, 22). As the highly homologous protein SLy1 has already been shown to be involved in MZ B-cell function, we expected this cell population to be affected by differential SLy2-expression. Surprisingly, the pool of MZ B cells was unaltered in the absence or overexpression of SLy2.

It is well-known that B-cell development and activation involves TLR-mediated signals (142). For instance, innate B-1 cells express high levels of TLR4 and TLR9 and are able to differentiate into plasma cells upon TLR-engagement (26, 44, 143, 144). Among others, common ligands for TLR4 and TLR9 include LPS and CpG DNA, respectively.

When we examined the *in vitro* proliferation and class-switch capacity of isolated peritoneal and splenic B-1a and B-1b cells from SLy2-deficient mice upon LPS-stimulation, we initially observed a dramatic shift of the B-1 cell population towards $CD5^-$ B-1b cells (134). Of interest, Savage et al. recently reported the down-regulation of the inhibitory co-receptor CD5 on B-1a cells in response to TLR-stimulation, matching our results (44). We further detected a significant upregulation of surface-expressed IgA and IgG_2 on B-1 cells through LPS-sensing. A combination of LPS plus IL-4 additionally induced class-switch to IgG_1. We were unable to detect any differences between Wt and SLy2-deficient cells in all conditions (134).

By contrast, a study by Wang et al. in 2010 reported increased proliferative responses of SLy2-deficient B cells towards stimulation with IL-4 plus anti-IgM or LPS (80). However, while we positively selected for CD43-expression on B-1 cells in our assays, Wang and colleagues isolated splenic B cells by CD43-negative selection. Therefore, distinct cell populations were examined, likely explaining the discrepancy of results. Innate and adaptive B cells greatly differ in their responsiveness towards BCR-ligation and TLR-stimulation (32). It is therefore reasonable to hypothesize that SLy2 might control the expansion of splenic B-2, but not B-1 cells *in vitro*.

Additionally, we performed stimulation experiments using the TLR9 ligand CpG DNA and investigated the resulting proliferative B-1 cell response. Supplementation of CpG DNA to the culture was sufficient to trigger extensive proliferation of splenic and peritoneal B-1a and B-1b cells. Our studies revealed fully comparable responses of both genotypes upon CpG-sensing (134).

TLRs commonly signal through their cytoplasmic domains by use of the adaptor protein myeloid differentiation factor 88 (MyD88) or TIR domain-containing adaptor-inducing interferon-ß (TRIF). While TLR9 solely relies on MyD88, TLR4 is able to signal through both effector molecules. Both pathways induce transcriptional changes that among others target the nuclear factor-κB (NFκB). Since both, the down-regulation of CD5 and the B-cell proliferation upon TLR-stimulation were fully comparable in the context of differential SLy2-expression, we can conclude that SLy2 is dispensable for these processes in B-1 cells.

Interestingly, when we analyzed the cell culture supernatants of the *in vitro* stimulation assays, we found differences in secreted antibody dependent on SLy2-expression. Both stimulation approaches (LPS alone and LPS plus IL-4) induced high amounts of IgM and IgG. However, B cells deficient for SLy2 secreted considerably more amounts of IgG_2 when stimulated with LPS plus IL-4, indicating that SLy2 is involved in IL-4-mediated antibody production, which is in accordance with previous studies (69, 134).

IL-4 signal transduction is mediated by various downstream molecules (145). In 2004, Zhu and colleagues demonstrated that IL-4-mediated upregulation of SLy2 was dependent on the expression of signal transducer and activator of transcription (STAT) 6 and involved NFκB signaling (69). Interestingly, activated STAT6 is known to translocate from the cytoplasm into the nucleus, as does SLy2 (146). Therefore, it would be interesting to examine whether they physically associate. Moreover, the application of PI3K-inhibitors interfered with IL-4-induced induction of SLy2, indicating that the adaptor protein may come into play downstream of PI3K-signaling (69). PI3K is well-known to be involved in lymphocyte development and proliferation and can be activated upon BCR and TCR antigen-ligation (147).

Interestingly, the highly homologous SLy1-protein is thought to be phosphorylated in a PI3K-dependent manner in T cells, and as a consequence is kept within the cytoplasm by 14-3-3 proteins (75, 131, 148). Furthermore, the third member of the family, SLy3/ SASH1, functions as a suppressor of the PI3K/ Akt signaling pathway in human pancreatic cancer cells (149). These shared implications of all three SLy/ SASH-adaptor proteins in PI3K-signaling point to a high conservation of their role in signal transduction (*Jaufmann et al., Review accepted February 2021*).

3.1.2. SLy2 in antibody responses to pneumococcal vaccination

In order to investigate TI antibody responses towards pneumococcal antigens, we immunized SLy2-deficient mice with the pPS vaccine P23. As expected, these mice produced significantly higher levels of P23-specific IgM. These results match to our previous findings on SLy2-Tg mice (81, 134).

Capsular pPS are classical TI type-2 antigens, as their repetitive structure is able to cross-link multiple BCRs on the cell surface (150). As a consequence, P23 is a vaccine mainly inducing innate responses that are mediated by B-1 and MZ B cells. By contrast, the conjugate-vaccine PCV13 additionally triggers long-lived adaptive responses since it contains a diphtheria carrier-protein (59). PCV13 is the vaccine of choice when it comes to high risk candidates such as DS patients. As we propose a role of SLy2 for the immune deficiency that is linked to DS, we were also interested in the TD antibody response towards PCV13 in the context of differential SLy2-expression.

The injection of PCV13 led to a marked reduction in peritoneal B-1 cells 7 days after immunization (134). B-1 cells are characterized by great mobility as they migrate from the peritoneal cavity through the omentum into the blood and secondary lymphoid organs (151). After activation, they are known to traffic towards the spleen and BM where they release antibodies into the circulation (43, 152, 153). Our findings demonstrate that intraperitoneal application of PCV13 induces activation and emigration of peritoneal B-1 cells. Conveniently, the frequency of splenic and BM-resident B-1 cells was greatly increased 14 days after immunization. As already observed prior to vaccination, SLy2-deficient mice harbored significantly more B-1b cells in the BM than their Wt counterparts (134). Notably, we recently analyzed B-cell precursors in the BM of SLy2-Tg mice in order to elucidate whether SLy2 is involved in B-cell developmental processes. Indeed, we found significantly reduced total counts of pro-B, pre-B and immature B cells in the context of SLy2-overexpression (154). Therefore, one likely explanation could be reinforced reconstitution of B-1b cells by BM precursors in the absence of SLy2, which would explain both the increased population size as well as elevated levels of natural IgM in SLy2-deficient animals. Another possibility that remains to be investigated is the modulation of B-1b cell-intrinsic survival factors. One interesting target would be a proliferation-inducing ligand (APRIL), which has implications in B-1 cell homeostasis but is also an

important constituent of the B-cell niche in the BM where it among others promotes plasma cell survival (5, 155).

Immunization with pneumococcal conjugate-vaccine rapidly induced huge amounts of PCV13-specific IgM antibodies in SLy2-Tg and SLy2-deficient mice. Of interest, the secretion of IgM was unaltered in both experimental mouse models if compared to their Wt littermates (134, 154). This is in contrast to what we have previously observed in the context of pure polysaccharide vaccine. More precisely, while P23-induced IgM antibodies are controlled by SLy2-expression levels; those triggered by the conjugate-vaccine PCV13 are not. These results demonstrate that TI and TD antibody responses to pneumococcal antigens are mediated by distinct signaling pathways and/ or types of B cells. What should also be kept in mind is the contribution of other immune cells, since PCV13 additionally contains protein antigens that stimulate adaptive T-cell responses.

IgM secretion was followed by the production of PCV13-specific IgG antibodies as soon as 14 days upon injection. These markedly peaked at day 21 post-immunization in Wt animals. In SLy2-Tg mice however, IgG_1 and IgG_3-levels dropped and did not exceed the amounts that were produced at day 14. This difference between the genotypes was statistically significant. Conveniently, we found markedly decreased percentages and absolute numbers of $CD138^+TACI^+$ plasmablasts in the BM of SLy2-Tg mice (154). This is in accordance with a previous study, demonstrating the negative modulation of CD138-expression on B cells by SLy2 (69).

On the contrary, SLy2-deficiency favored IgG_2 antibodies specific for PCV13, which were not produced in Wt controls (134). These observations highlight that the homozygous deletion of SLy2 does not necessarily reflect the exact opposite of its overexpression. In its absence, more IgG_2 was produced, whereas its overexpression led to impaired IgG_1 and IgG_3 responses. In simplified terms, the adaptor protein SLy2 differentially controls IgG antibody responses towards pneumococcal conjugate-vaccine.

As introduced in detail above, TD antibody responses generally involve the interaction of B and T cells within GCs that lead to the formation of highly specific plasma cells. For this reason, we also investigated the population of GC B cells in the spleen of mice upon PCV13-vaccination. However, the GC B-cell population size was

normal in both, SLy2-deficient and SLy2-Tg animals. Nevertheless, it is still possible that SLy2 is implicated in GC reaction efficacy and outcome. One of the most important signals derived from cognate T helper cells during GC reaction is IL-4 and as discussed above, SLy2 regulates the IL-4-induced IgG_2 secretion in B cells (5, 134).

Besides, the master regulator BCL-6 is essential to GC formation, as it prevents ASC-differentiation and keeps the B cells in the GC stage (156). For consequent differentiation into ASCs, BCL-6 needs to be down-regulated by Blimp-1, which in turn defines the plasma cell identity of a B cell (157). The tight regulation of both, BCL-6 and Blimp-1 signaling is essential to the formation of GCs and resulting ASC-differentiation. Our presented data strongly indicate that SLy2 is involved in the regulation of ASCs, as they are significantly reduced in the spleen and BM of SLy2-Tg mice (154). Consequently, it might be possible that SLy2 participates in BCL-6 and/ or Blimp-1-dependent signal transduction.

Further, based on the severe reduction of BM precursors in SLy2-Tg mice, expression analyses on PAX5 should be performed. PAX5-expression is present throughout B lymphopoiesis and defines the mature B-cell identity, while its inactivation induces a reversion towards the progenitor state (158). Additionally, Blimp-1-dependent repression of PAX5 is needed to allow differentiation of B cells into IgM-secreting plasma cells (159, 160). Thus, SLy2 might be implicated in B-cell development and differentiation by regulation of PAX5-expression levels or PAX-5 related pathways.

In conclusion, SLy2 is a component of B-cell signaling cascades and future studies on direct interaction partners of the adaptor could give valuable indications about its exact role in B cells.

3.1.3. SLy2 in pneumococcal lung infection

Clinical manifestations of pneumococcal infection include otitis media, meningitis, sepsis and pneumonia, with the latter making up for the largest burden of disease in humans (161). Therefore, we aimed to dissect the role of SLy2 during acute pneumonia and established a murine model of *S. pneumoniae*-induced lung infection. To this end, mice were intranasally infected with Pneumococci of the serotype 3 either without or after preceding P23-immunization, followed by 7 day-survival analysis.

SLy2-deficient mice and corresponding Wt controls benefitted from P23-vaccination, since their survival rate was increased as compared to the non-vaccinated animals. However, the deletion of SLy2 did not confer any survival advantages in the context of infectious pneumonia. More precisely, the percentage of survivors was highly comparable between the genotypes (134).

The same applies to the infection experiments with SLy2-Tg animals, as there were no statistically significant differences detected. However, when infected with an intermediate dose of bacteria 14 days after P23-immunization, 83% of Wt animals, but only 29% of the SLy2-overexpressing animals survived (154). This tendency of impaired survival might be caused by the reduced production of pPS-specific antibodies in response to vaccination in SLy2-Tg mice (81). Nevertheless, when infected with a high dose of *S. pneumoniae*, this tendency was abolished and SLy2-Tg mice even displayed higher survival rates (55%) that their Wt littermates (33%), indicating the presence of compensatory mechanisms (154).

There are three main anatomical sites of pneumococcal infection in humans: the nasopharynx/ upper respiratory tract, where *S. pneumoniae* can be asymptomatically carried; the lung/ lower respiratory tract, where it can cause severe pneumonia and the blood as site of bacterial sepsis (162). Various authors have investigated the immune response following nasopharyngeal colonization of *S. pneumoniae* in mice. These studies clearly show that both, antibody- and T cell-mediated mechanisms come into play upon bacterial exposure. More specifically, pneumococcal colonization triggers anti-pPS and anti-protein antibodies as well as $CD4^+$ T helper (T_H) cells (162). At mucosal sites, T_H cells mediate pathogen clearance by the secretion of the inflammatory cytokine IL-17, which rapidly recruits neutrophils towards the airways. Of interest, nasopharyngeal colonization prior to pneumonia is

an effective immunizing event, as it leads to a marked reduction of the bacterial burden in lungs of infected mice. For this protective effect, B cells and $CD4^+$ T_H helper have been shown to be responsible (163, 164). Intriguingly, in μMT mice lacking both B-cell and antibody responses, clearance of *S. pneumoniae* from the nasopharynx was still fully functional (165). In other words, humoral immune responses are negligible to inhibit primary pneumococcal colonization after intranasal infection, which instead is mediated by $CD4^+$ Th_{17} cells (166). By contrast, the prevention of septicemia occurred in a T-cell independent manner while it absolutely required $CD138^+$ plasma cells in a murine model (167, 168).

Collectively, these findings demonstrate that anti-pneumococcal immunity depends on both, B and T cells. Thereby, antibodies are especially important to prevent sepsis but not bacterial colonization at mucosal surfaces after intranasal challenge. Thus, it is reasonable to speculate that the outcome in our murine model of pneumonia did not solely rely on pPS-directed antibodies which are modulated by SLy2. Very likely, other immune compartments compensated for the decreased antibody titers; hence we were unable to detect significant differences between the genotypes. This is in accordance with the fact, that SLy2-Tg mice do not display any defects in T-cell development and function (81). Further, when analyzing the bacterial burden in the blood of mice, we found that the infection was exclusively restricted to the airways and did not involve invasion of the blood. Therefore, it would be of high interest to infect these mice intravenously instead of intranasally, to evaluate the role of SLy2 during *S. pneumoniae*-induced sepsis, which highly depends on antibody-mediated mechanisms (167).

In conclusion, SLy2 modulates the antibody response towards both, pneumococcal polysaccharide vaccine P23 and conjugate-vaccine PCV13. Future studies should focus on the role of SLy2 in the context of pneumococcal sepsis to enhance our understanding of humoral immune responses against *S. pneumoniae*-infections.

3.2. Role of CXCR1 for B-cell responses

Chemokine receptors are multifunctional proteins by providing guidance to migrating cells and at the same time regulate immune cell development, survival, and effector function (103). Among others, this applies to the compartment of developing and mature B cells. For instance, deletion of the chemokine receptor CXCR4 causes premature exit of B-cell progenitors from the BM, as they depend on its expression to retain within the developmental niche in a CXCL12-dependent manner (169). Upon maturation, follicular B-cell homing is guided by CXCL13-sensing via CXCR5 (170). Within the B-cell follicles themselves, the tight regulation of CXCR4-expression levels on B cells controls the dynamics of GC formation (21). Correspondingly, CXCR4-deficient GC B cells are locally restricted to the light zone and display low rates of SHM. On the other hand, the fixed placement of MZ B cells within the marginal sinus of the spleen depends on the receptor CXCR7 (171). Taken together, chemokines and their respective receptors play a pivotal role throughout the life span of a B cell.

As introduced above, differential expression patterns of the chemokine CXCL8/ IL-8 and its receptors CXCR1 and 2 are frequently linked to inflammatory diseases of the respiratory tract in humans. For this reason, the role of CXCR1 and 2 for neutrophil migration and effector function has been extensively studied in both, mice and men (115, 116, 122, 172). By contrast, their significance for B-cell immune responses remains poorly understood.

An early study in 1994 has revealed a nearly 10-fold increase of lymph node-resident B220^{+} B cells in mice globally lacking CXCR2-expression (173). Subsequently, these mice were studied in a model of allergen-induced pulmonary inflammation. Interestingly, while the number of recruited neutrophils was decreased within the airway lumen, the authors detected a significantly greater proportion of recruited B cells in CXCR2-deficient mice. The latter was accompanied by increased levels of total and allergen-specific serum IgE in the absence of CXCR2 (174). Collectively, these studies point to a role of the IL-8-receptor CXCR2 for B-cell responses.

Various independent investigations have highlighted functional differences between the highly homologous receptors CXCR1 and 2 regarding their ligand affinity, expression pattern and downstream signal transduction (172). However, the relevance of CXCR1 in terms of humoral immunity has not yet been dissected. For

this reason, we aimed to investigate antibody responses in mice that globally lack CXCR1-expression.

3.2.1. CXCR1 in innate antibody responses

In the first place, we found the innate B-cell repertoire being skewed in CXCR1-deficient mice as compared to their Wt littermates. More precisely, while splenic MZ B cells were unaltered, the proportion of B-1 cells was greatly diminished within the pool of $CD19^+$ splenocytes (175). In order to find out whether innate antibody responses might be impaired in these mice, we immunized them with the classical TI type-2 antigen TNP-Ficoll. Surprisingly, the amounts of circulating IgM against the epitopes NP-7 and NP-14 were comparable between the genotypes, indicating that the TI generation of antibodies was still fully functional in the context of CXCR1-deficiency. Since B-1 cells of the spleen are established as specialized responders to pPS, we next performed immunization studies with the polysaccharide vaccine P23 (176). As already observed for TNP-Ficoll, all mice were able to produce significant amounts of P23-, pPS4-, pPS6B- and pPS19F-specific serum antibodies in response to vaccination. Of note, analysis of pre-existing serum IgM under homeostatic conditions also revealed similar concentrations in Wt and CXCR1-deficient animals. In addition, the *in vitro* production of intracellular IgM by isolated B-1 cells was equally productive in both experimental groups (175). Added together, these findings suggest that CXCR1 is dispensable for the secretion of pre-antigenic and TI-induced IgM.

As shortly introduced above, innate B-1 cells are mainly derived from fetal origin early in ontogeny and subsequently maintain their population size by self-replenishment. Primarily, this cellular turnover occurs in an IL-5/ IL-5Rα-dependent manner. Hence, the expression of IL-5Rα on mature B-1 cells is required for the preservation of their population size and function (83, 177). We consequently wondered whether IL-5/ IL-5Rα-signaling might be affected in CXCR1-deficient mice, leading to the observed reduction in B-1 cell frequencies. Thus, we isolated peritoneal and splenic B-1 cells and analyzed their IL-5Rα-surface expression prior to and after *in vitro* stimulation with a combination of IL-4, α-CD40 and IL-5. Interestingly, the total percentage of $IL\text{-}5R\alpha^+$ B-1 cells was increased within the peritoneal; but decreased within the splenic cell fraction of CXCR1-deficient mice as compared to Wt controls. Although

the observed phenotype was subtle and largely compensated upon addition of the stimulants, this might point to an accumulation of activated B-1 cells within the peritoneum, which as a consequence would lack in the spleen (175).

It is established that stimulated B-1 cells egress from the peritoneum to enter the BM, the lymph nodes, and the spleen where they secrete protective antibodies (23, 43). Thereby, chemokines and corresponding receptors play an essential role in directing their migration. It is therefore tempting to speculate that CXCR1 might be involved in the regulation of B-1 cell entry into the spleen or egress from the body cavities. Specifically, B-1 cell homing and trafficking were shown to be regulated by the CXCR5-ligand CXCL13, as is B-cell homing into splenic follicles in general (170, 178).

Since the appearance of chemokine receptors on the surface of immune cells is tightly coordinated and often interconnected, we were interested in CXCR5-expression levels on B-1 cells. Indeed, we found a marked reduction of the CXCR5 receptor on the surface of peritoneal B-1 cells derived from CXCR1-deficient mice as compared to Wt littermates (175). This reduction could impair the homing into the spleen and lead to an accumulation of activated B-1 cells in different localizations. However, to support this idea, further data needs to be assessed including detailed assays on B-1 cell migratory behavior in the absence of CXCR1. In addition, it would be of utmost interest to investigate lymph node-resident B-1 cell populations in the context of CXCR1-deficiency.

To sum this part up, both spontaneous as well as TI antibody production were normal in the absence of CXCR1. Besides, *in vitro* responses of B-1 cells seemed to be unaffected. Nevertheless, our findings indicate that CXCR1 might be implicated in normal splenic B-1 cell positioning in mice, potentially by affecting CXCR5-expression levels (175). B-1 cells are a cell population of great mobility and the pathways regulating their organ distribution remain poorly understood. Therefore, further studies on relevant chemokines and chemokine receptors are required to expand our knowledge. Based on the preliminary results presented in this work, CXCR1 should be considered as a promising candidate when it comes to the regulation of B-1 cell migration.

3.2.2. CXCR1 in adaptive antibody responses

In contrast to innate B-1 cells, the frequency of adaptive FO B cells was significantly enriched within the spleen of CXCR1-deficient mice (175). This is analogous to the increased numbers of $B220^+$ cells that were previously found in the context of CXCR2-deficiency, indicating potentially related functions of the two murine receptor homologues for splenic B cells (173). To evaluate their redundancy, future studies should additionally consider the examination of CXCR1 and 2 double-deficient mice.

The marked increase in adaptive B cells made us wonder whether TD antibody responses might be improved in the absence of CXCR1. Indeed, when vaccinated with the TD protein antigen TNP-chicken gamma globulin (CGG), CXCR1-deficient mice produced significantly greater amounts of specific serum IgG_1 7 and 14 days after injection. By contrast, IgM and IgG_3 responses were comparable between the genotypes (175). As IgG_1 is the main isotype induced during GC reaction, we analyzed splenic GC B cells 14 days after immunization. Even though some CXCR1-deficient mice tended to harbor increased percentages of GC B cells, these differences did not reach statistical significance (175).

There exist different possible explanations for our findings. After successful rearrangement of their Ig heavy and light chains within the BM, transitional B-2 cells enter the circulation and traffic towards secondary lymphoid organs. Depending on various internal and external stimuli including the expression of key transcription factors (e.g. Notch2) and BCR-signaling strength, these cells are able to differentiate into MZ B or FO B cells (179). One possibility would be that CXCR1 is negatively controlling the follicular fate of development, which would in turn explain the increase in FO B cells upon its deletion. For instance, this could involve CXCR1-mediated recruitment of the B cells to other sites than the splenic B-cell follicles or might even occur through direct suppression of entry into the spleen. Alternatively, CXCR1 could locally regulate B-cell survival or proliferation in a negative fashion. As a consequence, its loss would promote local FO B-cell expansion and therewith the production of specific antibodies upon antigen encounter.

On the other hand, our observations could as well be the result of an indirect systemic effect. As multiply mentioned above, CXCR1-expression is indispensable for neutrophil effector functions. At the same time, it is well-known that neutrophils and lymphocytes interact with each other (107, 180). Since the impact of CXCR1-

deficiency is not restricted to B cells in our model, it is very likely that myeloid cell-derived signals contribute to the observed B-cell phenotype. The same applies to certain T-cell populations that were shown to express CXCR1 on their surface (181-183).

Speaking of T cells, the generation of high-affinity IgG_1 antibodies also depends on survival signals derived from specialized T_{FH} cells that drive the affinity maturation and selection of plasma cells within the B-cell follicles (21, 29). Thus, we complementarily inspected splenic T-cell subsets. While the frequency and counts of all $CD3^+$ and $CD4^+$ T cells were comparable between the groups, rates of CXCR5-expressing T_{FH} cells were significantly increased in mice lacking CXCR1 (175). This is of utmost interest, since T cells need to upregulate CXCR5 on their surface in order to enter the B-cell follicles and participate in GC reaction (28, 184). Accordingly, the deletion of CXCR5 results in severe defects regarding SHM and class-switch events (185). In other words, CXCR5 drives the interaction between cognate T and B cells. Consequently, the reinforced CXCR5-expression on T cells might be one reason for the improved IgG_1 response in CXCR1-deficient mice. Collectively, these observations further suggest that the expression of the chemokine receptors CXCR1 and CXCR5 might be interconnected by mutual regulation.

Apart from CXCR5, additional markers are nowadays recommended for complete identification of highly specialized T_{FH} cells, for example the transcription factors Bcl-6, PD-1 and ICOS (186). Based on our results, future experiments should set a special focus on the role of CXCR1 for the phenotype and function of T_{FH} cells.

Although the underlying mechanisms remain to be solved, our work identifies CXCR1 as a novel modulator of TD IgG_1 responses. This is also relevant in clinical terms, since autoimmunity frequently goes ahead with a hyper activation of GC reaction (187, 188). Furthermore, aberrant expansion of CXCR5-expressing T_H cells is linked to human autoimmune diseases including rheumatoid arthritis and systemic lupus erythematosus (189, 190).

To resolve whether CXCR1 exerts direct influence on B-cell development and function, or if these observations rely on indirect effects (for example mediated through signals derived from myeloid cells and T cells) further studies are needed. The expression of CXCR1 by B cells still remains a matter of debate, with only a few

number of reports providing evidence for B cell-specific CXCR1-expression (110, 119, 191). Furthermore, existing data on the functionality of murine CXCR1 are controversial. More specifically, one publication demonstrated that none of the known CXC ligands from mice and men were able to stimulate recombinantly expressed murine CXCR1 in an experimental model with transfected insect cells (123). By hard contrast, Fan et al. reported successful activation of murine CXCR1 by mouse CXCL6/ GCP-2 and human IL-8 in stably transfected murine IL-3-dependent pro-B (Ba/F3) cells (118). Hence, there's an urgent need for further clarification in the context of murine CXCR1 expression and functionality. It should also be kept in mind that CXCR1 could be activated by a yet undiscovered CXC chemokine in mice.

In order to investigate the expression of CXCR1 and 2 by ourselves, we performed RT-PCR analyses on the total RNA content of isolated CD19^{+} splenocytes. We were indeed able to detect CXCR1-expression in Wt samples, whereas it was almost absent in CXCR1-deficient mice (175). Moreover, CXCR2 mRNA was present at similar levels in both genotypes.

From this we can conclude that both, murine CXCR1 and 2 are expressed in splenic B cells at least on the transcriptional level. Thus, a direct influence of CXCR1 and 2 on innate and adaptive B-cell responses is likely and should be subject to future research.

4. References

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Appendix: Publications in thesis

Paper 1

Jaufmann J, Tümen L, Schmitt F, Schäll D, von Holleben M and Beer-Hammer S. (2020) SLy2-Deficiency promotes B-1 Cell Immunity and triggers enhanced Production of IgM and IgG_2 Antibodies against Pneumococcal Vaccine. *Immun Inflamm Dis. 2020; 8: 736– 752. DOI:10.1002/iid3.365.*

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ORIGINAL RESEARCH

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SLy2-deficiency promotes B-1 cell immunity and triggers enhanced production of IgM and IgG_2 antibodies against pneumococcal vaccine

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Abstract

Background: Despite the benefits of existing vaccines, *Streptococcus pneumoniae* is still responsible for the greatest proportion of respiratory tract infections around the globe, thereby substantially contributing to morbidity and mortality in humans. B-1 cells are key players of bacterial clearance during pneumococcal infection and even provide long-lasting immunity towards *S. pneumoniae*. Previous reports strongly suggest an essential role of the immunoinhibitory adapter Src homology domain 3 lymphocyte protein 2 (SLy2) for B-1 cell-mediated antibody production. The objective of this study is to evaluate *S. pneumoniae*-directed B cell responses in the context of SLy2 deficiency.

Methods: B-1 cell populations were analyzed via flow cytometry before and after pneumococcal immunization of SLy2-deficient and wild-type control mice. Global and vaccine-specific immunoglobulin M (IgM) and IgG antibody titers were assessed by enzyme-linked immunosorbent assay. To investigate survival rates during acute pneumococcal lung infection, mice were intranasally challenged with *S. pneumoniae* (serotype 3). Complementary isolated splenic B cells were stimulated in vitro and their proliferative response was assessed by fluorescent staining. In vitro antibody secretion was quantified by LEGENDplex.

Results: We demonstrate increased frequencies of B-1 cells and elevated titers of preantigenic IgM in SLy2-deficient mice. In addition, these mice produce significantly more amounts of IgM and IgG_2 upon pneumococcal vaccination. Knocking out SLy2 did not induce survival advantages in our murine model of acute pneumonia, indicating the presence of compensatory mechanisms.

Abbreviations: BM, bone marrow; BMSC, bone marrow stromal cells; DS, Down syndrome; Ig, immunoglobulin; IPD, invasive pneumococcal disease; Ko, knockout; LPS, lipopolysaccharide; P23, Pneumovax 23; PCV13, Prevenar 13; pPS, pneumococcal polysaccharide; SLy2, Src homology domain 3 lymphocyte protein 2; TD, thymus/T cell-dependent; Tg, transgenic; TI, thymus/T cell-independent; TLR, Toll-like receptor.

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Conclusion: Our results reveal reinforced specific antibody responses towards pneumococcal polysaccharides and enhanced IgG_2 secretion as a consequence of SLy2 deficiency, which could be relevant to the development of more efficient vaccines.

KEYWORDS

antibody responses, B-1 cells, natural IgM, pneumococcal vaccination, pneumonia, Src homology domain 3 lymphocyte protein 2, *Streptococcus pneumoniae*

1 | INTRODUCTION

Src homology domain 3 lymphocyte protein 2 (SLy2) is an immunoinhibitory adapter that is encoded on human chromosome 21 and belongs to a group of three highly homologous protein family members (SLy1, SLy2, and SASH1). It is expressed broadly throughout several tissues including heart, muscles, brain, and the hematopoietic system.[1,2] Differential expression of SLy2 has been brought into context with a variety of human diseases such as solid tumors, multiple myeloma, and Down syndrome (DS).[3–6]

SLy2 is localized in both the cytoplasm and the nucleus, suggesting nucleocytoplasmic shuttling of the protein. This hypothesis is further supported by the fact that it contains a bipartite nuclear-localization signal at its N-terminal end.[7] In addition, SLy2 holds an Src homology 3 and a sterile alpha motif domain, both of which are crucial to its function. As a classical adapter protein, SLy2 mediates the formation and localization of protein complexes, thereby contributing to the transmission of intracellular signaling cascades. It has been reported to colocalize with huge complexes that control gene transcription and was shown to be involved in the regulation of actin dynamics and cell spreading.[7–9]

In immunological terms, studies collectively point to a role of SLy2 as an inhibitor of B-1 cell activation and function. SLy2-overexpressing transgenic (Tg) mice display normal T-cell development, regular numbers of monocytes, dendritic cells, and granulocytes. However, these mice hold a significantly lower proportion of B-1 cells, accompanied by reduced levels of natural serum IgM. Moreover, SLy2 overexpression attenuates interleukin (IL) 5-dependent antibody production of stimulated B-1 cells in vitro.[10]

B-1 cells constitute an innate-like B-cell population, phenotypically and functionally differing from conventional (B-2) B cells. They are mainly localized in the peritoneal and pleural cavity, possess the ability of self-renewal, and display a limited repertoire of B-cell receptor specificities. B-1 cells constitutively secret natural IgM, targeting autoantigens on apoptotic, potentially damaging cells and toxic metabolites. In addition, they are key players of innate immunity, since they act as the main defense against numerous bacterial pathogens. Upon antigen recognition, B-1 cells rapidly respond in a T-cell independent (TI) manner and differentiate into short-lived plasma cells. In mice, B-1 cells are clearly defined as $CD19^+CD43^+IgM^+CD23^-$ and can be subdivided into B-1a and B-1b cells, being $CD5^+$ and $CD5^-$, respectively.[11,12] The phenotype of human B-1 cells is still controversially discussed; however, there exists a B-1 cell-like subset in humans sharing the functional and phenotypical characteristics of murine B-1 cells.[13]

B-1 cells substantially contribute to the clearance of pneumococcal antigens and even confer long-lasting immunity against *Streptococcus pneumoniae*.[14,15] *S. pneumoniae* (Pneumococcus) is a commensal of the upper respiratory tract in humans, asymptomatically carried by a majority of the population. Upon stable colonization and immune evasion, *S. pneumoniae* can cause severe infectious diseases associated with high mortality rates such as pneumonia, otitis media, and sepsis. The risk of developing the invasive pneumococcal disease (IPD) is especially given in infants, the elderly and immune-deficient subjects such as patients with DS.[16,17] DS goes ahead with significantly reduced levels of serum IgM and high susceptibility towards infections. A Swedish study identified infectious pneumonia as a leading cause of death in patients with DS.[18] Intriguingly, SLy2 is amongst a small group of nine genes additionally amplified in DS.[3] This led us to hypothesize that the overexpression of SLy2 in patients with DS may contribute to their increased susceptibility towards pneumococcal infection, by suppressing B-1 cell responses directed against *S. pneumoniae*. Indeed, when immunized with the pneumococcal vaccine, SLy2-Tg mice show significantly impaired antibody responses towards pneumococcal polysaccharides (pPS).[10]

Routine vaccinations of adults with the polysaccharide vaccine Pneumovax 23 (P23) and children with the conjugate-vaccine Prevenar 13 (PCV13) promote

herd immunity against *S. pneumoniae* by reducing nasopharyngeal colonization within the population. However, immune responses to pneumococcal vaccine decline with increasing age, and the overall efficacy of immunization in preventing adult pneumococcal lung infection is estimated to be weak.[19–21] Hence, it is important to intensify our understanding of underlying innate and adaptive immune responses towards pneumococcal antigens as a pre-requisite for the development of advanced vaccines.

To investigate whether B cell responses towards pneumococcal antigens are improved in the absence of SLy2, we generated a SLy2-knockout (Ko) mouse model. We analyzed B-1 cell populations, performed B-cell stimulation experiments in vitro, and measured antibody levels of these mice before and after immunization with pneumococcal vaccine. Complementary, mice were challenged with *S. pneumoniae* to evaluate their survival in the course of acute lung infection.

Our data reveal increased frequencies of bone-marrow (BM) resident B-1b cells in SLy2-Ko mice, accompanied by elevated levels of preantigenic immunoglobulin (Ig) M. In addition, Ko mice produced significantly more amounts of specific IgM and IgG_2 antibodies upon vaccination, demonstrating an inhibitory role of SLy2 in the response towards P23 and PCV13. Surprisingly, the loss of SLy2 did not improve the survival rate of mice during acute lung infection with *S. pneumoniae* serotype 3, possibly due to the superiority of antibody-independent mechanisms in our model.

2 | MATERIALS AND METHODS

2.1 | Generation, breeding, and maintenance of SLy2-Ko mice

For the generation of SLy2-Ko mice, we applied a fosmid derived cloning strategy, which makes use of fosmid vectors consisting of a long stretch of genomic DNA (~40–60 kb) cloned into a plasmid, allowing for propagation in *Escherichia coli* in the presence of chloramphenicole. To generate the targeting construct, a *neoR* cassette was inserted into the target site via homologous recombination (Red/ET cloning system; Gene Bridges). The first step of the cloning reaction was the electroporation of the fosmid-carrying *E. coli* with an inducible bacterial expression plasmid (pBAD) for the λ- recombinases Redα and Redβ that contains a temperature-sensitive origin of replication (*oriR101*), allowing the propagation of the plasmid only up to 30°C. This rendered the bacteria chloramphenicole/tetracycline double resistant. These double resistant clones were picked, grown, and expression of the recombinase was induced by the addition of 1 M L-arabinose solution and incubation of the cells at 37°C. Subsequently, 200 ng of the *neoR*-cassette containing short arms (50 bp on either side) homologous to the site of insertion were electroporated into the bacteria (primer sequence forward: CGCAGCAGCAGTTTTGGGA ATTTTGACCGTTTTCGGAATAATTCCGTATCGCCTTAA CGTTGGAAAAGCTG; reverse: CTCTTCTCCTGCTTCTGG GGACCTTTATCTTCTTAGGAGCTGCTTCCTCTTCCG AT CGCCTAGGGGTAACC). As the *neoR*-cassette contains a kanamycine resistance gene, bacteria harboring a successful recombination event were double-resistant against chloramphenicole and kanamycine. Double resistant clones were picked, and recombination was assessed by polymerase chain reaction (PCR), restriction digest, and finally by southern blot. The *HSV-TK* cassette was inserted 3′ of the homology region with the same method, using an expression cassette with an additional ampicilline resistance gene (primer sequence forward: GGATCCCCGGGTACCGAG CTCGAATTCGCCCTATAGTGAGTCG TATTACAATCGA GCAGTGTGGTTTTGC; reverse: GGTAACGCCAGGGTTT TCCCAGTCACGACGTT GTAAAACG ACGGCCAGTGAA GGTCATGAGATTATCAAAAAGG).

E14.1 embryonal stem (ES) cells were transfected by electroporation with the SLy2 targeting construct. To this end, 5×10^7 cells were mixed with 200 μg of the linearized targeting construct in 1 ml phosphate-buffered saline (PBS). Eight hundred microliters of this mix were electroporated in a Biorad Gene pulser II at 340 V and 250 μF. After 10 min of incubation on ice, cells were resuspended in prewarmed ES medium and the content of each cuvette was plated on two 10-cm dishes with feeder cells. Selection of recombinant ES cells after electroporation was performed by supplementation of 200 μg/ml neomycine and 2 μM ganciclovir, favoring cells that had incorporated the targeting construct in a homologous recombined fashion, without incorporation of the HSV-TK gene. C57BL/6 donor blastocysts were injected each with homologous recombined ES cells and blastocysts were implanted into CD1 foster mothers in a state of mock pregnancy. Chimeric mice were mated to C57BL/6 animals and the resulting offspring was analyzed for germline transmission of the mutation by southern blot and PCR.

All mice were kept and bred under specific pathogen-free conditions in open cages. The SLy2-Ko line was backcrossed to its C57BL6/N background for at least six generations. For immunization studies, 9–13-week-old female or male age-matched littermates were used. For a ranasal challenge with *S. pneumoniae*, 16–17-week old mice were utilized. All animal work was performed according to the German animal care regulations and animal experiments were approved by the local ethics committee (AZ G58/06; AZ 29.03.2017; PH5/11; PH1/14; and PH2/19).

2.2 | Immunization and preparation protocols

To study cellular and humoral immune responses towards a pneumococcal vaccine, SLy2-wild-type (Wt) and Ko littermates were immunized with either 1 µg P23 (SanofiPasteurMSD) or 3 µg PCV13 (Pfizer) in 100 µl PBS intraperitoneally (ip). Complementarily, mice were immunized with 2 µg trinitrophenyl hapten (TNP)–lipopolysaccharide (LPS) in 200 µl PBS. For P23 and LPS, blood was collected before and 4, 7, 14, and 21 days after immunization (retrobulbar blood sampling). For PCV13 studies, mice were killed before and 7, 14, and 21 days after immunization to collect blood, peritoneal cells, spleen, and BM.

All blood samples were collected in Microtainer® blood collection tubes (BD Bioscience). After at least 30 min of incubation at room temperature (RT), tubes were centrifuged at 15,000g for 90 s to collect the serum in the supernatant. Sera were stored at −20°C. Peritoneal lavage (PL) was performed with 5 ml of ice-cold PBS. Femurs were flushed out with 5 ml of ice-cold PBS to harvest BM cells. After one washing step, PL and BM cells were directly used for further analysis. Spleens were homogenized using a 70 µm cell strainer and subsequently incubated with erythrocyte lysis buffer to get rid of red blood cells before analysis.

2.3 | Infection experiments

For the challenge of mice with *S. pneumoniae* (ATCC strain 6303, serotype 3), an inoculated ring from a Roti®-Store Cryo tube was transferred from −80°C storage into 5 ml sterile brain heart infusion medium and incubated at 37°C overnight. The morning after, an OD_{600} of 0.5–0.7 defined a bacterial density of $\sim 30 \times 10^7$ colony-forming unit (CFU)/ml in the original culture. Slightly anesthetized, age-, sex-, and weight-matched mice were infected with 3.5×10^6 CFU in 25 µl sterile PBS by the intranasal application. Upon infection, mice were continuously monitored for 168 h. To estimate the degree of disease burden and to guarantee a consistent, well-defined endpoint, weight, temperature, behavior, posture, and appearance of mice were assessed at least every 6 h during the first 3 days. If necessary, additional inspections during the acute phase of infection took place every 3 h. Mice surviving the first 3 days of infection were subsequently controlled at least two times a day according to their health status. Mice losing 15% of their starting weight or displaying a body temperature of less than 34.5°C were killed immediately. One hundred and sixty-eight hours postinfection, all mice were killed, lungs were harvested after perfusion, and freezed at −80°C for histological examination.

2.4 | Enzyme-linked immunosorbent assay

Basal IgM levels were assessed in sera, supernatants of peritoneal washouts, and splenic single-cell suspensions. To this end, high-binding 96-well plates were coated with 5 µg/ml purified anti-mouse IgM in a coating buffer overnight. The other day, the plate was blocked for 1 h with an enzyme-linked immunosorbent assay (ELISA) blocking buffer. Samples were diluted in sample buffer and incubated for 2 h on precoated plates at RT. For analysis of PCV13-, P23-, or pPS-specific Ig titers in the sera, plates were coated with either 1 µg PCV13, P23, pPS4, pPS6B, or pPS19F in a coating buffer overnight at 37°C. Before sample incubation, sera were diluted in a sample buffer containing 10 µg/ml cell wall polysaccharides to capture unspecific antibodies. Dilutions were incubated on precoated plates for 3 h at 37°C. To detect TNP–LPS-induced antibodies, plates were coated overnight with 10 µg/mL NP14-bovine serum albumin (BSA).

In all cases, the detection was performed using biotinylated anti-mouse IgM, IgG_1, IgG_{2a}, or IgG_3 antibody (BD Bioscience) followed by the addition of streptavidin–horseradish peroxidase (HRP) conjugate (Bio-Techne). HRP-reaction was induced with 3,3′,5,5′-tetramethylbenzidine substrate (Thermo Fisher Scientific) and stopped by adding sulfuric acid. Chemoluminescent readout was done at 450/570 nm. All results were normalized to the mean value of Day 0 preimmunization for analysis of the fold changes in specific antibody levels upon immunization.

2.5 | Cell culture

For in vitro stimulation of splenic cells, $CD19^+$ cells were isolated via Magnetic Activated Cell Sorting using anti-CD19 microbeads and magnetic separation columns (Miltenyi Biotech). Subsequently, enriched B cells were stained with the CellTrace™ Carboxyfluorescein Succinimidyl Ester (CFSE) Proliferation Kit (Invitrogen) at 37°C for 20 min and the reaction was stopped by the addition of complete medium (Roswell Park Memorial Institute medium supplemented with 10% fetal calf serum, 1% L-glutamine, 1% penicillin/streptomycin (P/S), and 0.05 mM ß-mercaptoethanol). 2×10^6 $CD19^+$ splenic cells were cultured in 24-well plate inserts in 500 µl medium either unstimulated (US), with 25 µg/ml LPS only or

with 25 µg/ml LPS plus 10 ng/ml IL-4 at 37°C and 5% CO_2. After 48 h, cells were harvested, washed, and analyzed via flow cytometry. Cell culture supernatants were immediately frozen and stored at −20°C.

2.6 | LEGENDplex™ (Multi-Analyte Flow Assay Kit)

To determine the amounts of antibody secreted by isolated splenic B cells in cell culture, the respective supernatants were analyzed with the LEGENDplex™ Mouse Ig Isotyping Panel (BioLegend). To this end, all probes were used both pure and in a 1:5 dilution. The assay was performed in a V-bottom plate according to the manufacturer's protocol and data acquisition was done using the FACS Canto II flow cytometer (BD Bioscience). BioLegend's LEGENDplex™ Data Analysis Software was applied for analysis (www.biolegend.com/legendplex).

2.7 | Flow cytometry

For ex vivo analysis of B-1 cells, 1×10^6 single cells from PL, BM, or spleen were incubated with anti-mouse CD16/32 (Biolegend) for 15 min on ice for blocking of unspecific Fc-binding sites. Subsequently, cells were stained with anti-CD19 FITC, anti-CD43 PE-Cy7, anti-CD5 APC, anti-IgM APC-Cy7, and anti-CD138 PE antibodies (Biolegend and BD Bioscience) for 15 min on ice. To assess cell death, cells were incubated with 7-aminoactinomycin D (BD Bioscience) before analysis for at least 15 min, but no longer than 60 min.

To analyze proliferation and surface immune globulins after in vitro stimulation, CFSE-stained cells were incubated with anti-CD19 V450, anti-CD43 PE-Cy7, anti-CD5 APC, anti-IgM APC-Cy7, anti-IgD-PerCP, and anti-IgG_1 PE (LPS/IL-4) or with anti-CD19 APC-Cy7, anti-CD43 PE, anti-CD5 APC, anti-IgA BV421, anti IgG_{2ab} BB700, and anti-IgG_3 PE-Cy7 (LPS only) (Biolegend and BD Bioscience).

All measurements were performed at the BD FACS Canto II and biaxial gating was done with FlowJO Version 10.

2.8 | Real-time PCR

Bone marrow stromal cells (BMSCs) were separated from the pool of total BM cells based on adherence as previously described.[22] After 48 h of culture in minimum essential medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum, 1% P/S, 1% L-glutamine, and 0.05 mM ß-mercaptoethanol, all cells were harvested by trypsinization and total RNA contents were isolated using the ExtractMe Total RNA Kit (Blirt). Complementary DNA (cDNA) synthesis was done with the TranscriptMe cDNA Kit (Blirt) according to the manufacturer's protocol. Subsequently, real-time (RT)-PCR was performed with the Sensi-fast SYBR No-Rox Kit (Bioline). For each approach, 20 ng cDNA in 10 µl SYBR mix was applied and ß-actin was used as a reference gene. RT-PCR primer was designed via qpcr.probefinder.com and produced by biomers (IL-9 sequence forward: GCCTCTGTTTTGCTCTTCAGTT/IL-9 sequence reverse: GCATTTTGACGGTGGATCAT). The RT-PCR was run in the Light Cycler® 480 (Roche) and an Advanced Relative Quantification was performed with the Light Cycler® 480 software.

2.9 | Western blot

Splenocytes were either directly subjected to protein lysis in whole-cell lysis buffer or further processed by CD19-MACS to isolate a pure fraction of $CD19^+$ B cells. Subsequently, $CD19^+$ cells were lysed US or after 24 h of in vitro stimulation with 10 ng/ml IL-4. All protein lysates were diluted in Roti® Load1 (Roth) 4:1 and boiled at 95°C for 5 min before the performance of sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Ten percent of gels were prepared and loaded with 1×10^6 cells per sample. For band definition, Precision Plus Protein™ Dual Color Standard Marker (Bio-Rad) was used. After electrophoresis, the proteins were transferred from the gel onto a nitrocellulose membrane by wet blotting. Membranes were blocked in 5% BSA for 1 h at RT and subsequently incubated with rabbit anti-SAMSN1 antibody (Novus Biologicals) or mouse anti-GAPDH antibody (HyTest), followed by secondary incubation with HRP-conjugated anti-rabbit IgG light chain (Abcam) or Easy Blot anti-mouse IgG (GeneTex), respectively. Band detection was performed by adding Westar Supernova Luminol-enhancer solution (7BioScience) and readout in the VersaDoc (Bio-Rad).

2.10 | Statistics

For evaluation and graphical illustration of the data, GraphPad Prism Version 7 was utilized. Statistical testing was performed as indicated in corresponding figure legends.

3 | RESULTS

3.1 | Increased frequencies of BM-resident B-1b cells and enhanced levels of natural IgM in SLy2-Ko mice

We previously reported decreased frequencies of B-1 cells and pPS-specific antibodies in SLy2-overexpressing mice, identifying SLy2 as an immunoinhibitory adapter protein.[10] To analyze the impact of SLy2-deficiency on immune cell subsets and responses, we have generated Ko mice, globally lacking the expression of SLy2 (Figure S1). On the basis of earlier findings, we expected improved B-1 cell immunity in the absence of SLy2.

Flow cytometry analysis of T cell populations of the thymus and splenic B-2 cells revealed equal proportions in SLy2-Ko mice as compared to Wt littermates. Furthermore, these mice hold normal numbers of dendritic cells, neutrophils, and macrophages/monocytes (Figure S2). In addition, we found the total organ cell counts in peritoneum, spleen, and BM of SLy2-Ko mice being largely unaltered, accompanied by comparable frequencies of B-1 cells in peritoneum and spleen (Figure 1A). However, SLy2-Ko mice displayed an increased proportion of B-1 cells in the BM, referable to an increase in B-1b cells with B-1a cell numbers remaining equal (Figure 1A). Figure S3 exemplarily shows our flow cytometry gating strategy for B-1 cells, defined as $CD19^{+}CD43^{+}IgM^{+}$ and $CD5^{+/-}$.

Since BM-resident B-1 cells are assumed to be the main producers of natural protective antibody,[23] we subsequently examined IgM levels in peritoneal washouts, supernatants of splenic single-cell suspensions, and serum of mice at steady state. While concentrations of global IgM were comparable in peritoneal washouts of Wt and Ko littermates, they were significantly increased in both, spleen and serum of SLy2-deficient mice (Figure 1B).

3.2 | LPS and IL-4 induced in vitro production of IgG_{2b} is favored in isolated splenic B cells from SLy2-Ko mice

Proceeding from ex vivo analysis of immune cell populations and natural IgM, we subsequently performed in vitro assays to assess the function and responsiveness of B cells upon stimulation. To this end, splenic B cells were cultured with 25 µg/ml LPS for 48 h or left US as a control. As shown in Figure 2A, the addition of LPS induced extensive proliferation (upper panel left) and a proportional enrichment of $CD19^{+}CD43^{+}IgM^{+}$ B-1 cells (upper panel right). Moreover, we observed a significant shift from $CD5^{+}$ B-1a cells towards $CD5^{-}$ B-1b cells within the overall B-1 cell population upon stimulation (Figure 2A, lower panel). This observation is in accordance with a recent publication, proposing the downregulation of surface-CD5 on B-1a cells in response to Toll-like receptor (TLR)-mediated activation.[24]

Since IgM antibodies have been shown to be increased in both, spleen and serum of SLy2-Ko mice, we were wondering whether SLy2 might be involved in the regulation of class-switch events. Therefore, splenic B cells were again stimulated with LPS alone or with LPS + IL-4 for in vitro induction of class-switch as previously reported.[25] Afterwards, the expression of six surfaces Ig on B-1 cells was examined. As depicted in Figure 2B, LPS-stimulation significantly induced the expression of surface IgA and IgG_{2ab}, whereas IgG_3 expression remained unaltered (upper panel). Simultaneous addition of LPS and IL-4 triggered downregulation of surface IgD and upregulation of IgG_1, while the expression of IgM on the surface remained constant (lower panel).

Complementary, we assessed the concentration of secreted antibodies in these culture supernatants in the absence or presence of stimulation. While levels of secreted IgM, IgG_1, IgG_{2a}, and IgG_3 were comparable between the genotypes, SLy2-Ko B cells tended to produce higher amounts of IgG_{2b}, with differences being significant upon stimulation with LPS + IL-4 (Figure S4).

In summary, proliferation, proportional changes within the B-1 cell population, and surface Ig class-switch are equally efficient in SLy2-Wt and Ko B cells derived from the spleen. However, in SLy2-Ko mice, the production of IgG_{2b} antibodies was favored in all three conditions as compared to Wt controls, with a significant difference in response to addition of IL-4 (Figure S4).

3.3 | Increased production of specific IgM antibodies towards TI pneumococcal vaccine in SLy2-Ko mice

The importance of SLy2 regarding B-1 cell responses towards pneumococcal antigens has been previously pointed out by the investigation of SLy2-overexpressing mice in our group. These mice displayed significantly decreased levels of specific antibodies after injection with the pneumococcal vaccine.[10] Accordingly, we wanted to find out whether

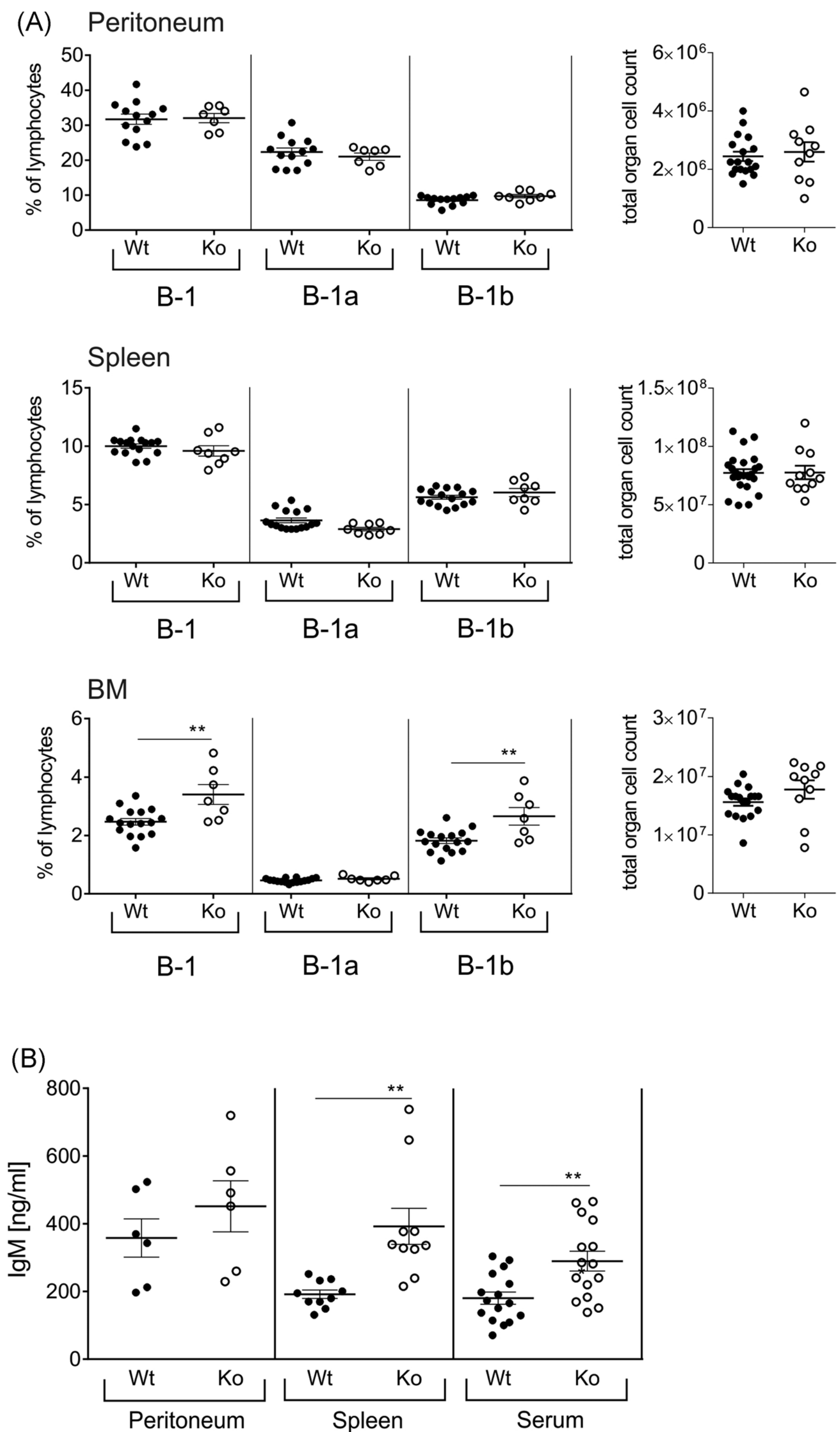

FIGURE 1 B-1 cell frequencies and natural IgM titers in SLy2-Wt and Ko mice at a steady state. (A) Frequency of overall B-1, B-1a, and B-1b cells in peritoneum, spleen, and BM of mice, analyzed via flow cytometry and given as the percentage of all single living lymphocytes. In addition, total cell numbers of all three organs are depicted at the right. B-1 cells were defined as $CD19^+CD43^+IgM^+$, with B-1a cells being $CD5^+$ and B-1b cells being $CD5^-$, respectively. Data represent $n = 7–16$ mice pooled from three independent experiments and are shown as mean ± *SEM*. Indicated significances were determined by Student's *t* test and a *p*-value of less than .05 was considered statistically significant ($*p < .05$, $**p < .01$). (B) Natural IgM levels in peritoneum, spleen, and serum of mice at steady state, determined by ELISA and given in ng/ml. Data represent $n = 6–15$ mice per genotype pooled from two to three independent experiments and are shown as mean ± *SEM*. Significance was determined by Student's *t* test and a *p*-value of less than .05 was considered statistically significant ($*p < .05$, $**p < .01$). BM, bone marrow; ELISA, enzyme-linked immunosorbent assay; IgM, immunoglobulin M; Ko, knockout; Wt, wild-type

SLy2-deficiency improves the immune response towards pneumococcal immunization.

P23 is a pure mixture of 23 pPS serotypes, triggering TI immune responses and leading to the rapid production of IgM.[17] We immunized SLy2-Wt and Ko littermates with 1 µg P23 and measured serum IgM levels at 4, 7, 14, and 21 days postimmunization (see vaccination timeline in Figure 3A). Figure 3B illustrates the amount of P23-specific IgM over time and normalized to day 0 (the fold of control). IgM titers were significantly increased in SLy2-deficient mice as compared to Wt 7 days postimmunization, indicating an improved TI antibody response upon KO of SLy2 (Figure 3B). Ancillary, mice were immunized with the TI antigen TNP–LPS. Figure 3C shows NP14-specific serum IgM levels as fold of preimmune titers. Again, SLy2-deficient mice tended to produce more amounts of specific IgM upon vaccination as compared to their Wt counterparts (Figure 3C).

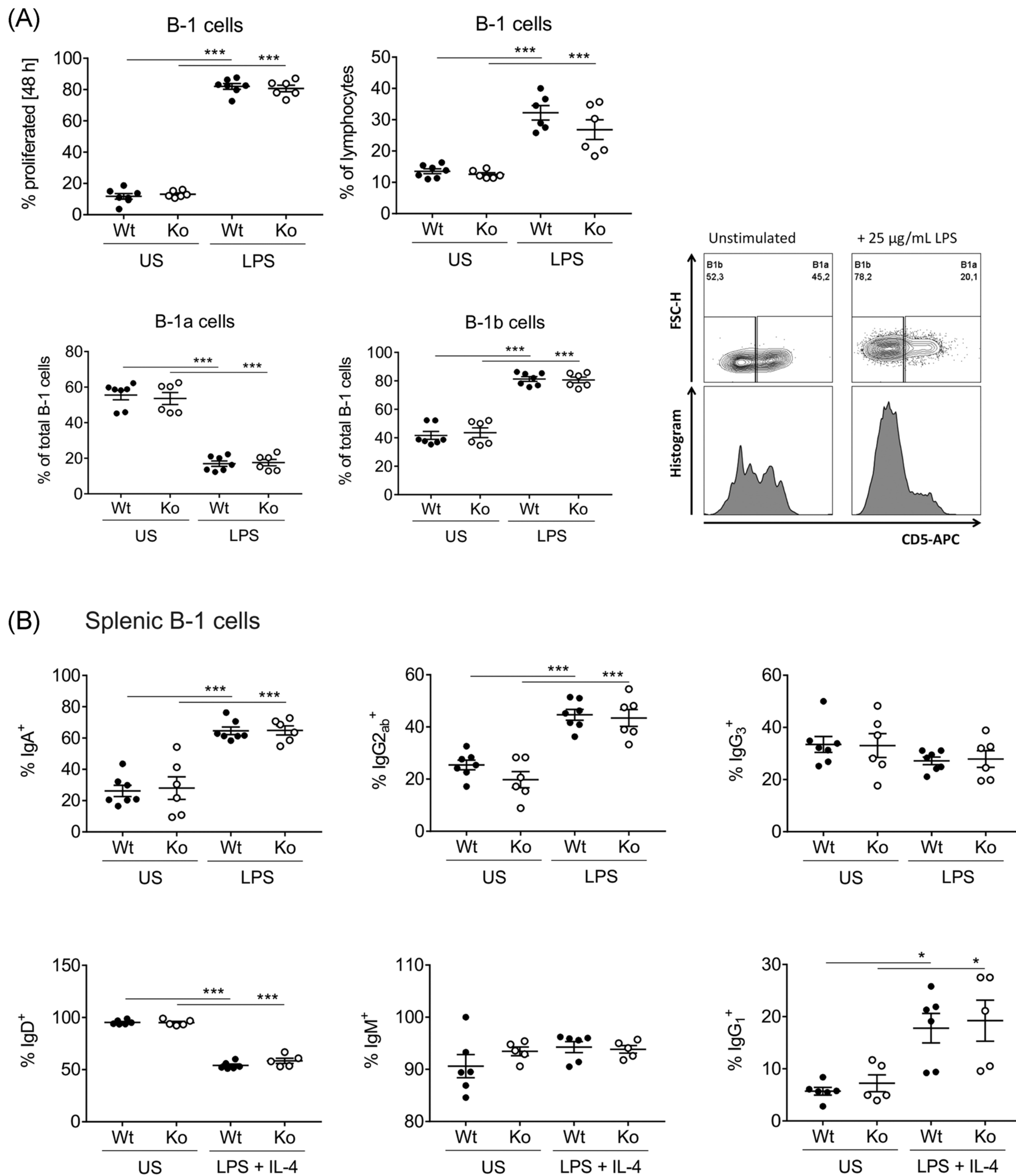

FIGURE 2 In vitro analysis of proliferation and class switch of isolated splenic B cells upon stimulation with LPS/IL-4. (A) Isolated splenic B cells were stimulated with 25 µg/ml LPS for 48 h. For assessment of the proliferation rate, CFSE staining was performed before cultivation, and cells were analyzed by flow cytometry after 48 h. Cell ratios are given as a percentage of all single lymphocytes or relative to the whole B-1 cell population. (B) CFSE-stained B cells were stimulated with 25 µg/ml LPS only or with LPS + 10 ng/ml IL-4 for 48 h. Subsequently, class-switch was investigated by flow cytometry staining of indicated surface immunoglobulins (IgA, IgG_{2ab}, IgG_3, IgD, IgM, and IgG_1) on all B-1 cells defined as $CD19^+CD43^+$. Data represent $n = 6$ mice from two independent experiments and error bars depict the mean ± *SEM*. Significance was determined by one-way analysis of variance with multiple comparisons and a *p*-value of less than .05 was considered statistically significant (*$p < .05$, ***$p < .001$). CFSE, carboxyfluorescein succinimidyl ester; FSC-H, forward scatter height; IL-4, interleukin-4; Ko, knockout; LPS, lipopolysaccharide US, unstimulated; Wt, wild-type

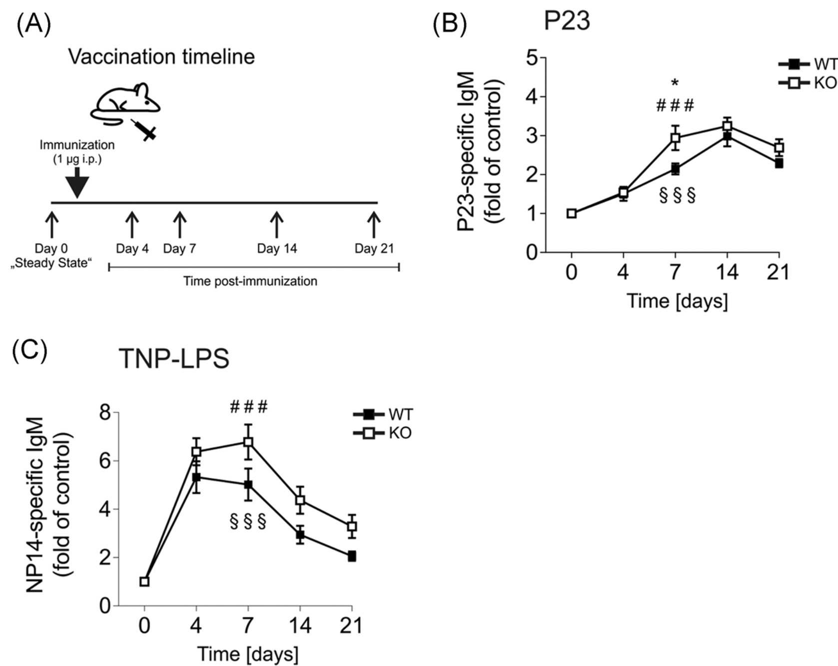

FIGURE 3 Pneumovax 23 (P23)- and TNP–LPS-specific IgM titers in SLy2-Wt and Ko mice upon vaccination. (A) Vaccination timeline, depicting the time points of blood sampling: before and 4, 7, 14, and 21 days after immunization. (B) P23- and (C) TNP–LPS-specific IgM responses in the serum of mice after immunization, presented as fold of preimmune titer. Data represent $n = 7–16$ mice per genotype pooled from two independent experiments and are shown as mean ± *SEM*. Significance was determined by a two-way analysis of variance with multiple comparisons and a *p*-value of less than .05 was considered statistically significant ($^*p < .05$, $^{**}p < .01$). IgM, immunoglobulin M; Ko, knockout; LPS, lipopolysaccharide; TNP, trinitrophenyl hapten; Wt, wild-type

3.4 | Enhanced B-1 cell frequencies and pPS-specific IgG_{2a}-levels in SLy2-Ko mice upon immunization with pneumococcal conjugate vaccine

Immunization with P23 successfully elicits immune responses in healthy adults. Nevertheless, it has been shown to provide only limited and temporally transient protection in high-risk groups which is problematic, since especially infants, the elderly and immunocompromised patients suffer from increased incidence of IPD.[26] Thus, the conjugate-vaccine PCV13 was introduced and is routinely recommended for high-risk candidates. It contains 13 pPS-serotypes coupled to a carrier protein, thereby additively inducing thymus/T-cell-dependent (TD) responses, providing reinforced protection.[27] As we were interested in the response of SLy2-Ko mice towards the TD vaccine, we immunized them with 3 µg PCV13 and subsequently investigated serum Ig responses and B-1 cells in three different organs at 7, 14, and 21 days postimmunization. The percentages of B-1 cells are presented as curves over time (Figure 4A) and as dot plots for direct comparison of Wt and SLy2-deficient individuals (Figure 4B).

As a consequence of PCV13 injection, peritoneal B-1 cell frequencies markedly declined in both genotypes after 7 days, most likely attributable to the emigration of activated B-1 cells from the peritoneum (Figure 4, upper panel). While rates of splenic B-1 cells were irregular in Wt animals, they increased in Ko mice over time, reaching significance after 14 days as compared to preimmune conditions (Figure 4, middle panel). Percentages of BM-resident B-1 cells significantly increased over time in both, Wt and Ko littermates, with B-1 cell proportions being substantially higher in Ko mice 7 and 14 days postimmunization (Figure 4, lower panel).

Considering the profound alterations in B-1 cell frequencies, we next measured concentrations of IgM and IgG antibodies, specifically produced upon exposure to PCV13. PCV13-specific antibody levels are depicted as fold of preimmune titers over time in curves (Figure 5A) or as dot plots for direct comparison of genotypes (Figure 5B). Immunization rapidly induced significant levels of specific IgM from Day 7 postvaccination on, with titers being identical in both genotypes (Figure 5A,B). Conjugate immunization also stimulated the production of IgG_1 and IgG_3 starting after 14 days, indicating the formation of a germinal center response and class-switch events (Figure 5A,B).[28] SLy2-Ko mice tended to produce

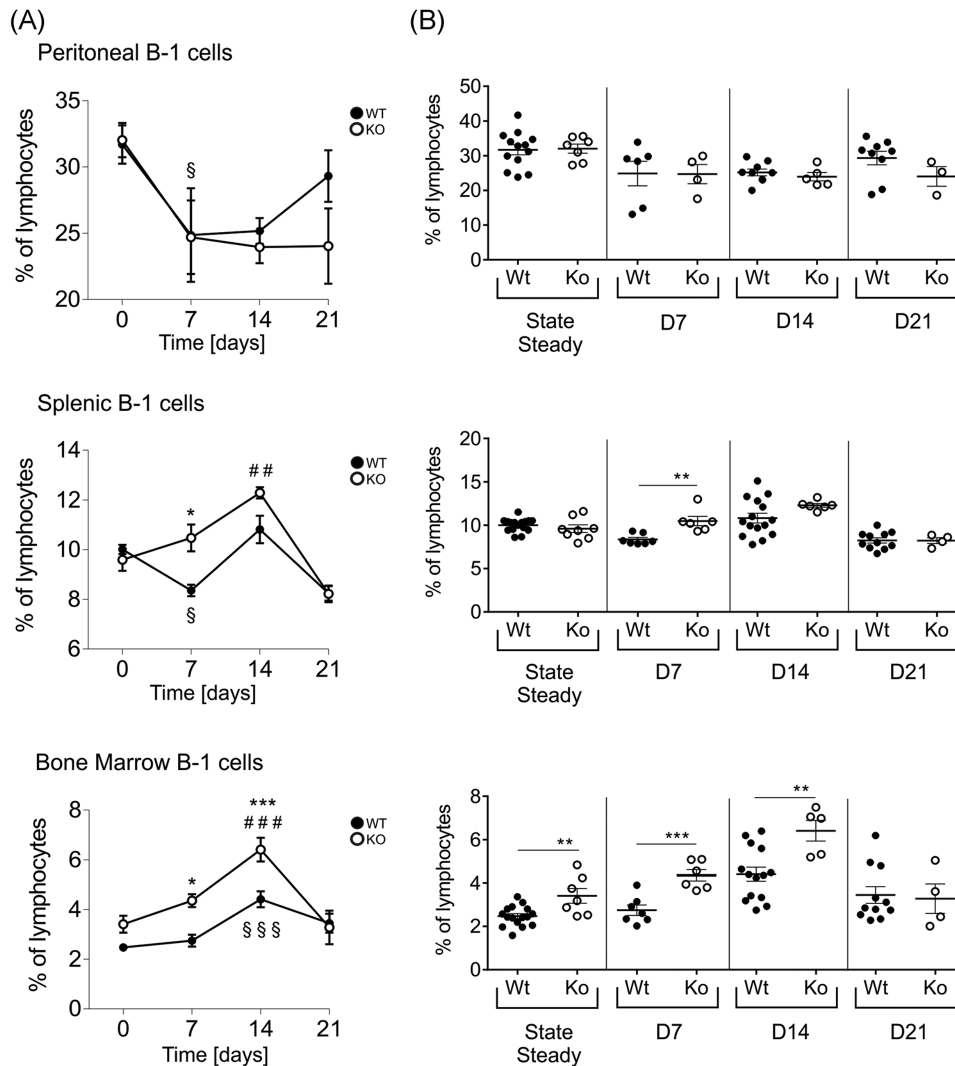

FIGURE 4 Frequency of B-1 cells in the peritoneum, spleen, and BM of mice before and after injection with Prevenar 13. (A) Over time progression of B-1 cell frequencies in SLy2-Wt and Ko mice from Day 0 preimmunization to Days 7, 14, and 21 postimmunization. Animals were killed at all four-time points for collection of peritoneal washouts, spleen, and BM. Cell percentages are given related to all single living lymphocytes and represent $n = 4$–16 mice per genotype pooled from two to four independent experiments. Error bars indicate the mean ± *SEM* and significance was determined by two-way analysis of variance with multiple comparisons. "§" indicates the significance of Wt curves and "#" of Ko curves in comparison to Day 0, and asterisks reflect significant differences between the genotypes. A *p*-value of less than .05 was considered statistically significant (§, #, **p* < .05; §§, ##, ***p* < .01; §§§, ###, ****p* < .001). (B) Direct comparison of B-1 cell frequencies at respective days, shown as dot plots and given as a percentage of all single living lymphocytes. Data represent $n = 4$–16 mice per genotype pooled from two to four independent experiments. Error bars indicate the mean ± *SEM* and significance between the genotypes was determined by Student's *t* test. A *p*-value of less than .05 was considered statistically significant (***p* < .01, ****p* < .001). BM, bone marrow; Ko, knockout; LPS, lipopolysaccharide; Wt, wild-type

fewer amounts of IgG_1 and more IgG_3 than Wt littermates, but those differences did not reach statistical significance.

While the amounts of IgG_{2a} produced by Wt animals were negligible, it seemed to be strongly favored in some SLy2-deficient individuals. As illustrated in Figure 5A,B, PCV13-specific IgG_{2a} was produced in Ko mice from Day 7 on, reaching significance at Days 14 and 21 postvaccination. Differences between the genotypes were statistically significant. Complementarily, we evaluated IgG_{2a} titers against the pneumococcal serotypes 3, 4, 6B, and 19F, all of which are highly virulent serotypes of *S. pneumoniae*, frequently found in clinical contexts. We found increased serum concentrations of pPS3-, 6B-, and 19F-directed IgG_{2a} antibodies in Ko mice after 14 and 21 days.

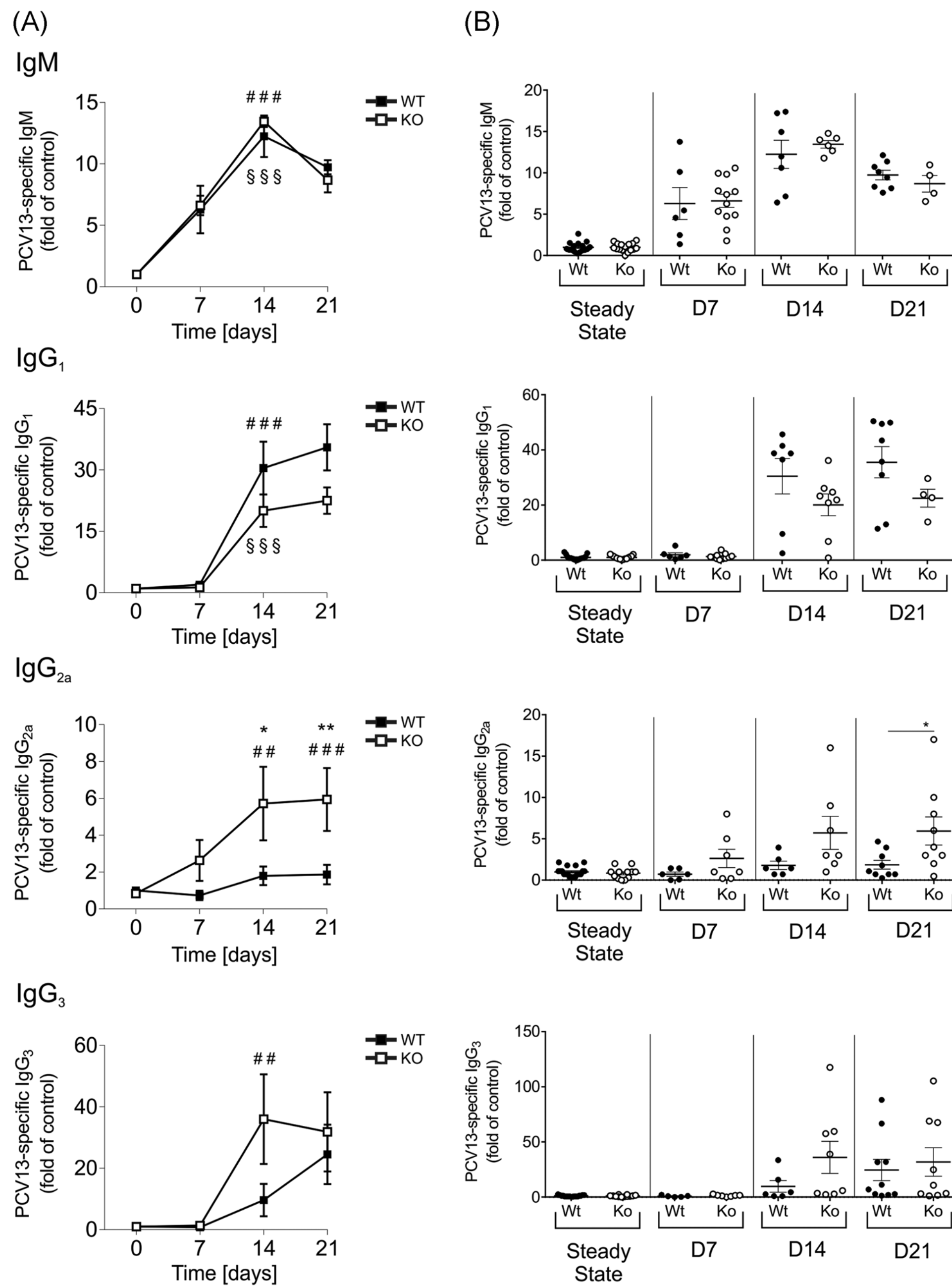

FIGURE 5 Prevenar 13 (PCV13)-specific IgM and IgG in the serum of mice upon immunization. (A) Over time progression of PCV13-specific IgM, IgG_1, IgG_{2a}, and IgG_3 levels in the serum of mice from Day 0 preimmunization to Days 7, 14, and 21 postimmunization. All values are given as fold of preimmune titers and were assessed within two to four independent experiments per day. Data represent $n = 4$–16 mice and error bars depict the mean ± *SEM*. Significance was determined by a two-way analysis of variance (ANOVA) with multiple comparisons. "§" indicates the significance of Wt curves and "#" of Ko curves in comparison to Day 0, and asterisks reflect significant differences between the genotypes. A *p*-value of less than .05 was considered as statistically significant (§, #, *$p < .05$; §§, ##, **$p < .01$; §§§, ###, ***$p < .001$). (B) Direct comparison of PCV13-specific Ig-titers in SLy2-Wt and Ko mice. Data are shown as dot plots and significances within genotypes at different time points were assessed by Student's *t* test. A *p*-value of less than .05 was considered statistically significant (*$p < .05$). (C) Over time progression of pPS3, pPS4, pPS6B, and pPS19F-specific IgG_{2a} serum antibody-titers (fold of Day 0). Data represent $n = 9$–16 mice and error bars indicate the mean ± *SEM*. Significance was determined by one-way ANOVA with multiple comparisons. "#" indicates the significance of Ko curves in comparison to day 0, and asterisks reflect significant differences between the genotypes. A *p*-value of less than .05 was considered statistically significant (#, *$p < .05$; ##, **$p < .01$; ###, ***$p < .001$). IgM, immunoglobulin M; Ko, knockout; pPS, pneumococcal polysaccharide; Wt, wild-type

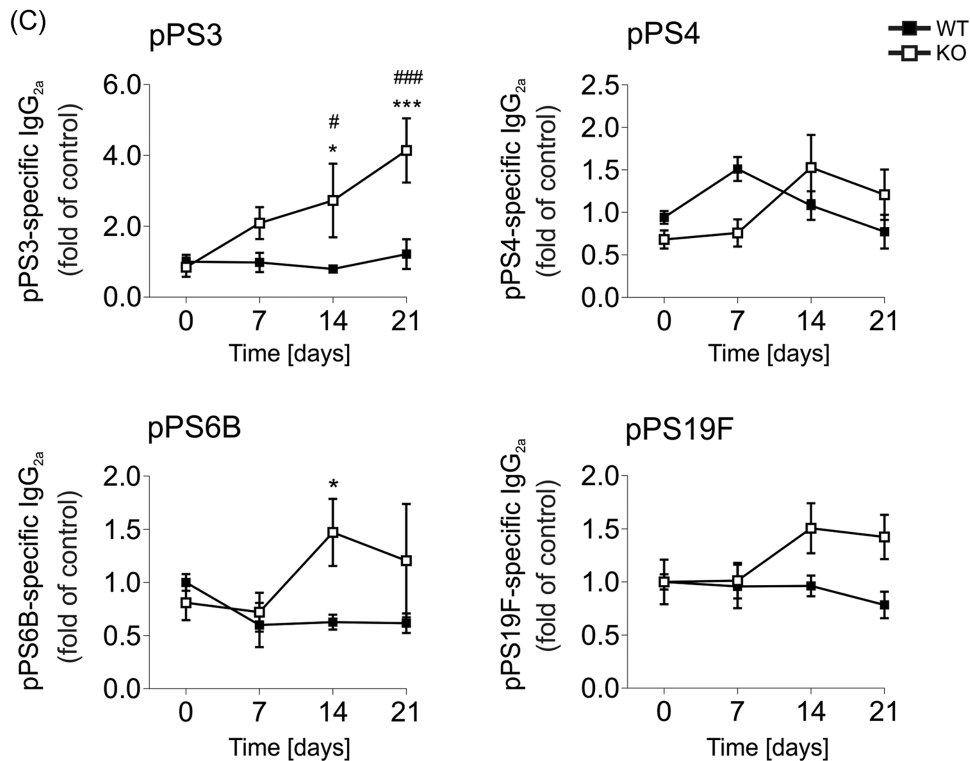

FIGURE 5 (Continued)

These differences were significant for pPS3 and 6B (Figure 5C).

3.5 | Unaltered survival rate of SLy2-Ko mice in the course of acute pneumococcal lung infection

Proceeding from the marked differences in antibody responses towards P23 and PCV13, we decided to assess the survival rate of Wt and Ko animals during acute pneumococcal infection. Since the ELISAs revealed the most pronounced differences for pPS3, we decided to use *S. pneumoniae* serotype 3 for our infection experiment. Mice were intranasally challenged with 3.5×10^6 CFU and subsequently monitored for 7 days. The burden of disease was estimated using precise physiological criteria (e.g., weight, temperature, and appearance) to guarantee constant and ethical endpoint definition. 24–72 h upon challenge, mice developed symptoms of the disease including dropping temperature, fast breathing, and weight loss. Intranasal application of *S. pneumoniae* induced pronounced symptoms of pneumonia such as severe consolidation of the lung tissue as compared to healthy controls. Figure 6A exemplarily shows hematoxylin and eosin (HE)-stained lung cryosections of one healthy mouse (top), and one sick mouse (bottom), with the latter being killed during acute infectious disease. *S. pneumoniae*-infection induced intra-alveolar accumulation of protein-rich fluids, visualized as light-pink material in the HE-staining (indicated by asterisks, Figure 6A). Moreover, we could observe immune cell infiltration into the infected tissue, as visible by blue-colored nuclei within alveolar spaces (indicated by arrowheads, Figure 6A).

As shown in Figure 6B, WT and KO mice were infected in parallel either without preceding immunization (upper panel) or after P23-vaccination (lower panel). We could not detect significant differences between the survival rates of Wt and SLy2-deficient mice during acute infection. The injection of P23 14 days before challenge improved overall survival in both experimental groups, indicating that immunization conferred immune protection in a genotype-independent manner (Figure 6B, lower panel).

To sum up, we found increased vaccine-specific antibody responses in SLy2-deficient mice in the context of both, TI and TD pneumococcal vaccination. However, these were not sufficient to improve the survival of mice during acute pneumococcal pneumonia.

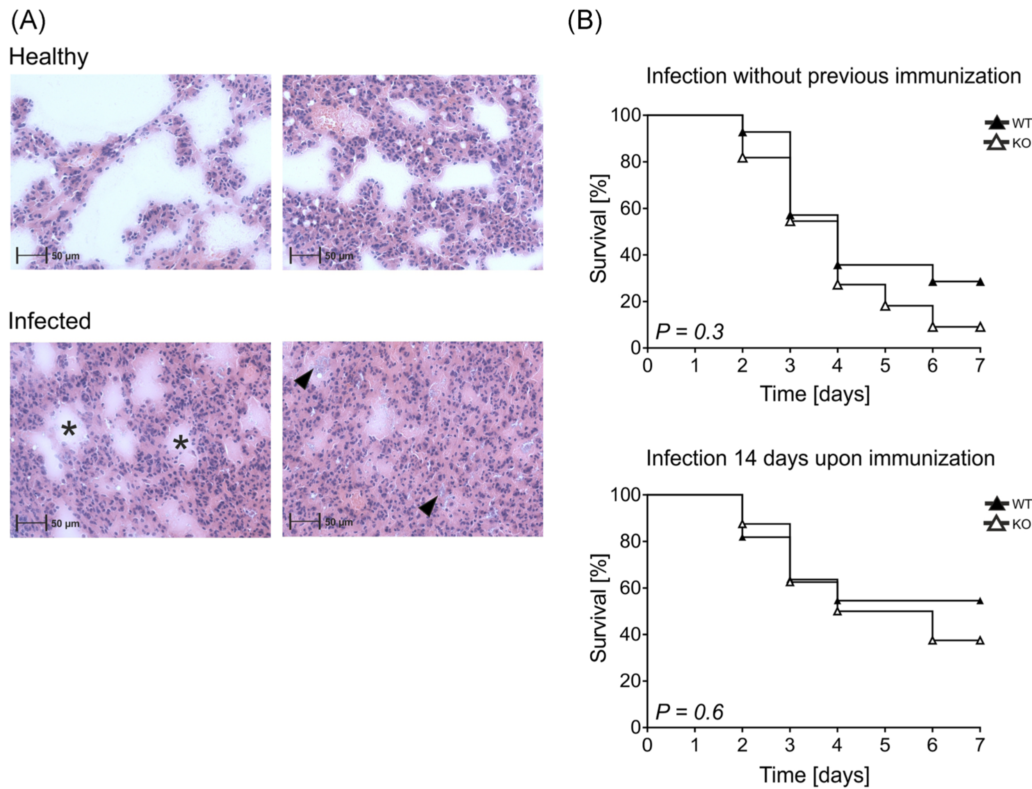

FIGURE 6 Histology and survival of SLy2-Wt and Ko mice upon intranasal challenge with *Streptococcus pneumoniae* serotype 3. (A) Exemplary histological images of lung tissue from one healthy mouse (pictures on the top) and one sick mouse (pictures on the bottom) (magnitude ×100). Lungs were shock-frosted in liquid nitrogen upon perfusion with PBS and stored at −80°C. For all four pictures, 12 µm cryosections of lung tissues were performed and stained with hematoxylin and eosin solution. The asterisks mark protein-rich edema within alveolar spaces and arrowheads indicate immune cell infiltrations as visualized by their blue-stained nuclei. (B) Seven days survival analysis of SLy2-Wt and Ko mice upon intranasal infection with 3.5×10^6 CFU/mouse without preceding immunization or after intraperitoneal vaccination with 1 µg P23 14 days before infection. Data are shown in Kaplan–Meier survival graphs and represent $n = 14$ mice per genotype pooled from three independent infection experiments. Indicated significances were determined using the logrank (Mantel–Cox) test. CFU, colony-forming unit; Ko, knockout; PBS, phosphate-buffered saline; Wt, wild-type

4 | DISCUSSION

S. pneumoniae is a major bacterial pathogen in humans, being responsible for a huge burden of disease worldwide. One of the most frequent manifestations of pneumococcal infection is pneumonia, accounting for around 1 million deaths of children annually.[29–31] Beyond its substantial contribution to child mortality, *S. pneumoniae* also represents a significant health problem in the elderly and immune-deficient patients.[17,30,32] By secretion of toxic mediators, *S. pneumoniae* efficiently induces host inflammation and evades the immune system through a variety of mechanisms.[16] The outer pPS capsule is the most immunogenic structure of *Pneumococci* and nowadays, more than 90 different serotypes are known.[26] P23 and PCV13 cover 23 and 13 of the most virulent and prevalent serotypes of *S. pneumoniae*, respectively. However, many patients do not mount or sustain adequate levels of pPS-specific antibodies upon immunization. Thus, severe clinical manifestations of pneumococcal infection remain common despite vaccination.[21,33]

Existing studies independently highlight the importance of innate B-1 cells in the context of infection caused by microbial pathogens such as *S. pneumoniae*.[34,35] Particularly B-1b cells represent an important first-line defense upon pneumococcal infection by the production of pPS-specific antibodies. They are substantially involved in the response towards a pneumococcal vaccine and were shown to mediate long-lasting immunity against *S. pneumoniae*.[14,15]

Here, we demonstrate increased frequencies of B-1b cells in the BM of SLy2-deficient mice (Figure 1A). The enrichment in BM B-1b cells is likely to explain the significantly elevated levels of circulating IgM measured in both, serum and spleen of these mice (Figure 1B).

Conveniently, B-1 cells of the BM have been identified as the major source of spontaneously secreted IgM in the absence of antigen.[23] The BM is a well-known niche for antibody-secreting cells, providing survival factors and allowing the deposition of immune globulin into the blood.[36,37] Recently, IL-9 has been reported to be an important growth factor specifically for B-1b cells and it is known to be produced in the BM.[38,39] Since increased production of IL-9 could drive survival and expansion of B-1b cells in SLy2-deficient mice, we analyzed IL-9 expression levels in isolated BMSCs. We observed a tendency of SLy2-Ko mice to produce more IL-9, however not in a statistically significant manner (Figure S5).

Notably, while we observed unaltered numbers of peritoneal B-1 cells, a previous report by Wang and colleagues revealed increased rates of $B220^{low}/CD5^{+}$ or $IgM^{+}/CD5^{+}$ peritoneal B-1a cells SLy2-Ko mice.[40] Importantly, here we defined B-1 cells by the simultaneous surface expression of CD19, CD43, and IgM, as previously published by Baumgarth.[11] The profound differences in B-1 cell phenotype definition explain the divergence of results between these two studies. When we performed additional staining using solely IgM, B220, and CD5, we also found increased percentages of $B220^{+}/IgM^{+}$ $CD5^{+}$peritoneal B-1a cells in our mouse model (data not shown).

Following ex vivo analysis of cell populations, we wanted to address whether the proliferation of SLy2-deficient B cells may be altered in response to TLR engagement. In vitro LPS-stimulation revealed a similar proliferative capacity of both Wt and Ko cells, implicating that SLy2 is not involved in B-1 cell LPS-sensing pathways such as TLR-4-dependent signaling.[41] Since B-1 cells are known to respond towards TLR-9-ligands, we further investigated the proliferation of peritoneal and splenic B-1 cells from Wt and Ko mice upon CpG stimulation (Figure S6).[42,43] However, there were no differences seen between the genotypes, supporting the assumption that SLy2 might be dispensable for functional TLR-signaling in B-1 cells.

On the other hand, the enhanced secretion of IgG_2 antibodies by isolated splenic B cells from SLy2-Ko mice indicates an intrinsic function of the adapter for IgG_2-isotype formation. The given differences only reached statistical significance upon IL-4-addition (Figure S4). Since IL-4 is the main cytokine produced by T follicular helper cells in the course of the germinal center reaction, these results indicate a specific role of SLy2 not only for TI responses but also for the generation of IgG_{2b} antibodies in cooperation with T-cell help.[44]

Upon immunization with the pPS vaccine P23, Ko mice produced elevated levels of specific IgM (Figure 3B). This observation matches to the dampened immune responses towards P23 recently found in SLy2-Tg mice.[10] Thus, these data collectively strengthen the hypothesis of SLy2 as an inhibitor of B-1 cell-mediated antibody production against pPS in a TI setting.

On the basis of these findings, we got also interested in the responsiveness of SLy2-Ko mice towards pneumococcal vaccine under TD conditions. As a consequence of the ip injection of PCV13, B-1 cells in the peritoneum markedly declined. This drop was attributable to a rapid and significant reduction of B-1a cells over time, with the B-1b cell population being increased after 21 days (data not shown). At the same time, we observed an enrichment of B-1 cells in the BM with cell percentages being significantly higher in Ko mice (Figure 4A,B). It is known that activated peritoneal B-1 cells rapidly migrate to the omentum and peripheral lymphoid tissues to produce and spread protective antibodies.[45,46] Therefore, our observation points to the efficient activation of peritoneal B-1 cells through the administration of PCV13. Whether the continuous increase in BM-resident B-1 cells upon vaccination is attributable to BM homing or merely a result of local proliferation, is a question of high interest that remains to be solved.

Investigation of PCV13-specific serum Ig revealed a fast induction of IgM in both SLy2-Wt and Ko animals on a highly comparable level. This is in contrast to what we have found earlier regarding P23, indicating that SLy2 might not be involved in the regulation of IgM responses under TD conditions.

Moreover, we demonstrate that SLy2-deficiency specifically favored the production of PCV13- and pPS-specific IgG_2 antibodies, which is in accordance with the increased production of IgG_2 upon IL-4 stimulation previously observed in vitro (Figures 4 and S4). Of all four IgG subclasses known, IgG_2 is the one that is primarily induced in response to polysaccharide antigens.[47,48] Interestingly, phenotypic manifestations of human Trisomy 21 include impaired responses towards a pneumococcal vaccine and a specific lack of IgG_2.[49,50] Since SLy2 is overexpressed in peripheral blood cells of patients with DS, our data allow the suggestion that excessive SLy2 might interfere with the formation of proper IgG_2 responses in DS patients.

S. pneumoniae infection experiments did not reveal any difference in survival of SLy2-Ko mice as compared to Wt, suggesting that increased levels of natural and pPS-specific antibodies were not sufficient to induce survival advantages. It is well-established that the outcome of infectious pneumonia depends on the proper activation of neutrophils, alveolar macrophages (AM) and natural killer cells.[51,52] For example, disruption of AM function has been reported to increase susceptibility

and mortality of mice in the context of *S. pneumoniae* infection.[53,54] However, differential expression of SLy2 only affects B-1 cells, but not T cell or myeloid cell populations (Figure S2).[10,40] When assessing the bacterial blood burden in our mice 24 or 48 h postinfection, we detected only low to zero numbers of CFUs (data not shown), suggesting that the survival outcome in our infection model mainly relied on the effectiveness of cell-mediated immune responses in the lung. Possible benefits through increased antibody titers might be undermined by a predominance of antibody-independent immune mechanisms in our infection model. Yet, the impact of SLy2-deficiency during systemic pneumococcal infection (e.g., during sepsis) remains to be investigated.

5 | CONCLUSION

To sum up, our study demonstrates improved pPS-specific antibody responses in the absence of SLy2 expression in mice. While knocking out SLy2 reinforced IgM responses under TI conditions, it specifically promoted IgG_2 production in the context of TD conjugate vaccination; suggesting that the role of the adapter protein highly depends on the type of B-cell stimulation that is given. The in vitro analysis of antibody secretion further points to a B-cell intrinsic role of SLy2 in the formation of IgG_2 responses upon TD stimulation via IL-4.

Considering the suboptimal immune response of many patients towards both, TI and TD pneumococcal immunization, the elevated production of pPS-specific IgM and IgG_2 as a result of SLy2-deficiency is of utmost interest. Differential expression levels of SLy2 in humans could at least in part explain the huge differences seen in the formation of antibody responses towards pneumococcal antigens. Thus, SLy2 should be considered as a potential target for future therapeutic interventions, aiming to improve pneumococcal vaccine-induced B cell immunity.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Jennifer Jaufmann designed and carried out the experiments, analyzed the data, and wrote the manuscript. Leyla Tümen, Fee Schmitt, Daniel Schäll, and Max von Holleben performed experiments. Sandra Beer-Hammer designed experiments, provided experimental and conceptual advice, and wrote the manuscript. All authors discussed the data and edited the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Paper 2

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ORIGINAL RESEARCH

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Enhanced IgG_1-mediated antibody response towards thymus-dependent immunization in CXCR1-deficient mice

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ICEPHA Mouse Clinic

Abstract

Background: Chemokine receptors and their corresponding ligands are key players of immunity by regulation of immune cell differentiation and migration. CXCR1 is a high-affinity receptor for CXCL8. Differential expression of CXCR1 is associated with a variety of human pathologies including cancer and inflammatory diseases. While various studies have highlighted the importance of CXCR1-mediated CXCL8-sensing for neutrophil trafficking and function, its role in B-cell responses remains unsolved. Therefore, our aim was to investigate innate and adaptive antibody responses in CXCR1-deficient mice.
Methods: Cell populations of the spleen and the peritoneal cavity were identified and quantified via flow cytometry. To investigate thymus-independent (TI) and thymus-dependent (TD) antibody responses, mice were immunized intraperitoneally with TNP-Ficoll, Pneumovax23, and TNP-Chicken Gamma Globulin. Mice were bled before as well as 7 and 14 days after vaccination to collect serum. Serum antibody levels overtime were analyzed according to their specificity by enzyme-linked immunosorbent assay. B-1 cell functionality was examined by IL-5/IL-5Rα-dependent stimulation of peritoneal and splenic cells in vitro. To analyze CXCR1/2-expression, $CD19^+$ splenocytes were enriched by magnetic-activated cell sorting before isolation of total RNA contents, followed by reverse transcription and real-time polymerase chain reaction.
Results: The distribution of natural B-1 cell populations was disturbed in the absence of CXCR1, while their responsiveness towards TI antigens and in vitro stimulation remained functional. Besides, CXCR1-deficiency was accompanied by increased frequencies of follicular B-2 cells in the spleen. Interestingly, these mice produced elevated levels of antigen-specific IgG_1 upon TD immunization and harbored a significantly enlarged proportion of CXCR5-expressing T helper (H) cells. CXCR1-expression was detectable in $CD19^+$ splenocytes derived from wild-type, but not CXCR1-deficient mice.

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Conclusion: Our data demonstrate a previously unknown relevance of CXCR1 for the production of specific IgG_1 in response to vaccination. These findings identify CXCR1 as a promising candidate for future studies on the regulation of adaptive antibody responses.

KEYWORDS

B-1 cells, B-2 cells, chemokine receptors, CXCR1, germinal center reaction, innate and adaptive antibody responses, vaccination

1 | INTRODUCTION

Chemokines are a large family of cytokines initially described as chemotactic molecules, since they are key regulators of immune cell positioning and lymphocyte trafficking. They execute their function by binding to respective chemokine receptors, which mainly belong to the group of G-protein-coupled receptors (GPCRs).[1] Beyond attracting immune cells towards sites of infection and inflammation, chemokines also contribute to embryonic development, regulate angiogenesis and promote proliferation, and metastasis of tumor cells.[2,3] Thus, chemokines and their receptors are potential candidates for the development of novel immunotherapeutic approaches.

Human CXCR1 and CXCR2 are high affinity receptors for the CXC-chemokine ligand 8 (CXCL8/interleukin-8 [IL-8]). Both of them are highly expressed on the surface of neutrophils, but also on macrophages, T cells and normal as well as malignantly transformed B cells.[4–6] In humans, CXCL8 is rapidly produced under inflammatory conditions and leads to the attraction of neutrophils into inflamed microenvironments, such as the airways, where they get activated and provide defense against bacterial pathogens.[7,8]

Whereas CXCR2 mainly mediates neutrophil transmigration, studies suggest an important role for CXCR1 in the course of bacterial sensing and killing. In 2014, Carevic et al.[9] reported reduced Toll-like receptor (TLR) 5-expression and impaired reactive oxygen species-production of neutrophils during *Pseudomonas aeruginosa* infection in CXCR1-deficient mice. In humans, CXCR1 gene variants and expression levels have been shown to correlate with cystic fibrosis lung disease and proteolytic cleavage of CXCR1 impaired antibacterial neutrophil host defense functions.[7,10] In summary, these studies suggest an essential role of CXCR1 in airway infection and innate immunity.

While the role of CXCR1 for neutrophil function is subject of various studies, its role for B-cell biology remains largely unknown. B cells are essential mediators of both innate and adaptive immunity by providing humoral protection.[11] Generally, they can be divided into conventional B-2 and innate-like B-cell populations, with the latter comprising natural B-1 and marginal zone (MZ) B cells.

Thymus-independent (TI) antigens such as pneumococcal polysaccharides (pPS) rapidly trigger the activation of innate-like B cells without the need for T-cell help, for example, via B-cell receptor crosslinking and TLR-engagement.[12] This response results in short-lived plasma cell differentiation, accompanied by low levels of somatic hypermutation and inducing antibodies of rather broad specificity.[12,13] Innate B cells are characterized by a unique expression of surface markers, the ability of self-replenishment and their specific anatomical localization. In mice, MZ B cells are restricted to the spleen where they interact with blood-borne antigens and produce protective immune globulin (Ig).[14,15] By contrast, natural B-1 cells are enriched within the pleural and peritoneal body cavities and can also be found in the spleen.[16] Under homeostatic conditions, B-1 cell populations are maintained by self-renewal and constitutively secrete autoreactive antibodies, clearing apoptotic cells and toxic metabolites. Besides, they respond towards common danger-associated epitopes, thereby substantially contributing to the clearance of encapsulated bacteria.[17–22]

On the other hand, long-lived adaptive B-cell responses towards thymus-dependent (TD) protein antigens are usually driven by follicular (FO) B-2 cells in cooperation with cognate T helper (T_H) cells during a process called germinal center (GC) reaction. It comprises several rounds of affinity maturation and selection, eventually resulting in highly specific and class-switched effector B cells.[23] Meanwhile, the function of T cells is to provide signals essential to the proliferation, survival, and differentiation of maturing B cells. GC formation is a dynamic process that critically depends on coordinated changes in chemokine receptor expression, for instance affecting CXCR4, CXCR5, and CCR7.[24] Tight regulation of these processes is crucial to maintain self-tolerance and aberrant adaptive antibody responses are associated with severe autoimmune pathologies in humans.[25,26]

In the present study, we analyzed innate and adaptive B-cell populations in the absence of CXCR1 in mice.

Concomitantly, we evaluated in vivo antibody responses to immunization with TI and TD antigens, considering different Ig isotypes.

While frequencies of FO B cells were enhanced in the spleen of CXCR1-deficient mice, the population of innate-like B-1 cells was significantly reduced. CXCR1-deficiency favored the production of specific serum IgG_1 towards the TD antigen TNP-CGG, accompanied by increased percentages of CXCR5-expressing T_H cells.

In conclusion, our results reveal a novel role of CXCR1 during the formation of TD antibody responses.

2 | MATERIALS AND METHODS

2.1 | Mice

CXCR1-deficient mice were generated by P. Murphy/NIH as described previously and purchased from The Jackson Laboratory (strain B6.129-*Cxcr1*^{tm1Msli}/J).[9] Mice were bred at the animal facility of the Institute of Pharmacology and Toxicology in Tübingen under specific pathogen-free conditions in open cages. For all experiments, age- and sex-matched littermate controls were used (10–15 weeks old). All animal work was performed according to the German animal care regulations and animal experiments were approved by the local ethics committee (AZ 04.01.2018; PH2/18).

2.2 | Organ preparation and cell culture

Peritoneal cells were harvested via lavage with 10 ml of ice cold PBS. Spleens were isolated and homogenized using a 70-µm cell strainer, followed by erythrocyte lysis for 3 min to get rid of red blood cells. Subsequently, single cell suspensions were analyzed by flow cytometry either directly or after in vitro stimulation.

For in vitro assays, 2×10^6 cells were cultured in 500-µl cell culture medium (RPMI 1640 supplemented with 10% FCS, 1% L-glutamine, 1% penicillin/streptomycin, and 0.05-mM β-mercaptoethanol) in 24-well plates. For stimulation, 10-ng/ml IL-4, 2-µg/ml α-CD40, and 10-ng/ml IL-5 were supplemented and cells were incubated for 48 h at 37°C and 5% CO_2.

2.3 | Flow cytometry

To investigate B-1 cell populations pre- or poststimulation, splenocytes and peritoneal cells were stained with CD19-V450, CD43-PE, CD5-APC, IgM-PE-Cy7, and CD125-FITC. For analysis of B-1 cell intracellular IgM-expression after stimulation, cells were initially stained on the surface with CD19-V450, CD43-FITC, CD5-APC, and IgM-PE. Fixation, permeabilization, and intracellular staining were performed using the Transcription Buffer Set (BD Bioscience) and IgM-PE-Cy7 antibody. To analyze splenic GC B cells and T_{FH} cells, splenocytes were stained with B220-PerCP, CD21-FITC, CD23-BV510 and GLY7-PE, or CD3-FITC, CD4-PacificBlue, CXCR5-APC and GLY7-PE, respectively. MZ and FO B cells were stained using B220-PerCP, CD23-BV510, CD21-FITC, IgM-APC-Cy7, and IgD-V450. All fluorchrome-conjugated antibodies were purchased from BD Bioscience or Biolegend. Before surface staining, unspecific F_C-binding sites were blocked by incubation with anti-CD16/32 (Biolegend) for 15 min. Measurements were performed at the BD FACSCanto II and gating was done using FlowJo Version 10. To ensure correct gating, fluorescence minus one controls were applied.

2.4 | Immunization protocol

Mice were immunized intraperitoneally with either 1-µg Pneumovax23 (P23; SanofiPasteurMSD) in 100-µl PBS, 100-µg TNP-CGG (Biosearch Technologies) precipitated in 100-µl Alum + PBS (1:1) or 5-µg TNP-Ficoll (Biosearch Technologies) in 100-µl PBS. Blood sampling was performed before, as well as 7 and 14 days after immunization. Blood was collected in Microtainer® SST™ Tubes (BD Bioscience) and kept at room temperature for 30–60 min. Subsequently, the serum was separated by centrifugation (90 s at 15,000 g) and stored at −20°C.

2.5 | Enzyme-linked immunosorbent assay

To measure concentrations of global serum IgM, high-binding 96-well plates were coated with purified rat anti-mouse IgM capture antibodies (BD Pharmingen) at 4°C over night. The other day, the plate was blocked for 1 h with blocking buffer. Diluted sera were incubated on precoated plates for 2 h at room temperature. Purified mouse κ IgM isotype control (BD Pharmingen) was used as a standard in serial dilutions.

For detection of P23- and pPS-specific antibodies in the sera of mice, high-binding 96-well plates were coated over night at 37°C with 1-µg/ml P23, pPS3, 4, 6B, or 19F (SSI Diagnostica). Antibody responses towards TNP-Ficoll/-CGG were evaluated by coating of 10-µg/ml NP7-BSA or NP14-BSA (Biosearch Technologies) at 4°C over night. Sera were diluted in sample buffer and P23-probes were freshly supplemented with 10-µg/ml cell wall polysaccharides (SSI Diagnostica) to capture unspecific

antibodies. Serum dilutions were incubated on precoated, blocked plates for 2 h at 37°C (P23 and pPS) or for 1 h at room temperature (TNP-Ficoll/CGG).

Specifically bound antibodies were detected by incubation with biotin-conjugated anti-mouse IgM, IgG_1, IgG_{2a}, or IgG_3 (BD Pharmingen).

For all enzyme-linked immunosorbent assays (ELISAs) subsequently, streptavidin-conjugated horseradish peroxidase (Bio-Techne) was incubated and chemoluminescent reaction was induced by addition of 3,3′,5,5′-tetramethylbenzidine substrate (Thermo Fisher Scientific). The reaction was stopped with sulfuric acid and measurement was performed at a wavelength of 450 nm. To allow the direct comparison of samples without specific standard, all probes were measured on the same ELISA plate.

2.5.1 | Real-time polymerase chain reaction

$CD19^+$ B cells were isolated from the pool of total splenocytes by performance of magnetic-activated cell sorting using anti-CD19 micro beads and magnetic separation columns (Miltenyi Biotech) for positive selection. Subsequently, RNA-contents were isolated with the ExtractMe Total RNA Kit (Blirt). Four hundred nanograms of RNA were transcribed using TranscriptMe cDNA Kit (Blirt). Subsequently, real-time polymerase chain reaction (RT-PCR) was performed with the Sensi-fast SYBR No-Rox Kit (Bioline) in the Light Cycler® 480 (Roche). The final amount of cDNA was 50 ng/plate well. CXCR1 and CXCR2 primers were used as previously described by Fu et al.[27] and produced by Biomers (CXCR1 forward: 5′-GCTGC CCACTGGAGATTATTTC-3′, CXCR1 reverse 5′-TATGCC TGGCGGAAGATAGC-3′; CXCR2 forward: 5′-ATGCTG TTCTGCTACGGG-3′; CXCR2 reverse: 5′-ATGGATGAT GGGGTTAAG-3′). Amplification was performed under following conditions: preincubation at 94°C (2 min), followed by 30 cycles of each 94°C (30 s), 55°C (30 s), and 72°C (1 min). Advanced relative quantification was done with the Light Cycler® 480 software and β-actin served as reference gene.

3 | RESULTS

3.1 | Skewed repertoire of B-cell populations in CXCR1-deficient mice

Initially, we investigated splenic B-2 cell populations in CXCR1-deficient mice as compared to wild-type (WT) littermate controls. While the number of splenocytes, the frequency of $B220^+$ cells and MZ B cells were comparable between the genotypes, the proportion of FO B cells was significantly increased in the context of CXCR1 deficiency (Figure 1).

Subsequently, we analyzed innate-like B-1 cells residing in peritoneal cavity and spleen (Figure 2A,B). While peritoneal B-1 cells were mostly unaltered, we observed a severe reduction of splenic B-1 cells in CXCR1-deficient mice, especially affecting the population of $CD5^-$ B-1b cells (Figure 2B). For flow cytometry gating strategies applied to identify corresponding B-cell populations, the reader is referred to Figure S1.

Since B-1 cells are well-established as major producers of circulating IgM under homeostatic conditions, we evaluated the concentration of global serum IgM in these mice. Surprisingly, there were no differences detectable (Figure S2).

3.2 | Normal antibody response towards TI antigens in CXCR1-deficient mice

Given the diminished pool of splenic B-1 cells in CXCR1-deficient mice, we further examined their antibody response towards TI antigens. To this end, mice were immunized with the TI antigen TNP-Ficoll and blood sampling was performed before, 7 and 14 days after immunization. As depicted in Figure 3A, the induction of specific IgM targeting the epitopes NP7 (left panel) and NP14 (right panel) was equally productive in both, CXCR1-deficient and WT control mice.

B-1 cells are known to be essentially involved in the antibody-mediated response towards pPS. In this regard, they represent an important first line defense but also contribute to long-lasting immunity against *Streptococcus pneumoniae*.[18,28,29] Thus, we were interested in the antibody response of CXCR1-deficient mice towards the pure polysaccharide vaccine P23, provoking TI responses. Figure 3B displays the analysis of specific IgM targeting P23 as well as four common serotypes of *S. pneumoniae*, frequently associated with human disease (pPS3, 4, 6B, and 19F). As illustrated in Figure 3B, the TI antibody production in response to P23-immunization was unaltered in CXCR1-deficient mice.

3.3 | IL-5/IL-5Rα-dependent stimulation of B-1 cells

The IL-5/IL-5R-signaling pathway is known to be a key regulator of B-1 cell survival and homeostatic proliferation. IL-5 binding is mediated through the IL-5Rα chain, which is constitutively expressed on B-1 cells.[30]

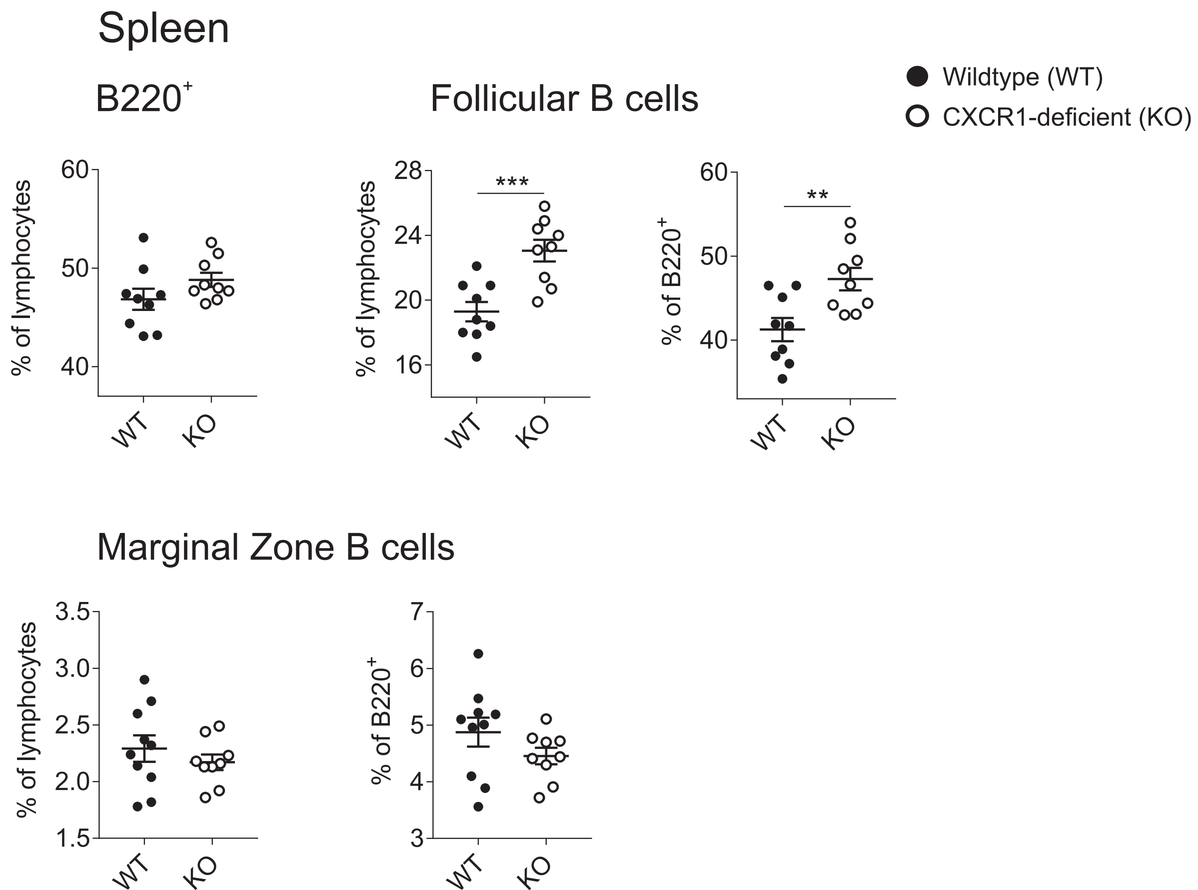


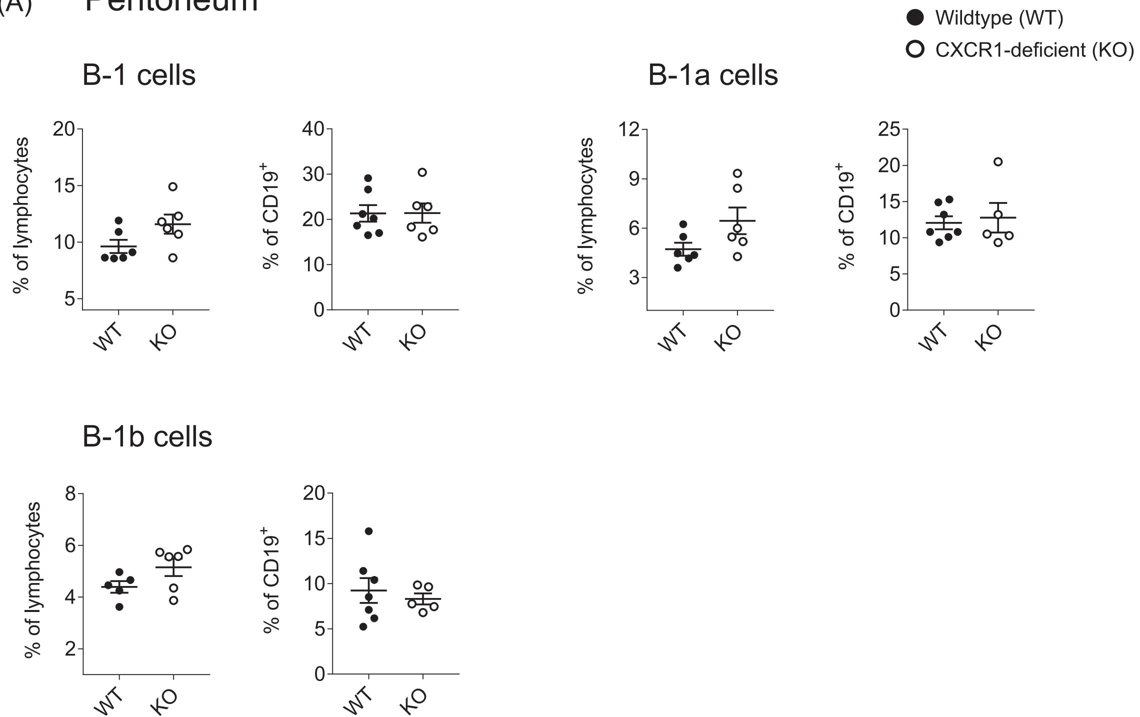
FIGURE 1 Enriched proportions of follicular (FO) B cells in the spleen of CXCR1-deficient mice. Splenic single cell suspensions were fluorescently stained with B220-PerCP, CD23-BV510, CD21-FITC, IgM-APC-Cy7, and IgD-V450 and subsequently analyzed by flow cytometry. FO B cells were defined as $CD23^{+}CD21^{low}IgD^{+}IgM^{low}$ and marginal zone B cells as $CD23^{-}CD21^{high}IgM^{+}IgD^{low}$. Cell populations are shown as percentage of all single lymphocytes and percentage within the fraction of $B220^{+}$ cells for wild-type (WT) and CXCR1-deficient (KO) mice. Data represent $n = 9–10$ mice per genotype from two independently performed experiments and error bars indicate mean ± *SEM*. Significances were determined by Student's *t*-test and a $p < .05$ was considered statistically significant (*p <.05, **p < .01, ***p < .001)

Accordingly, depletion of the IL-5Rα chain results in a strong decrease of the B-1 cell population in mice.[31] We, therefore, wondered whether the IL-5 pathway might be affected in the context of CXCR1-deficiency, leading to the massive reduction in splenic B-1 cells we could previously observe. Hence, we analyzed IL-5Rα expression on peritoneal and splenic B-1 cells before and after in vitro stimulation with IL-4, α-CD40, and IL-5. As illustrated in Figure 4A, the proportion of IL-5Rα^{+} peritoneal B-1 cells was significantly increased in the absence of CXCR1 (left panel), while being decreased within the population of splenic B-1 cells (right panel). Surprisingly, these differences were compensated to a great extent after 48 h of stimulation (Figure 4A).

Besides, IL-5 efficiently triggers differentiation of peritoneal B-1 cells into IgM-producing plasmablasts.[32] We thus complementarily analyzed the expression of intracellular IgM in peritoneal B-1 cells after 24 h of stimulation either without or with IL-5 supplementation. As shown in Figure 4B, intracellular IgM-expression was specifically induced by the addition of IL-5 on a highly significant level. There were no differences detected between the genotypes (Figure 4B).

3.4 | Improved IgG_1 response towards TD antigen in CXCR1-deficient mice

As we have found markedly increased populations of splenic B-2 cells in CXCR1-deficient mice, we next focused on their antibody response towards TD antigen. To this end, mice were immunized with TNP-CGG, and antibody reactions were measured at Days 0, 7, and 14. Apart from IgM, we additionally included IgG_1 and IgG_3 isotypes in our analysis. Again, we assessed serum Ig levels specifically targeting NP7 (left panel) and NP14 (right panel; Figure 5A). While IgM and IgG_3 responses were comparable between the genotypes, CXCR1-deficient mice



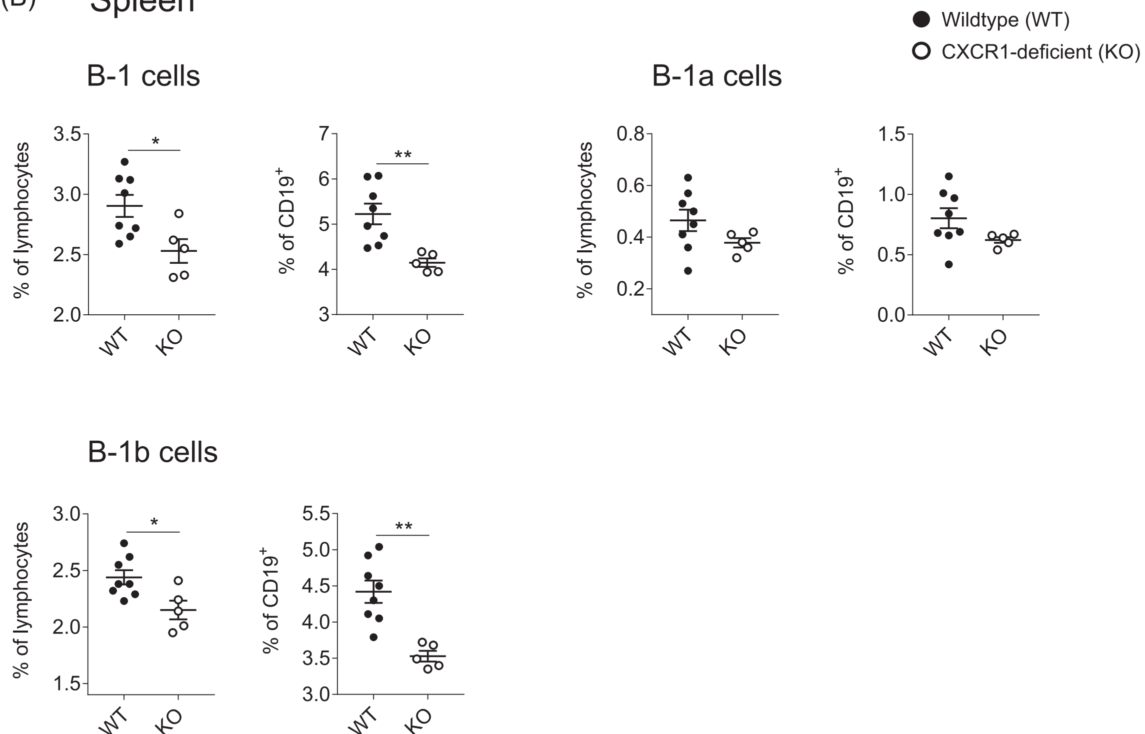


FIGURE 2 Decreased populations of splenic B-1 cells in CXCR1-deficient mice. Cells were fluorescently stained with CD19-V450, CD43-PE, CD5-APC, and IgM-PE-Cy7 and subsequently analyzed by flow cytometry. B-1 cells were defined as $CD19^+CD43^+IgM^+$ and subdivided into B-1a and B-1b cells, being $CD5^+$ or $CD5^-$, respectively. B-1 cell frequencies were assessed for wild-type (WT) and CXCR1-deficient (KO) mice in (A) peritoneal washouts and (B) the spleen and are given as both, percentage of all single lymphocytes and percentage within the $CD19^+$ population. Data represent $n = 5–8$ mice per genotype pooled from two independent experiments. Error bars show the mean ± *SEM* and significance was determined by Student's *t*-test. A $p < .05$ was considered statistically significant ($^*p < .05$, $^{**}p < .01$)

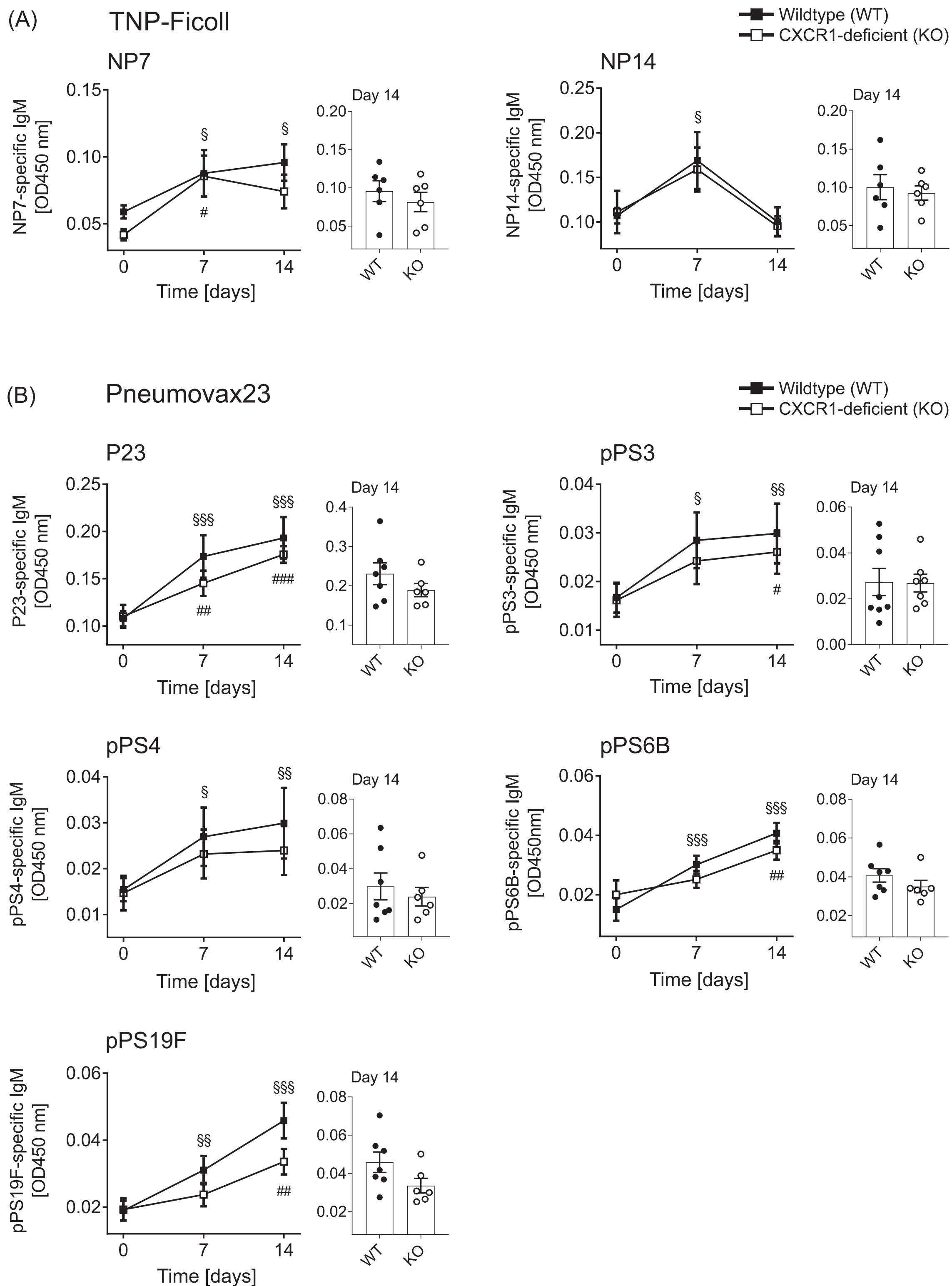


FIGURE 3 In vivo IgM responses towards the thymus-independent (TI) antigens TNP-Ficoll and Pneumovax23 (P23). For analysis of TI immune responses, wild-type (WT) and CXCR1-deficient (KO) mice were intraperitoneally immunized with (A) TNP-Ficoll or (B) P23. Blood sampling was performed before (Day 0) and 7 and 14 days postimmunization to measure NP7 and NP14 (TNP-Ficoll) or P23, pPS3, 4, 6B and 19F (P23)-specific antibodies by enzyme-linked immunosorbent assay (ELISA). Graphs depict the overtime progression of serum IgM levels given as optical density (OD) at 450 nm measurement wavelength. Accessorily, single data points are shown for Day 14. For every specific assay, all probes were analyzed on one identical ELISA plate in duplicates to allow direct comparison of samples. Curves represent $n = 5–8$ mice per genotype out of two independent experiments and error bars illustrate the mean ± *SEM*. Significances were determined by two-way analysis of variance multiple comparisons and a $p < .05$ was considered statistically significant. § indicates significance of WT curves and $^{\#}$ of KO curves ($^{§,\#}p < .05$, $^{§§,\#\#}p < .01$, $^{§§§,\#\#\#}p < .001$)

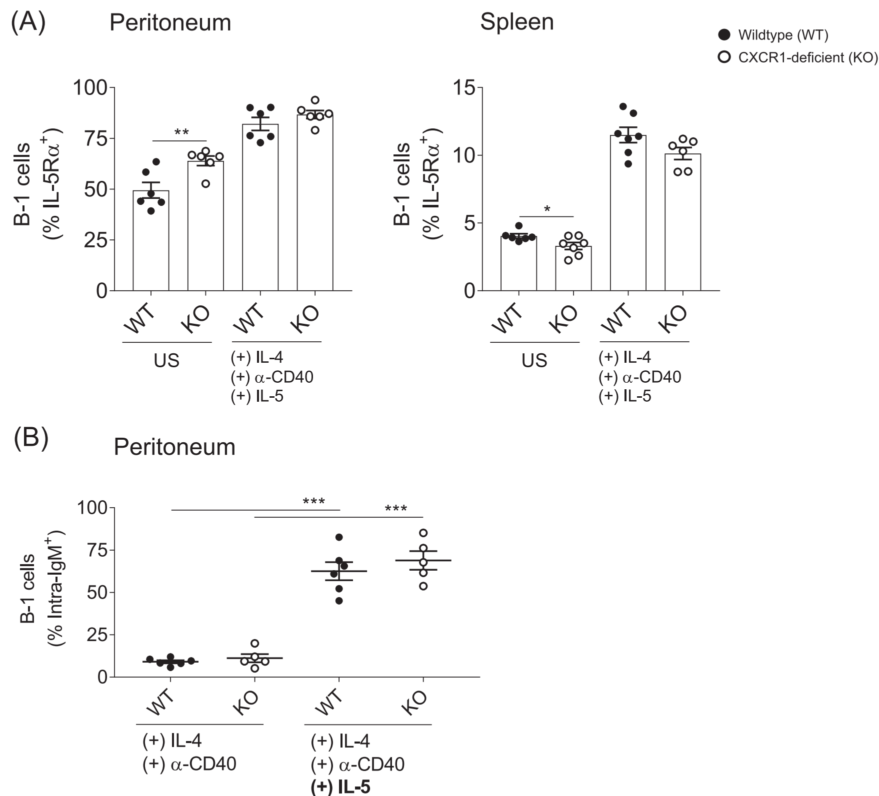


FIGURE 4 IL-5/IL-5Rα-dependent stimulation of B-1 cells in vitro. (A) Peritoneal (left panel) and splenic (right panel) cells were isolated and cultured in complete cell medium to investigate the surface expression of IL-5Rα on B-1 cells. To this end, 10-ng/ml IL-4, 2-µg/ml α-CD40, and 10-ng/ml IL-5 were supplemented and cells were incubated for 48 h at 37°C and 5% CO_2. Subsequently, cells were fluorescently stained with CD19-V450, CD43-PE, CD5-APC, IgM-PE-Cy7, and CD125/IL-5Rα-FITC for flow cytometry. Graphs depict the proportion of IL-5Rα-positive B-1 cells within unstimulated (US) controls and the stimulated cell fraction. (B) Peritoneal cells were stimulated either with IL-4 and α-CD40 alone or additionally with IL-5. Subsequently, cells were stained with CD19-V450, CD43-FITC, CD5-APC, and IgM-PE on the surface and intracellular with IgM-PE-Cy7 for flow cytometry. Graphs illustrate the percentual expression of intracellular IgM in wild-type (WT) and CXCR1-deficient (KO) B-1 cells in the absence or presence of IL-5. Data comprise $n = 5–6$ mice from each two independent experiments and error bars indicate mean ± *SEM*. Significances were determined by Student's *t*-test (A) or one-way analysis of variance multiple comparisons (B) and a $p < .05$ was considered statistically significant (*$p < .05$, **$p < .01$, ***$p < .001$)

produced significantly more amounts of NP7- and NP14-specific IgG_1 at both days postimmunization (Figure 5A).

Since TD IgG_1 antibody responses generally depend on GC reaction B cells in cooperation with CXCR5-expressing T_H cells, we investigated both cell types in the spleen of mice 14 days after TNP-CGG vaccination.[23]

First, we found the total cell numbers of splenocytes, $B220^+$ B cells, $CD3^+$ T cells, and $CD3^+CD4^+$ T_H cells being similar in both experimental groups (Figure S3A,B). Second, populations of GC reaction B cells did not statistically differ in CXCR1-deficient mice as compared to their WT counterparts (Figure 5B, upper panel).

However, when analyzing CXCR5-expressing T_H cells, we found that these cells were significantly enriched in the absence of CXCR1 within the overall lymphocyte population as well as within the fraction of all T_H cells (Figure 5B, lower panel). These findings were supported by reinforced median fluorescent index of CXCR5-APC on $CD3^+CD4^+$ T_H cells (Figure 3C). Figure S3D exemplarily shows the gating strategy applied for identification of $CXCR5^+$ T_H cells.

4 | DISCUSSION

Chemokine receptors and their corresponding chemotactic molecules play a pivotal role in immunity.[33] While CXCR1 is already established as an important regulator of neutrophil migration and function, its role in the context of antibody responses has not been studied before.

The present work reveals a novel relevance of CXCR1-expression with regard to TD antibody responses. Upon TNP-CGG vaccination, we found greatly increased levels of antigen-specific IgG_1 in the serum of CXCR1-deficient mice (Figure 5A). Besides, these mice harbored

increased rates of $CXCR5^+$ T_H cells in the spleen (Figure 5B). The absolute number of the overall T-cell population was unaltered, indicating that the loss of CXCR1 specifically favored CXCR5-expression on T_H cells (Figures S3B and 5B).

IgG_1 is the most abundant subclass of the IgG isotype and is classically induced in response to protein antigens.[34] This involves interactions of B and T cells during GC reaction in the B-cell follicles.[23] More precisely, specialized T cells drive the development of high-affinity GC B cells that eventually differentiate into antibody-secreting, class-switched plasma cells.[13]

It is well-known that activated T_H cells need to downregulate CCR7 and at the same time upregulate CXCR5 on their surface to get access to the B-cell follicle.[35] Moreover, CXCR5-expression by T cells is known

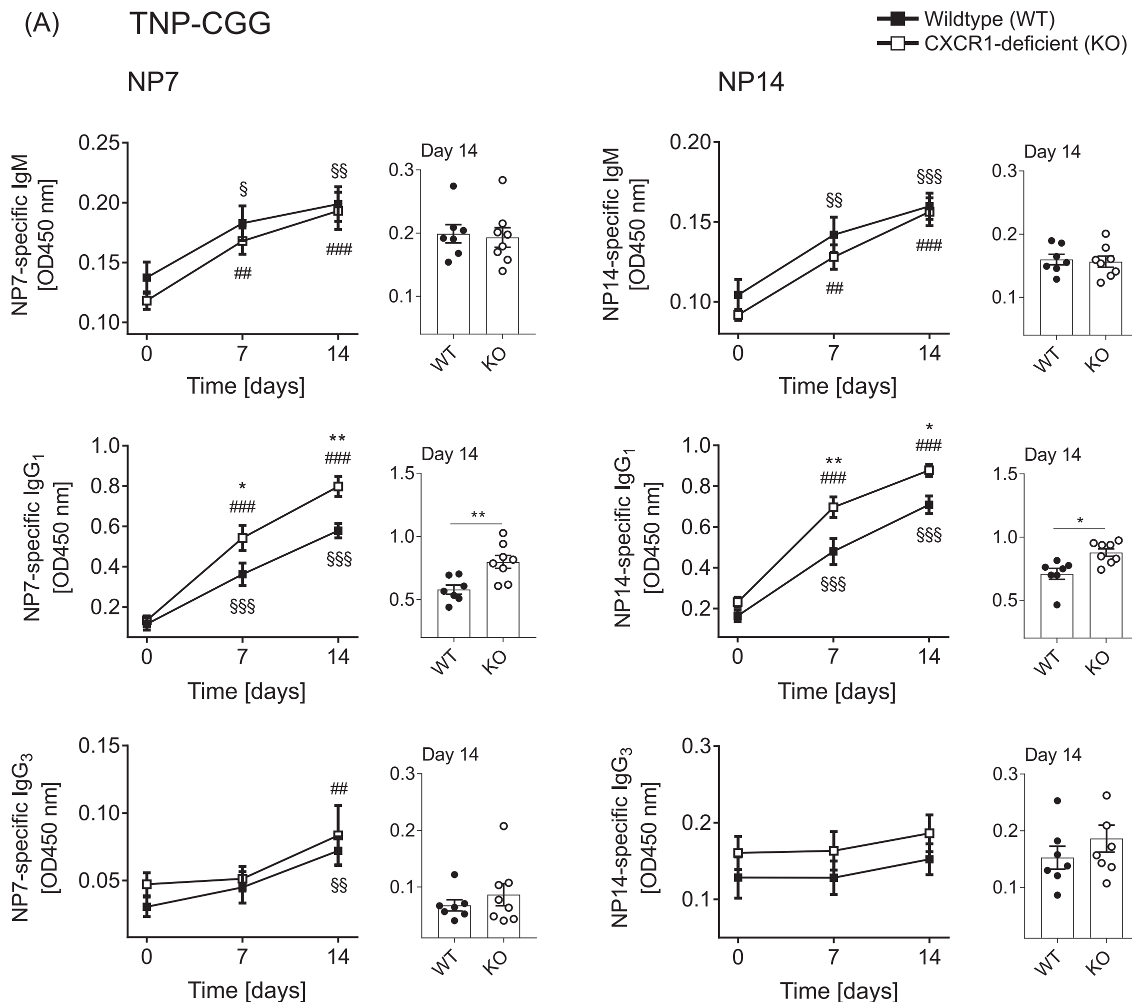


FIGURE 5 Improved in vivo B- and T-cell responses towards thymus-dependent (TD) antigen TNP-CGG in CXCR1-deficient mice. Wild-type (WT) and CXCR1-deficient (KO) mice were immunized intraperitoneally with TNP-CGG precipitated in alum and blood sampling was performed before (Day 0) and 7 and 14 days after vaccination to measure NP7 and NP14-specific antibodies by enzyme-linked immunosorbent assay (ELISA). For every immune globulin (Ig) and specificity, all probes were analyzed on one identical ELISA plate in duplicates to allow for direct comparison. After 14 days, mice were killed and splenocytes were analyzed by flow cytometry. (A) Overtime progression of the amount of specific serum IgM, IgG_1, and IgG_3 shown as optical density (OD) at 450 nm measurement wavelength. Curves represent $n = 7$–8 mice per genotype out of two independent experiments and error bars indicate ± *SEM*. Significances were determined by two-way analysis of variance multiple comparisons and a $p < 0.05$ was considered statistically significant. § indicates significance of WT curves; # of KO curves; and * reflect differences between the genotypes ($^{§,\#,*}p < .05$, $^{§§,\#\#,**}p < .01$, $^{§§§,\#\#\#,***}p < 0.001$). (B) Splenic germinal center reaction B cells and T_{FH} cells were defined as $B220^+GLY7^+$ and $CD3^+CD4^+CXCR5^+$, respectively. Results are depicted as rate of all single lymphocytes and percentage within the overall $B220^+$ or $CD3^+CD4^+$ T_H cell-population. In addition, the median fluorescent index of CXCR5-APC is shown for T_H cells. Data is shown for $n = 4$–8 mice and error bars represent mean ± *SEM*. Significances were determined by Student's *t*-test and a $p < .05$ was considered statistically significant ($^*p < 0.05$, $^{**}p < .01$)

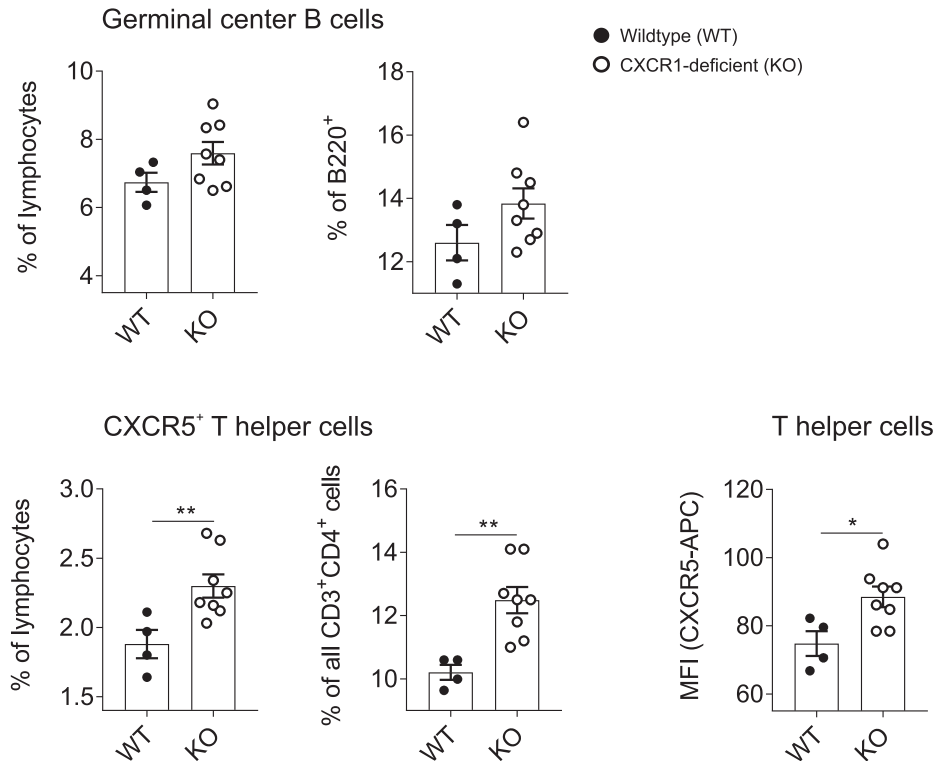


FIGURE 5 Continued

to increase the probability of direct interaction with their cognate antigen-specific B cells. Conversely, GC reactions are strongly affected by CXCR5-deficiency. Among others, its deletion is accompanied by impaired B-cell isotype class switching.[24,35–37] Based on this knowledge we speculate that increased CXCR5-expression levels on T_H cells might be one mechanism leading to the improved IgG_1 production in CXCR1-deficient mice. This might occur through reinforced T-cell entry into the follicles or by strengthened interactions of T and B cells. Another contributing point might also be the enriched FO B-cell population in these mice at steady state, being the foundation for enhanced responses after antigen encounter (Figure 1).

Importantly, subsequent studies are needed to clarify whether CXCR1-expression exerts direct influence on FO B and T_H cells, or whether our observations rely on indirect effects. Of note, by performing RT-PCR, we were able to detect expression of CXCR1 in $CD19^+$ splenocytes derived from WT mice, while its expression was absent in CXCR1-deficient mice. At the same time, the highly homologous receptor CXCR2 was expressed in B cells of both genotypes (Figure S4).

As a consequence, it is reasonable to speculate that CXCR1 expressed by B cells could directly regulate their function. However, to verify this hypothesis, in vitro proliferation and migration assays should be performed to study the role of CXCR1 in B-cell activation and trafficking. Immune responses are highly versatile and involve the interplay of various cell types. For example, neutrophil function is known to depend on CXCR1 and at the same time, cross-talk between neutrophils and lymphocytes has been reported.[38,39] Thus, the increased IgG_1 response in the context of CXCR1-deficiency might also rely on inflammatory signals derived from myeloid cells. In summary, our observations are of high interest and CXCR1 should be studied in detail in the context of GC reactions.

Interestingly, the frequency of splenic B-1b cells was drastically reduced in the absence of CXCR1 (Figure 2A). On the other hand, the enrichment of IL-5Rα-expressing cells in the peritoneum of CXCR1-deficient mice further points to a local accumulation of activated B-1 cells, which in turn were decreased within the splenic fraction (Figure 4A). Since the divergences in IL-5Rα-expression were largely compensated upon stimulation in vitro, these most likely do not result from any functional deficit in the absence of CXCR1. This idea is further supported by the fact that we were unable to detect any differences in natural serum IgM or intracellular IgM expression of

stimulated B-1 cells (Figures S2 and 4, respectively). The latter further indicates that CXCR1 is dispensable for IL-5-induced IgM production by peritoneal B-1 cells (Figure 4B). Moreover, there were no statistically significant differences in antibody production towards TI immunization (Figure 3).

Nevertheless, it is possible that CXCR1-signaling is involved in the regulation of B-1 cell distribution under homeostatic conditions. B-1 cells are characterized by great mobility, as they continuously traffic between the blood and the peritoneal space, passing the omentum.[40] Moreover, upon activation, they rapidly egress from the peritoneum to enter the spleen.[41,42] One possibility would be that CXCR1 is required for B-1 cell homing into the spleen, which would in turn explain the markedly reduced population of splenic B-1 cells on the background of CXCR1-deficiency. Strikingly, B-cell migration and entry into B-cell follicles is known to involve CXCR5–CXCL13-mediated signaling and CXCR5 has been shown to be highly expressed on B-1 cells.[40] Accordingly, the increased expression of CXCR5 on T_H cells in CXCR1-deficient mice made us wonder whether CXCR5-expression might also be altered on B-1 cells. Indeed, we found significantly lower CXCR5-expression levels on peritoneal B-1 cells in the absence of CXCR1, while splenic samples were comparable (Figure S5). Among others, homeostatic B-cell positioning is regulated by CXCR5.[1] The dampened CXCR5-expression on B-1 cells of CXCR1-deficient might prevent their entry into the spleen, leading to an accumulation of these cells in other locations. However, more data needs to be assessed to support this idea, especially since mechanisms of B-1 cell trafficking remain poorly understood.

5 | CONCLUSION

In summary, our data reveals a previously unknown role of CXCR1 during the formation of TD antibody responses in mice. Since we were able to detect expression of CXCR1 in $CD19^+$ splenocytes, it is possible that CXCR1 might exert direct influence on B-cell responses. Moreover, our findings allow the assumption that CXCR1-mediated signaling might take over a regulatory role for T_H cells by controlling CXCR5-expression levels. These findings could be useful since adaptive antibody responses are commonly hyperactivated in human autoimmune diseases.[43,44] Besides, the accumulation of CXCR5-expressing T_H cells positively correlates with disease severity in patients suffering from systemic lupus erythematosus, Sjögren's syndrome, and rheumatoid arthritis.[45–47]

Based on our novel observations, CXCR1 should be subject to future studies in the context of GC dynamics and B-cell migration.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Jennifer Jaufmann designed and carried out the experiments, analyzed the data, and wrote the manuscript. Melanie Carevic performed experiments and provided experimental advice. Derya Eliacik and Leyla Tümen performed experiments. Fee Schmitt designed experiments. Dominik Hartl provided experimental and conceptual advice. Sandra Beer-Hammer designed experiments, provided experimental and conceptual advice and wrote the manuscript. All authors discussed the data and edited the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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Paper 3

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ORIGINAL RESEARCH

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SLy2-overexpression impairs B-cell development in the bone marrow and the IgG response towards pneumococcal conjugate-vaccine

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Abstract

Background: Infections with *Streptococcus pneumoniae* can cause severe diseases in humans including pneumonia. Although guidelines for vaccination have been established, *S. pneumoniae* is still responsible for a serious burden of disease around the globe. Currently, two pneumococcal immunizations are available, namely the pure polysaccharide vaccine Pneumovax23 (P23) and the conjugate-vaccine Prevenar13 (PCV13). We recently reported impaired thymus-independent antibody responses towards P23 in mice overexpressing the immunoinhibitory adapter SLy2. The purpose of this study was to evaluate adaptive B-cell responses towards the thymus-dependent vaccine PCV13 in SLy2-overexpressing mice and to study their survival rate during pneumococcal lung infection. Moreover, we investigated B-cell developmental stages within the bone marrow (BM) in the context of excessive SLy2-expression.

Methods: B-cell subsets and their surface immune globulins were investigated by flow cytometry. For class-switch assays, isolated splenic B cells were stimulated in vitro with lipopolysaccharide and interleukin-4 and antibody secretion was quantified via LEGENDplex. To study PCV13-specific responses, mice were immunized and serum antibody titers (immunoglobulin M, immunoglobulins IgG_1, IgG_2, and IgG_3) were examined by enzyme-linked immunosorbent assay. Survival rates of mice were assessed within 7 days upon intranasal challenge with *S. pneumoniae*.

Results: Our data demonstrate impaired IgG_1 and IgG_3 antibody responses towards the pneumococcal conjugate-vaccine PCV13 in SLy2-overexpressing mice. This was accompanied by reduced frequencies and numbers of BM-resident plasmablasts. In addition, we found drastically reduced counts of

Abbreviations: BM, bone marrow; DS, Down syndrome; FO, follicular; GC, germinal center; Ig, immunoglobulin; IL, interleukin; Ko, knockout; LPS, lipopolysaccharide; MZ, marginal zone; P23, Pneumovax23; PCV13, Prevenar13; pPS, pneumococcal polysaccharide; SLy2, Src homology domain 3 lymphocyte protein 2; TD, thymus/T cell-dependent; Tg, transgenic; TI, thymus/T cell-independent; TLR, toll-like receptor.

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B-cell precursors in the BM of SLy2-Tg mice. The survival rate upon intranasal challenge with *S. pneumoniae* was mostly comparable between the genotypes.
Conclusion: Our findings demonstrate an important role of the adapter protein SLy2 in the context of adaptive antibody responses against pneumococcal conjugate-vaccine. Interestingly, deficits in humoral immunity seemed to be compensated by cellular immune effectors upon bacterial challenge. Our study further shows a novel relevance of SLy2 for plasmablasts and B-cell progenitors in the BM.

KEYWORDS
antibody responses, B cells, B-cell development, pneumococcal conjugate-vaccine, pneumonia, Src homology domain 3 lymphocyte protein 2, streptococcus pneumoniae

1 | INTRODUCTION

Streptococcus pneumoniae is an opportunistic pathogen in humans, asymptomatically colonizing the upper respiratory tract under normal conditions. Upon local spread, invasion of the lower airways or the bloodstream, *S. pneumoniae* can cause severe diseases such as sepsis, meningitis, otitis, and pneumonia.[1,2] It is the most common cause of community-acquired pneumonia and mortality due to pneumococcal infections is especially high in infants, the elderly and in immunocompromised individuals.[3] Despite the availability of vaccines, pneumococcal disease still constitutes a major global health problem.[2,4]

Nowadays, two different types of pneumococcal immunizations are available. Pneumovax23 (P23) is a pure mixture of *S. pneumoniae*-derived capsular polysaccharides and induces a thymus-independent (TI) B-cell response, relying on antibodies produced by innate B-1 cells.[5,6] These cells are mainly located within the peritoneal and pleural body cavities but can also be found in the bone marrow (BM) and the spleen, where they constitutively secrete natural immunoglobulin M (IgM). B-1 cell-derived antibodies fulfill an important housekeeping function as they clear apoptotic cells and toxic metabolites from the circulation.[7,8] Besides, they quickly respond towards infections, thus representing an important first line defense against microbial pathogens, such as *S. pneumoniae*.[9]

The second vaccine is termed Prevenar13 (PCV13) and consists of 13 pneumococcal polysaccharides (pPS) coupled to a diphtheria carrier-protein. Therefore, PCV13 accessorily induces thymus-dependent (TD) responses that are mediated by adaptive B-2 cells and provide long-lived protection.[10,11] Since P23 has been shown to provide only transient and inadequate protection in high risk groups, the conjugate-vaccine PCV13 is recommended for children less than 5 years, adults greater than 50 years and patients with immune defects.[5]

We recently reported a regulatory role of Src homology domain 3 lymphocyte protein 2 (SLy2), also termed HACS1 (hematopoietic adapter containing SH3 and SAM domains 1) or SAMSN1 (SAM domain, SH3 domain, and NLS 1), for B-cell responses towards pneumococcal antigens.[12,13] SLy2 is an immunoinhibitory adapter protein that is mainly expressed in lymphocytes.[14] It is a key player of intracellular signaling by recruiting and linking downstream effector molecules upon receptor-induced activation. To this end, SLy2 holds specific protein interaction modules and is able to shuttle between the cytoplasm and the nucleus.[15] In humans, SLy2 is encoded on chromosome 21q11.2, a region frequently disrupted by translocation events in the context of hematopoietic disorders.[16] Several studies have highlighted the role of SLy2 for B-cell responses.[12,14,17–19] More precisely, it is upregulated in murine and human B cells upon activation.[18]

The investigation of SLy2-deficient mice revealed an enlarged compartment of B-1 cells and enhanced proliferative B-cell responses in the absence of the adapter. Moreover, natural serum IgM levels are increased in these mice, accompanied by improved antibody responses to pPS.[13,19] On the contrary, SLy2-transgenic (Tg) mice, specifically overexpressing the protein in T and B cells, harbor decreased proportions of B-1 cells and significantly reduced titers of homeostatic IgM.[12,17] This was accompanied by impaired antibody responses towards the pPS-vaccine P23.[12] This is of high interest since SLy2 belongs into a group of 9 genes that are additionally amplified in Down syndrome (DS) patients and might contribute to the disease phenotype associated with Trisomy 21.[20] DS patients suffer from several immune defects including abnormal antibody production and an enhanced susceptibility to pneumococcal infections.[21,22]

Since TI B-cell responses towards P23 are impaired in SLy2-Tg mice, we were interested in their TD antibody production towards the conjugate-vaccine PCV13, which

is recommended for DS patients. Moreover, we evaluated the survival rate of SLy2-Tg mice in comparison to their wildtype (Wt) littermates during acute pneumococcal infection. Our results revealed reduced rates of $CD138^+TACI^+$ plasmablasts (PBs) and PCV13-specific immunoglobulin G (IgG) levels in SLy2-Tg mice. We were unable to detect significant alterations with regard to the survival of SLy2-Tg mice during acute pneumococcal lung infection, probably due to compensatory mechanisms. Interestingly, the numbers of BM B-cell precursors were greatly diminished in SLy2-Tg mice, which is of high interest as DS children also suffer from defects in B-cell developmental processes.[22,23]

2 | MATERIALS AND METHODS

2.1 | Mice

SLy2-transgenic (Tg; over-expressing) mice were generated as described previously and kept under specific pathogen-free conditions.[17] All animal work was performed according to the German animal care regulations and animal experiments were approved by the local ethics committee (AZ 29.03.2017; PH1/14 and PH2/19). For all experiments, age-matched littermate mice were used. For PCV13-vaccination studies, mice were 9–13 weeks old. For acute infection with *S. pneumoniae*, 16–17 weeks old mice were used.

2.2 | Immunization experiments

Wt and SLy2-Tg mice were intraperitoneally immunized with 3 μg Prevenar13 (Pfizer) in 100 μl phosphate-buffered saline (PBS). To analyze cell populations and serum antibody titers, mice were sacrificed before and 7, 14, and 21 days after immunization to collect blood, peritoneal lavage (PL), spleen, and BM.

2.3 | Organ preparation

Blood was collected in Microtainer blood collection tubes (BD Bioscience). After at least 30 min of incubation at RT, tubes were centrifuged at 15,000*g* for 90 s to collect the serum in the supernatant. Sera were stored at −20°C.

PL was performed with 5 ml of ice cold PBS. BM was collected by flushing out femurs with 5 mL of ice cold PBS. Spleens were homogenized with a 70 μm cell strainer and subsequently incubated in erythrocyte lysis buffer to get rid of red blood cells before analysis.

2.4 | Pneumococcal infection

S. pneumoniae (ATCC strain 6303, serotype 3) was stored at −80°C in inoculated Roti-Store Cryo tubes. The day before an infection, an inoculated ring was transferred into 5 ml sterile BHI medium and incubated at 37°C overnight without shaking. An OD_{600} of 0.5–0.7 defined a bacterial density of approximately 30×10^7 colony forming unit (CFU)/ml in the original culture. Mice were slightly anesthetized and infected with either 1–2 (intermediate dose) or 2.5–3 (high dose) $\times 10^6$ CFU in 25 μl sterile PBS by intranasal application. Upon infection, mice were continuously monitored for 168 h. To estimate the degree of disease burden and to guarantee a consistent, well-defined endpoint, weight, temperature, behavior, posture, and appearance of mice were assessed at least every 6 h during the first 3 days. If necessary, additional inspections during acute phase of infection took place every 3 h. Mice surviving the first 3 days of infection were subsequently controlled at least two times per day (more frequently if necessary), according to their health status and exactly as described above. Mice losing 15% of their starting weight or displaying a body temperature of less than 34.5°C were sacrificed immediately. One hundred and sixty-eight hours after infection, all mice were sacrificed. For corresponding disease score data sheet please refer to supplementary Figure 1.

2.5 | Enzyme-linked immunosorbent assay

For assessment of specific immunoglobulin titers in the sera of mice, high-binding plates were coated with 1 μg PCV13 in coating buffer overnight at 37°C. Before sample incubation, sera were diluted in sample buffer containing 10 μg/ml cell wall polysaccharides to capture unspecific antibodies. The samples were incubated on pre-coated plates for 3 h at 37°C. Afterwards, biotinylated antimouse IgM, IgG_1, IgG_{2a}, or IgG_3 antibody (BD Bioscience) were applied, followed by addition of streptavidin-HRP conjugate (Biotechne). HRP-reaction was induced with 3,3′,5,5′-tetramethylbenzidine substrate (Thermo Scientific) and stopped by addition of sulfuric acid. Chemoluminescent read out was done at 450 out of 570 nm.

2.6 | Cell culture

To analyze immune globulin class-switch of B cells, $CD19^+$ cells were isolated from the spleen by Magnetic Activated Cell Sorting using anti-CD19 micro beads and magnetic separation columns (Miltenyi Biotech). Before stimulation,

B cells were stained with the CellTrace CFSE Proliferation Kit (Invitrogen) at 37°C for 20 min and the reaction was stopped by addition of complete cell culture medium (RPMI medium supplemented with 10% FCS, 1% *L*-glutamine, 1% penicillin/streptomycine (P/S) and 0.05 mM β-mercaptoethanol). A total of 2×10^6 B cells were cultured in 24-well plate inserts in 500 µl medium either unstimulated, with 25 µg/ml lipopolysaccharide (LPS) only or with 25 µg/ml LPS plus 10 ng/ml interleukin-4 (IL-4) at 37°C and 5% CO_2. After 48 h, cells and supernatants were analyzed via flow cytometry and via LEGENDplex, respectively.

2.7 | LEGENDplex (multi-analyte flow assay kit)

To determine the amounts of antibody secreted by activated B cells in culture, supernatants were analyzed using the LEGENDplex Mouse Immunoglobulin Isotyping Panel (BioLegend). The assay was performed in a V-bottom plate according to manufacturer's protocol and data acquisition was done with the FACS Canto II Flow Cytometer (BD Bioscience). BioLegend's LEGENDplex Data Analysis Software was applied for analysis (www.biolegend.com/legendplex).

2.8 | Flow cytometry

For flow cytometry, 1×10^6 single cells from PL, BM, and spleen were incubated with anti-mouse CD16/32 (BioLegend) for 15 min to block unspecific Fc-binding sites. Subsequently, cells were fluorescently stained in two mixes to identify B-1 cells, CD138$^+$ cells, splenic B-2 cells, and BM subpopulations: B220-FITC, anti-CD5-PerCP-Cy5.5, anti-IgD-V450, anti-IgM-APC-Cy7, anti-CD43-PE, anti-CD138-APC and anti-CD23-BV510, anti-B220-PerCP, anti-CD21-FITC, anti-CD23-BV510, and GLY7-PE (BioLegend and BD Bioscience). For identification of PBs and plasma cells (PCs), fluorescent staining was performed using anti-B220-FITC, anti-CD19-V450, anti-TACI-APC, anti-CD138-PE, and 7-AAD for exclusion of dead cells.

To analyze surface immune globulins after in vitro stimulation, splenic B cells were incubated with anti-CD19-V450, anti-CD43-PE-Cy7, anti-CD5-APC, anti-IgM-APC-Cy7, anti-IgD-PerCP and anti-IgG$_1$-PE (LPS/IL-4) or with anti-CD19-APC-Cy7, anti-CD43-PE, anti-CD5-APC, anti-IgA-BV421, anti-IgG$_{2ab}$-BB700, and anti-IgG$_3$-PE-Cy7 (LPS only) (Biolegend and BD Bioscience). Proliferation was analyzed via carboxyfluorescein succinimidyl ester-staining.

All measurements were performed at the BD FACS Canto II and biaxial gating was done with FlowJo Version 10. For flow cytometry gating strategies the reader is referred to Figure S2.

2.9 | Statistics

All illustrations were designed with Graph Pad Prism Version 7. Statistical testing was done as indicated in corresponding figure legends.

3 | RESULTS

3.1 | Normal populations of adaptive B cells, but decreased rates of antibody-secreting cells in SLy2-Tg mice

Initially, we analyzed B-1 cell populations of the peritoneum, spleen and the BM in SLy2-Tg mice as compared to Wt littermates. As previously described by our group, the overexpression of SLy2 causes decreased proportions of innate B-1 cells (Figure S3).[12] The overall counts of splenocytes were comparable between the genotypes and adaptive B-cell populations isolated from the spleen remained unaltered, including transitional T1 B cells, follicular (FO) B cells, marginal zone (MZ) B cells and germinal center (GC) B cells (Figure 1A). By contrast, the total number of BM cells was significantly reduced in SLy2-Tg mice (Figure 1A).

We recently reported decreased titers of serum IgM in SLy2-Tg mice, indicating that excessive expression of the adapter protein impairs the antibody production in vivo.[12] Since murine antibody-secreting cells (ASCs) are known to express CD138 on their cell surface, we initially analyzed the proportion and number of CD138$^+$ B cells in the spleen and BM of mice.[7,24] As shown in Figure 1A, the frequency of CD138$^+$ cells was decreased in both organs of SLy2-Tg mice.

Important to mention, CD138$^+$ B cells constitute a heterogeneous population. For exact identification of PBs and PCs, additional markers, such as transmembrane activator and CAML interactor (TACI) are required.[25] For this reason, we went into more detail by examining CD138 and TACI double-positive cells, a subset that can be further subdivided into B220^{intermediate} PBs and B220^{low} PCs.[26] As depicted in Figure 1B, PBs and PCs tended to be reduced in the spleen of transgenic mice; however these differences did not reach statistical significance. By contrast, the absolute number and frequency of CD138$^+$TACI$^+$ Cells were strongly diminished in the BM of SLy2-Tg mice when compared to the Wt controls. The same applied to the specific fraction of CD138$^+$TACI$^+$B220^{int} PBs (Figure 1B). For flow cytometry gating strategies please refer to Figure S2.[26]

3.2 | In vitro proliferation, class-switch and antibody-secretion of isolated B cells

Since we found decreased ratios of ASCs in the spleen of SLy2-Tg mice, we performed in vitro proliferation and class-switch assays with isolated splenic B cells. Stimulation of $CD19^+$ cells with LPS alone or LPS + IL-4 significantly induced B-cell proliferation (Figure 2A). In addition, LPS-sensing triggered the upregulation of IgA and IgG_{2ab} on the surface of B cells on a highly comparable level in both groups (Figure 2A). Simultaneous supplementation of both stimulants led to a drastic reduction in IgD-surface expression. Both, Wt and SLy2-Tg B cells

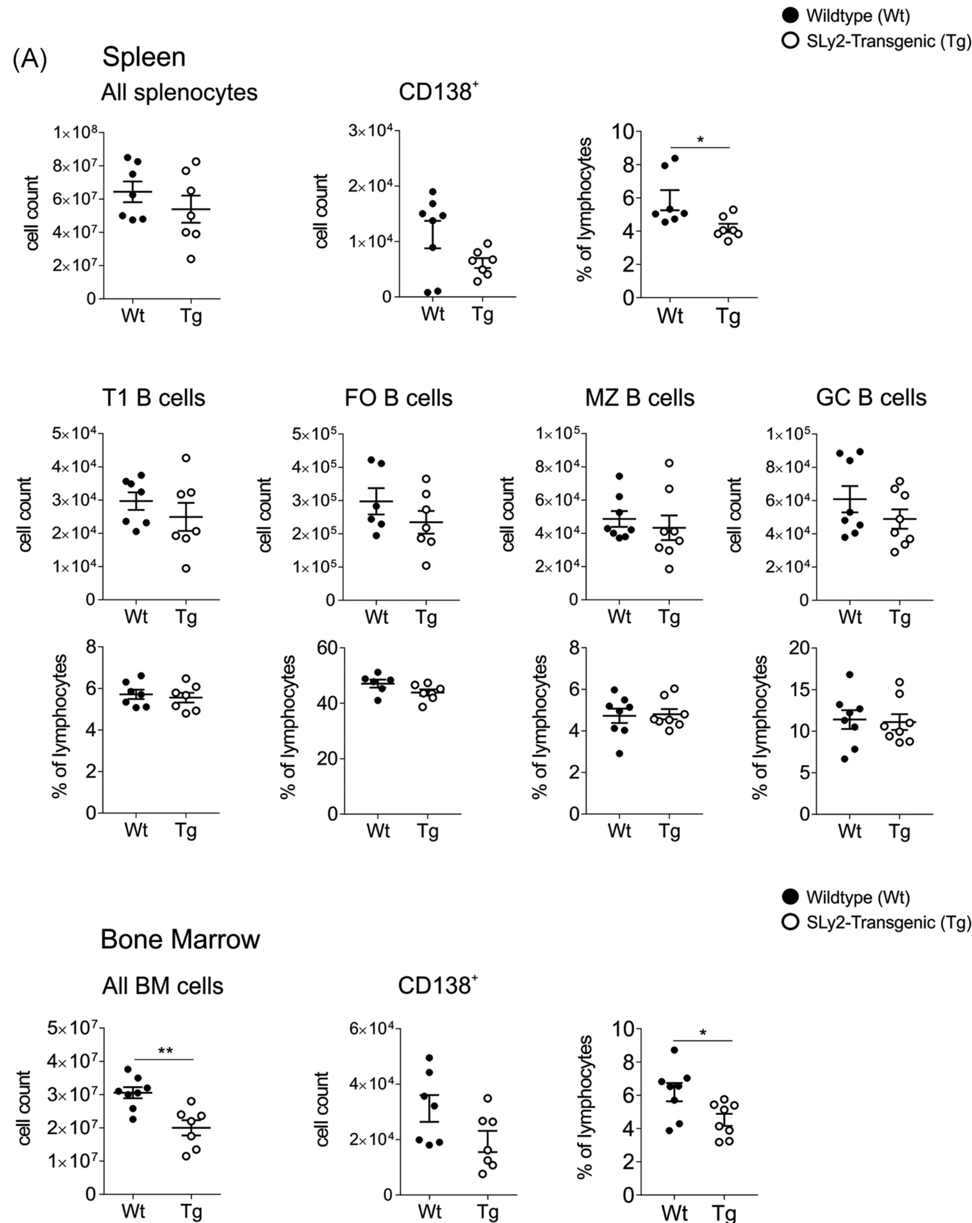

FIGURE 1 Frequency and count of B-2 cell subsets and $CD138^+$cells in the spleen and bone marrow (BM) of SLy2-Tg and Wt control mice. (A) Graphs depict the total number of splenocytes and the frequency and number of $CD138^+$ cells as well as following splenic B-2 cell subsets: $B220^+CD23^-IgM^{high}IgD^{low}CD21^{low}$ transitional (T1) B cells, $B220^+CD23^+IgM^{+/-}$ $CD21^{low}$ follicular (FO) B cells, $B220^+CD21^+IgM^+CD23^-$ marginal zone (MZ) B cells, and $B220^+GLY7^+$ germinal center (GC) B cells. In addition, the total count of BM cells is shown together with BM-resident CD138+ B-cell populations. (B) Graphs depict the TACI and CD138 double-positive cell populations of spleen and BM. These were further subdivided into $B220^{intermediate}$ plasmablasts and $B220^{low}$ plasma cells. Data were assessed via flow cytometry by analysis of $n = 6$–8 mice per genotype in two to three independent experiments. Error bars indicate the mean ± *SEM* and significance was determined by Student's *t* test with a *p* value of less than 0.05 considered statistically significant (*$p < 0.05$, **$p < 0.01$)

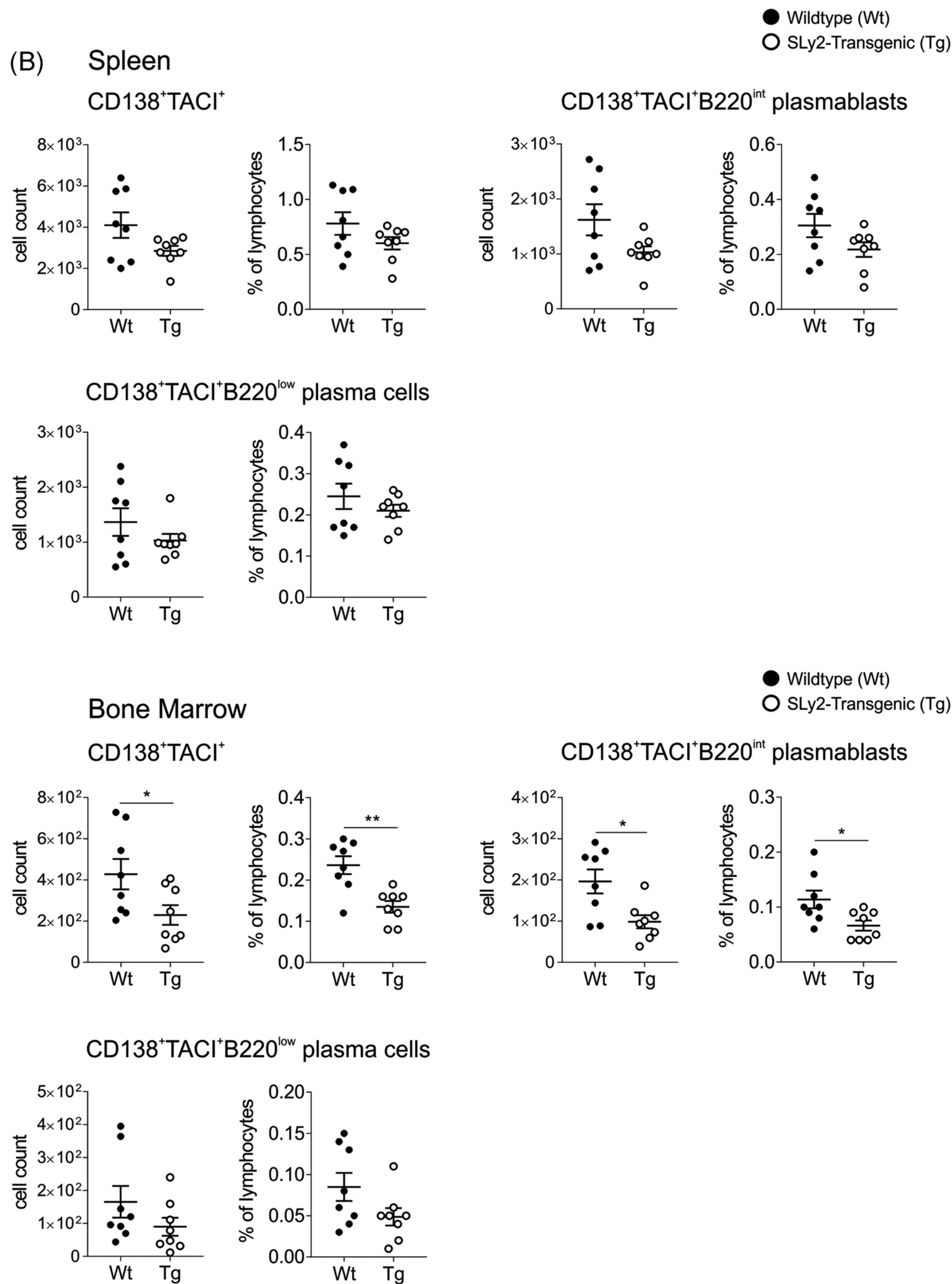

FIGURE 1 Continued

efficiently switched to IgG_1 in response to LPS + IL-4 (Figure 2A).

Consequently, we quantified the secreted immune globulins in the supernatants of the in vitro assays (Figure 2B). Both stimulation approaches induced high levels of secreted IgM and moderate levels of IgA, IgG_{2ab} and IgG_3. Upon supplementation with IL-4, the B cells released significant amounts of IgG_1 into the supernatants (Figure 2B). There were no differences between the genotypes.

3.3 | Impaired responses towards Prevenar13 in SLy2-Tg mice

Our previous studies revealed a regulatory role of SLy2 for TI antibody responses towards the polysaccharide vaccine P23, as they were significantly impaired in its absence. This occurred in an IL-5 reeceptor α (IL-5Rα)-dependent manner, since we could demonstrate decreased levels of IL-5Rα surface expression on B-1 cells.[12]

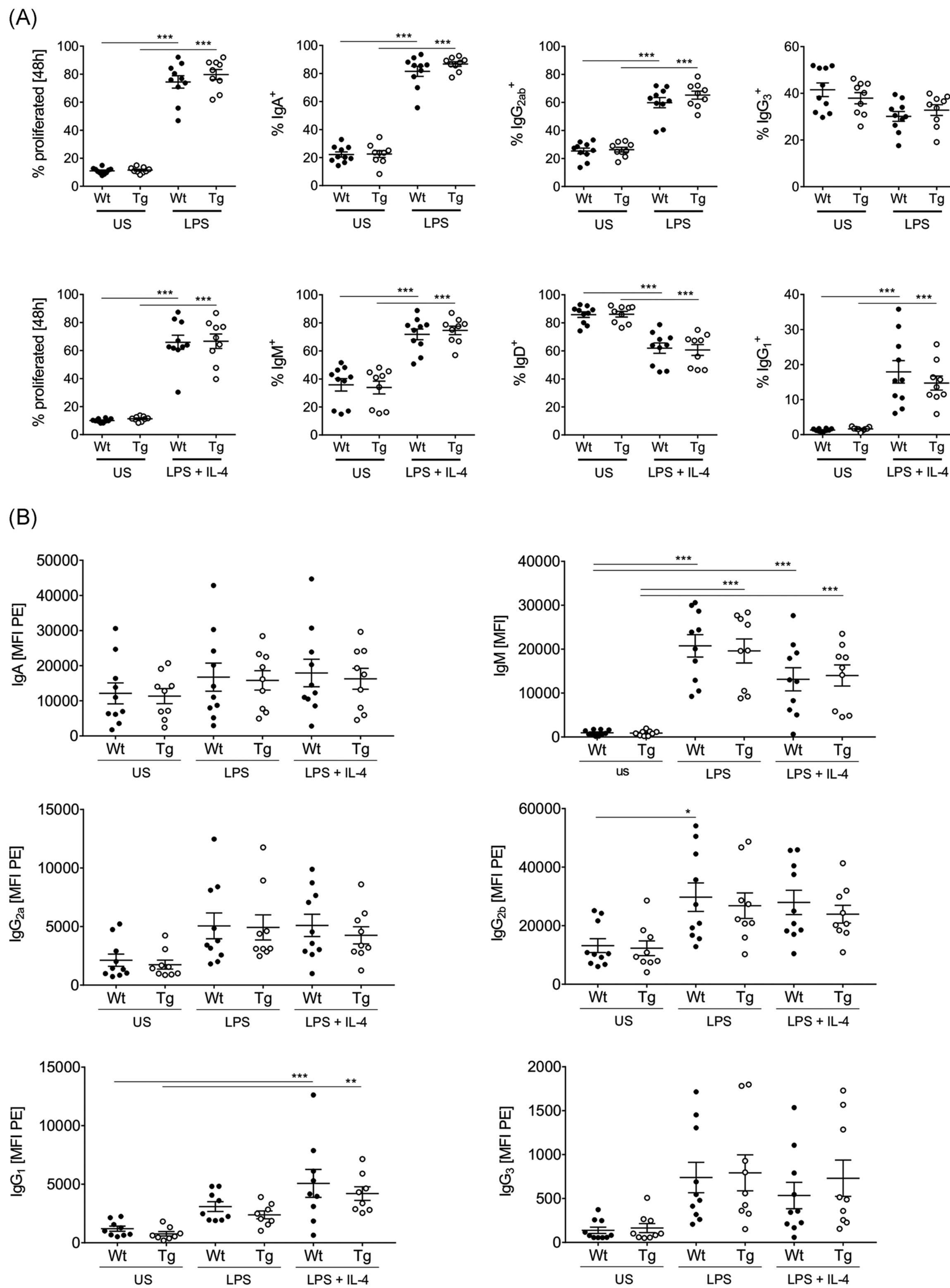

FIGURE 2 Surface immune globulin (Ig) class-switch and antibody secretion of B cells upon in vitro stimulation. (A) The proliferation rate of isolated splenic B cells after 48 h of stimulation with lipopolysaccharide (LPS) only or LPS + interleukin-4 (IL-4) is shown. In addition, percentages of IgA^+, $IgG_{2ab}{}^+$, $IgG_3{}^+$ (LPS, upper panel) and IgM^+, IgD^+, $IgG_1{}^+$ (LPS + IL-4, lower panel) B cells are depicted. (B) After 48 h of stimulation, immune globulins in the cell culture supernatants were quantified by LEGENDplex analysis. Graphs depict the mean fluorescent index (MFI) of PE-fluorescence, which corresponds to the concentration of either IgM, IgA, IgG_{2a}, IgG_{2b}, IgG_1, or IgG_3 in the supernatants. In every assay, an unstimulated (US) control was included. Data was assessed within three independent experiments using $n = 5$–9 mice per genotype. Significances were determined by one-way analysis of variance (ANOVA) with a p value of less than 0.05 considered as statistically significant ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$). Error bars represent the mean ± *SEM*. IgG, immunoglobulin G; IgM, immunoglobulin M; Wt, wildtype

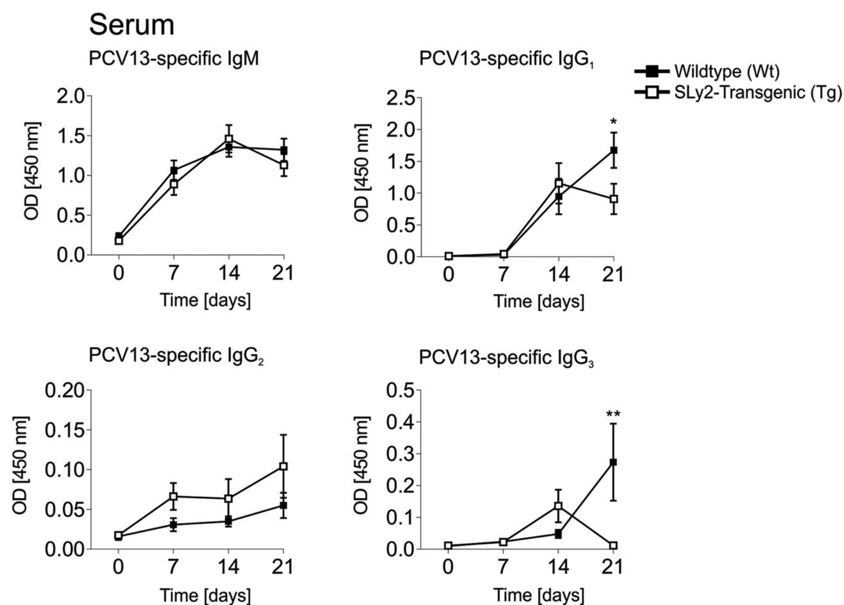

FIGURE 3 Prevenar13 (PCV13)-specific immune globulin levels in the serum of mice. IgM, IgG_1, IgG_2, and IgG_3 titers were determined by ELISA at Day 0 before immunization and 7, 14 and 21 days post-immunization with PCV13. Graphs depict the optical density (OD) at 450 nm measurement wavelength over time (570 nm reference wavelength). All sera were incubated on one plate to allow for direct comparison of the time points. Curves represent n = 8-11 mice per genotypes from two independently performed experiments. Error bars display the mean ± *SEM*. Significance was determined with two-way ANOVA with a *p* value of less than 0.05 was considered statistically significant ($*p < 0.05$, $**p < 0.01$). ELISA, enzyme-linked immunosorbent assay

We were therefore additionally interested in the TD antibody response of SLy2-Tg mice towards the conjugate-vaccine PCV13, which is recommended for high risk groups. As shown in Figure 3, PCV13-specific IgM and IgG_2 responses were comparable between the genotypes. However, 21 days post-vaccination, Wt mice produced more amounts of IgG_1 and IgG_3 (Figure 3).

Accompanying, we investigated the progression of BM-resident and splenic $CD138^+$ cells as well as adaptive B-2 cells in the spleen from Day 0 pre- to Day 21 post-immunization. While splenic MZ B and B-2 cell compartments were comparable, SLy2-Tg mice significantly lacked $CD138^+$ cells within the spleen and the BM at Day 14 post-immunization (Figure 4).

3.4 | Survival of SLy2-Tg and Wt control mice in the course of acute pneumococcal infection

As SLy2-Tg mice do not properly respond towards both, TI and TD pneumococcal vaccination, we next wanted to assess their survival rate during acute lung infection with *S. pneumoniae*. Thus, mice were infected without previous immunization (Figure 5A), as well as 14 days after injection of P23 (Figure 5B). For infection, we used two different doses of CFU: intermediate ($1–2 \times 10^6$ CFU/mouse) and high ($2.5–3 \times 10^6$ CFU/mouse).

Figure 5A depicts the survival curves of naïve mice after infection with the two varying doses of *S. pneumoniae*.

After exposure to an intermediate infection dose, 67% of all Wt mice and 40% of all SLy2-Tg mice survived (Figure 5A, left panel). The challenge with a high dose of bacteria resulted in lower rates of survival, being 0% and 20% for Wt and SLy2-Tg animals, respectively (Figure 5A, right panel). P23 vaccination before challenge conferred protection to Wt mice, as 83% of them survived the intermediate dose (Figure 5B, left panel) and 33% the high dose (Figure 5B, right panel). Surprisingly, SLy2-Tg animals infected with an intermediate dose of *S. pneumoniae* did not benefit from preceding immunization, as only 29% of them survived (Figure 5B, left panel). By contrast, upon administration of a high infection dose, SLy2-Tg mice were protected as well, with their survival rate being 55% (Figure 5B, right panel). There were no statistically significant differences in survival between the genotypes in all conditions examined.

3.5 | Decreased counts and proportions of B-cell precursors in the bone marrow of SLy2-Tg mice

Classically, B-2 cells develop within the BM from hematopoietic precursors, thereby undergoing a range of selection and differentiation steps. Meanwhile, they

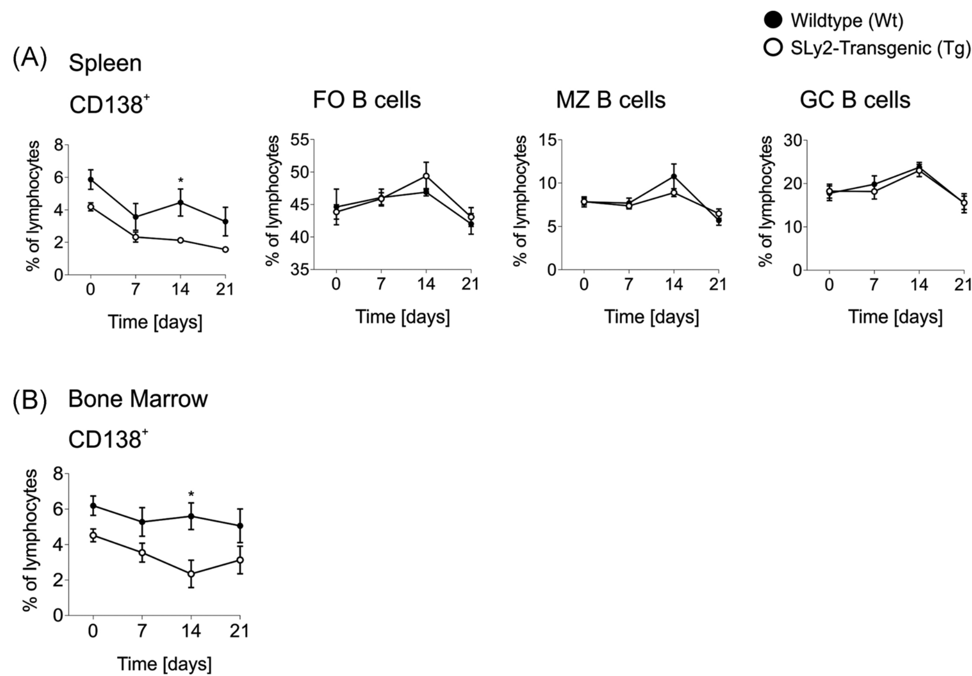

FIGURE 4 The over-time progression of B-cell populations upon vaccination with Prevenar13 (PCV13). Curves depict the over-time progression of B-cell frequencies within the pool of all lymphocytes as determined by flow cytometry. Populations shown are CD138$^+$ cells, B220$^+$CD23$^+$IgM$^{+/-}$ CD21^{low} FO, B220$^+$CD21$^+$IgM$^+$CD23$^-$ MZ B and B220$^+$GLY7$^+$ GC B cells in (A) the spleen and (B) the BM. Graphs represent $n = 7$–12 mice per genotype pooled from two or three independent experiments and error bars show the mean ± *SEM*. Significance was determined with two-way ANOVA with a *p* value of les than 0.05 was considered statistically significant ($^*p < 0.05$, $^{***}p < 0.001$). ANOVA, analysis of variance; BM, bone marrow; FO, follicular; GC, germinal center; IgM, immunoglobulin M; MZ, marginal zone

individually rearrange their immune globulin gene loci and finally express a unique B-cell receptor on their surface to leave the BM as mature naïve B cells.[27,28] While B-1a cells mainly derive from the fetal liver early in ontogeny, B-1b cells and adaptive B-2 cells can be reconstituted from BM precursors.[29–32]

Since the total number of BM cells was drastically reduced in SLy2-Tg mice, we next analyzed different precursor populations according to their surface marker expression. As shown in Figure 6, almost all stages of B-cell progenitors were impaired upon overexpression of SLy2. More precisely, the total cell counts of pro-B cells, pre-B cells as well as immature and mature B cells were significantly reduced (Figure 6). In addition, pre-B cells were also decreased in their ratio within the overall lymphocyte population.

4 | DISCUSSION

Our previous report on SLy2-Tg mice demonstrated decreased populations of innate B-1 cells and attenuated titers of natural antibodies caused by lymphocyte-specific overexpression of the adapter protein SLy2. This was accompanied by a significantly impaired TI response to the pure polysaccharide vaccine P23.[12] In the present work, we aimed to investigate TD responses towards the conjugate-vaccine PCV13 in the context of excessive SLy2-expression, with an emphasis on adaptive B-cell subsets.

TD protein antigens usually trigger a rapid extra-follicular response, shortly followed by the formation of a GC reaction within the B-cell follicles.[33,34] The GC is a highly dynamic process during which FO B cells undergo somatic hypermutation and are positively selected for increasing antigen affinity. This occurs in dependence of specialized cognate T follicular helper (T_{FH}) cells. Eventually, highly specific effector B cells arise, including class-switched plasma cells and long-lived memory B cells.[35,36] Classically, the immune globulin isotypes evoked during GC reaction are IgG_1 and IgG_3.[37,38]

Conveniently, injection of PCV13 triggered huge amounts of specific serum IgM, followed by IgG_1 and IgG_3 after 2 weeks (Figure 3). Surprisingly, the IgM serum concentrations were fully comparable within the genotypes, indicating that SLy2 controls IgM induction under TI, but not TD conditions (Figure 3). This is in accordance with recent findings regarding SLy2-deficient

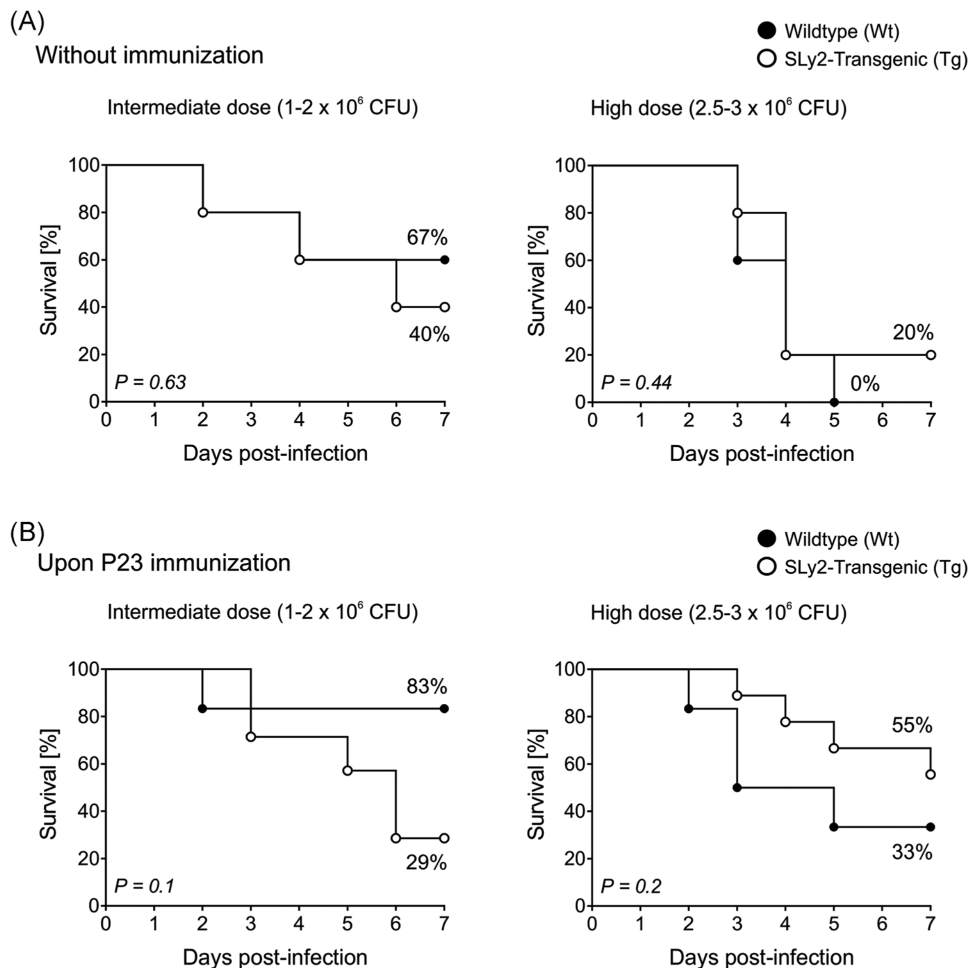

FIGURE 5 Survival analysis of SLy2-Tg and Wt mice upon intranasal infection with *Streptococcus pneumoniae*. Mice were intranasally infected with either 1–2 × 10^6 (intermediate) or 2.5–3 × 10^6 (high) CFU of *S. pneumoniae* serotype 3 in 25 µL PBS. This was done (A) without preceding immunization or (B) 14 days after intraperitoneal vaccination with 1 µg P23. Each single survival curve represents $n = 5$–9 wildtype or SLy2-transgenic mice as defined in the legends. Data is shown as Kaplan–Meier survival graph and indicated p values were determined by Log rank (Mantel Cox) test. CFU, colony forming unit

mice.[13] By contrast, SLy2-Tg mice produced significantly lower amounts of IgG_1 and IgG_3 than their Wt counterparts. More precisely, while PCV13-directed IgG_1 and IgG_3 levels peaked in Wt mice at Day 21 post-immunization, they already dropped after 14 days in the sera of transgenic animals (Figure 3).

The observation that SLy2-Tg mice still generated equal levels of IgG_1 and IgG_3 at Day 14 post-vaccination points to normal induction of GC reaction and class-switch in these mice. This thought is strongly supported by the fact that in vitro class-switch and Ig secretion of B cells were fully comparable between the genotypes (Figure 2A,B). Moreover, this points to a regulatory role of the adapter protein SLy2 specifically in the context of antibody responses to *S. pneumoniae*-derived polysaccharides.

The populations of splenic FO and GC B cells were unaltered in SLy2-Tg mice; both pre- and post-immunization (Figures 1 and 4). These results indicate that the overexpression of SLy2 might specifically impair extrafollicular TD responses.[39] On the other hand, the reduced serum IgG_1 and IgG_3 titers were preceded by markedly decreased ratios of splenic and BM-resident $CD138^+$ cells at Day 14 in the context of SLy2-overexpression (Figure 4). In addition, if compared to Wt littermates, these mice significantly lacked steady state $CD138^+TACI^+B220^{int}$ PBs (Figure 1B). Notably, further experiments are required to clarify whether these PBs are mainly derived from the B-1 or the B-2 cell compartment.

Collectively, these findings point to a possible implication of the adapter protein SLy2 in ASC differentiation and/or survival. It is well-established, that the initiation of the genetic ASC program depends on Blimp-1 mediated gene repression.[40,41] Hence, one possibility would be a SLy2-mediated repression of Blimp-1 and as a consequence, inhibition of ASC development. On the

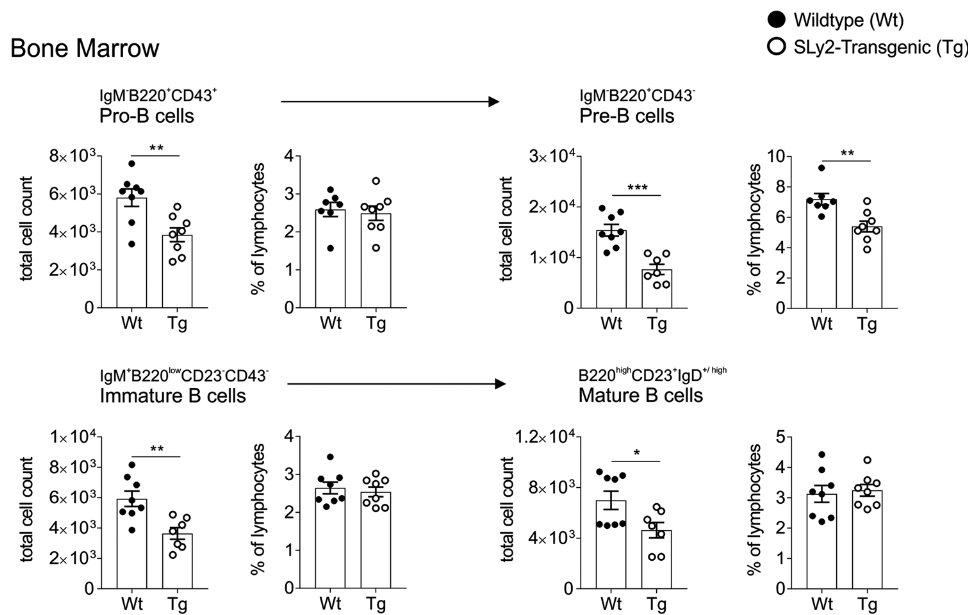

FIGURE 6 B-cell developmental stages within the BM of SLy2-Tg and Wt littermate mice. B-cell precursors of the BM were investigated by flow cytometry. The percentage within the overall lymphocyte population and the total count of cells are shown for $IgM^-B220^+CD43^+$ Pro-B cells, $IgM^-B220^+CD43^-$ Pre-B cells, $IgM^+B220^{low}CD23^-$ immature B cells and $B220^{high}CD23^+IgD^+$ mature B cells. Data represent $n = 7–8$ mice per genotype from two independent stainings. Error bars indicate the mean ± *SEM* and significance was determined by Student's *t* test with a *p* value of less than 0.05 considered statistically significant (**p* < 0.05, ***p* < 0.01, ****p* < 0.001). BM, bone marrow; IgM, immunoglobulin M; Wt, wildtype

other hand, XBP-1 is a master regulator of ASC activity, by driving the expression of Ig transcripts in plasma cells.[42] Thus, SLy2 could as well be implicated in XBP-1 mediated regulation of antibody production. Based on our findings, the role of SLy2 in ASC-specific signaling pathways should be subject to subsequent work.

Of interest, we recently reported improved PCV13-specifc IgG_2 responses in mice deficient for SLy2 and thus conclude, that the overexpression of the adapter protein does not necessarily reflect the exact opposite of its homozygous deletion.[13] Nevertheless, these data collectively demonstrate that SLy2 controls both, the generation of specific IgM and IgG towards TI and TD pneumococcal vaccine, respectively.

Surprisingly, despite the impaired antibody response of SLy2-Tg mice towards both pure pPS and conjugate-vaccine, they had no statistically significant survival disadvantage upon intranasal infection with *S. pneumoniae*. While SLy2-Tg mice tended to be more prone towards infection with intermediate units of bacteria (Figure 5B, left panel), they even displayed a better survival rate in the context of a high infection dose (Figure 5B, right panel). Immune responses to *S. pneumoniae* always involve both, humoral and cellular components: pPS- and protein-specific antibodies act as neutralizing and opsonizing agents, while $CD4^+$ T_H cells rapidly recruit phagocytes towards the airways by producing inflammatory cytokines.[43] While antibodies have been shown to be of upmost importance regarding septicemia, the response to pneumococcal colonization in the nasopharynx seems to solely depend on IL-17-secreting $CD4^+$ T_H cells. More precisely, the mucosal clearance of pneumococcal antigens upon intranasal application was fully functional in a study on μMT mice, lacking B cells and antibodies.[44] By contrast, immunity to septicemia absolutely required $CD138^+$ ASCs.[43,45] Based on this knowledge we hypothesize, that cellular components of the immune system compensated for the lack of antibodies in SLy2-Tg mice in our murine model of pneumonia, especially after facing a high infection dose (Figure 5). Previous experiments with SLy2-deficient mice further support this hypothesis, as these did not benefit from their increased serum antibody titers during pneumonia.[13] In addition, when assessing the bacterial burden in the blood, we found that our infection model was exclusively restricted to the lung (data not shown). We therefore plan to investigate the role of SLy2 during pneumococcal sepsis by intravenous infection of our mice.

The family of SLy/SASH-adapter proteins comprises three highly homologous members, namely SLy1, SLy2, and SLy3/SASH1.[46,47] Apart from SLy2, lymphocytes express high levels of SLy1, which has been shown to be indispensible for normal development of thymocytes.[47,48]

In the present work, we found greatly reduced numbers of B-cell precursors in the BM of SLy2-Tg mice as compared to Wt littermates (Figure 6). Importantly, this deficit applied to all stages examined, including pro-B, pre-B and immature B cells. This indicates that SLy2 not only interferes with one specific transition stage, but affects the overall progenitor population (Figure 6). B-cell development is a multistep process. Initially, hematopoietic stem cells give rise to common lymphoid progenitors, which are committed to the lymphocyte lineage.[49] Subsequently, the B-cell developmental program is driven by tight regulation of specific transcription factors. For example, the differentiation of pro-B and pre-B cells is controlled by the master regulator Pax-5.[50] Pax-5 is expressed throughout B lymphopoiesis and its inactivation leads to a reversion towards an uncommitted precursor state.[51] Negative regulation of Pax-5-expression by SLy2 could be one possible explanation for the severely reduced B-cell precursors in SLy2-Tg mice, hence this should be subject to future studies.

Interestingly, previous studies on mice expressing a mutated version of the highly homologous protein SLy1 revealed an impaired ability of the hematopoietic system to reconstitute the lymphocyte compartment.[47] Further, novel data assessed by our group demonstrate a role of the SLy1-protein downstream of the IL-7R and preTCR in developing thymocytes (unpublished data). While the percentages of pro-B cells and immature B cells were unaltered in SLy2-Tg mice, the proportion of pre-B cells within the overall lymphocyte population was diminished (Figure 6). This observation could point to a special role of SLy2 in pro-B to pre-B transition, which among others depends on the IL-7 signaling pathway.[52] Based on the high sequence similarity and conservation of the SLy/SASH-proteins, SLy2 should be considered as a potential player during IL-7/IL-7R-signaling in developing B cells. Collectively, these findings allow speculating that SLy2 might take over similar roles in lymphocyte development as does its homolog SLy1.

Of note, since the SLy2-transgene in our mouse model is expressed in both B and T cells, an additive inhibition of early T-cell progenitors should be debate of discussion; especially since our phenotype investigation did not include the CD19-marker (Figure 6). However, in B cells, the SLy2-transgene is under the control of the Eμ IgH enhancer and therefore already expressed in early pro-B cells, rearranging their heavy chain.[53] By contrast, T-cell specific SLy2-overexpression depends on the *lck*-promoter, which is initially activated in thymocytes.[54] Thus, our observations are likely to reflect a specific effect of excessively expressed SLy2 on developing B cells.

By interfering with B lymphopoiesis, SLy2 might negatively control the reconstitution of certain B-cell compartments in SLy2-Tg mice. This is interesting since SLy2 is significantly overexpressed in DS patients, which mount inadequate antibody responses to vaccination and suffer from B lymphocytopenia.[22,55] More precisely, fetuses with DS show a significant decrease in $CD19^+$ lymphocytes. Furthermore, while B cells usually undergo enormous expansion during the first year of life, this process was shown to be defective in children with DS when compared to healthy controls.[23,56] Hence, follow-up studies should focus on whether SLy2 is implicated in transcriptional regulation and key pathways during B-cell development. In this regard, Pax-5 and the IL-7-signaling pathway might be promising targets.

5 | CONCLUSION

In summary, the present study reveals impaired antibody responses to pneumococcal conjugate-vaccine in SLy2-Tg mice, accompanied by decreased populations of BM-resident plasmablasts. The overall survival rate upon acute pneumococcal lung infection was comparable between the genotypes, indicating that the impaired antibody-mediated protection in SLy2-Tg mice could be compensated by cellular effectors. Besides, our findings demonstrate a novel role of the immunoinhibitory adapter protein SLy2 for B-cell developmental processes, as reflected by severely reduced numbers of precursor cells within the BM.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Jennifer Jaufmann designed and carried out the experiments, analyzed the data and wrote the manuscript. Leyla Tümen performed experiments and analyzed the data. Sandra Beer-Hammer designed experiments, provided experimental and conceptual advice and wrote the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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