DISSERTATION

Unveiling a crucial role for ILC2s in B1 cell regulation via IL-5

Aufdeckung einer entscheidenden Rolle von ILC2s in der Regulierung von B1 Zellen durch IL-5

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Abkürzungsverzeichnis

AID ..... Activation-induced cytidine deaminase

AREG ..... Amphiregulin

BCR ..... B cell receptor

BSA ..... Bovine serum albumin

Btk ..... Bruton's tyrosine kinase

CD40L ..... CD40 ligand

cDNA ..... Complementary DNA

CLP ..... Common lymphoid progenitor

CSR ..... Class-switch recombination

DAMP ..... Danger-associated molecular pattern

FT ..... *Francisella tularensis*

GM-CSF ..... Granulocyte-macrophage colony-stimulating factor

H. polygyrus ..... *Heligmosomoides polygyrus*

HBSS ..... Hanks' Balanced Salt Solution

IFN-γ ..... Interferon-γ

IgG3 ..... Immunoglobulin G3

Ighv ..... *Immunoglobulin heavy variable*

Igkv ..... *Immunoglobulin kappa variable*

IgM ..... Immunoglobulin M

iILC2 ..... Inflammatory ILC2

IL17RB ..... Interleukin 17 receptor B

Il5 ..... *Interleukin 5*

ILC ..... Innate lymphoid cell

ILC1 ..... Group 1 innate lymphoid cell

ILC2 ..... Group 2 innate lymphoid cell

ILC2 KO ..... *Nmur1*$^{iCre\text{-}T2A\text{-}GFP}$ *Id2*$^{flox/flox}$ mice

ILC3 ..... Group 3 innate lymphoid cell

ILF ..... Isolated lymphoid follicle

JAK2 ..... Janus kinase 2

LTi ..... Lymphoid tissue inducer

MCMV ..... Murine cytomegalovirus

MHC ..... Major histocompatibility complex

N. brasiliensis ..... *Nippostrongylus brasiliensis*

nILC2 ..... Naturally occuring ILC2

NK ..... Natural killer

PAMP ..... Pathogen-associated molecular pattern

PBS ..... Phosphate – buffered saline

PD-L1–PD-1 ..... Programmed Cell Death Ligand 1- Programmed Cell Death Protein 1

PRR ..... Pattern recognition receptor

PtC ..... Phosphatidylcholine

qPCR ..... Quantitative real-time PCR

Rα ..... Receptor alpha subunit

S. pneumoniae ..... *Streptococcus pneumoniae*

sc ..... Single cell

SHM ..... Somatic hypermutation

SNN ..... Shared nearest neighbor

ST2 ..... Receptor for IL-33

STAT3 ..... Signal transducer and activator of transcription 3

STAT5 ..... Signal transducer and activator of transcription 5

tcrβ ..... T cell receptor beta

TD..... Thymus-dependent

TdT ..... Terminal deoxynucleotide transferase

TF ..... Transcritpion factor

Th1 .....T helper 1

Th17 .....T helper 17

Th2 .....T helper 2

TI ..... Thymus-independent

TLR..... Toll-like receptor

TNF ..... Tumor necrosis factor

TRAIL ..... TNF-related apoptosis-inducing ligand

UMAP .....Uniform manifold approximation and projection

Abstract

The immune system orchestrates a complex network of cellular interactions to maintain homeostasis and protect the host against various pathogens. Within this network, innate lymphoid cells (ILCs) represent a family of lymphocytes with pivotal roles in orchestrating both innate and adaptive immune responses. Strategically positioned at barrier tissues, they sense and react with different immune responses to disturbances in tissue homeostasis. Among ILCs, group 2 innate lymphoid cells (ILC2s) are important regulators of immune responses via type 2 cytokines, such as IL-5, amphiregulin and IL-13. However, shared effector functions of ILC2s with diverse immune cells raise the fundamental question of whether ILC2s are pivotal to the type 2 response or if their functions can be effectively taken over by alternative cellular players. In this thesis evidence is provided for a non-redundant function of ILC2s in supporting the development and function of B1 cells, an innate subset of B cells, responsible for generating natural antibodies important for the primary defence against pathogens and the preservation of tissue integrity. Using ILC2-deficient mice combined with flow cytometry and single-cell sequencing, a reduction and impaired proliferation of B1 cells and a notable decline in phosphatidylcholine-specific antibody-producing B1 cells in the absence of ILC2s was revealed. By employing a targeted deletion of *Il5* in ILC2s, the specific dependence of B1 cells on ILC2-derived IL-5 was successfully demonstrated. Ultimately, the IL-33 receptor on ILC2s was identified as an essential cell intrinsic regulator of IL-5 production.

Das Immunsystem koordiniert ein komplexes Netzwerk von zellulären Interaktionen, um die Homöostase aufrechtzuerhalten und den Wirt vor verschiedenen Krankheitserregern zu schützen. Innerhalb dieses Netzwerks stellen angeborene Lymphozyten (ILCs) eine Familie von Lymphozyten mit einer zentralen Rolle bei der Orchestrierung sowohl der innaten als auch der adaptiven Immunantwort dar. Strategisch in Geweben mit Barrierefunktion positioniert, nehmen sie Störungen der Gewebehomöostase auf und reagieren mit unterschiedlichen Immunantworten darauf. Unter den ILCs sind die angeborenen Lymphozyten der Gruppe 2 (ILC2s) wichtige Regulatoren der Immunantworten durch Typ-2-Zytokine wie IL-5, Amphiregulin und IL-13. Gemeinsame Effektorfunktionen von ILC2s mit verschiedenen Immunzellen werfen jedoch die grundlegende Frage auf, ob ILC2s für die Typ-2 Immunantwort entscheidend sind oder ob ihre Funktionen effektiv von alternativen zellulären Akteuren übernommen werden können. In dieser Arbeit wird eine nicht-redundante Funktion von ILC2s bei der Unterstützung der Entwicklung und Funktion von B1 Zellen, innate B Zellen, nachgewiesen, welche für die Generierung natürlicher Antikörper zur primären Abwehr von Krankheitserregern und Erhaltung der Gewebeintegrität verantwortlich sind. Unter Verwendung von ILC2-defizienten Mäusen in Kombination mit Durchflusszytometrie und Einzelzellsequenzierung ergab sich in Abwesenheit von ILC2s eine Verringerung und Beeinträchtigung der Proliferation von B1 Zellen und ein bemerkenswerter Rückgang der B1 Zellen, die spezifisch mit ihren Antikörpern Phosphatidylcholin binden. Durch die gezielte Deletion von *Il5* in ILC2s konnte die spezifische Abhängigkeit der B1 Zellen von IL-5 aus ILC2s nachgewiesen werden. Letztendlich wurde der IL-33 Rezeptor auf ILC2s als essentieller zellintrinsischer Regulator der IL-5 Produktion identifiziert.

1 Introduction

1.1 Potential role of ILC2s in regulation of innate antibody production

To generate protective antibodies against thymus-dependent (TD) antigens, follicular B cells, also called B2 cells, rely on T cell help via the expression of CD40 ligand (CD40L). The interaction between T and B cells constitutes a quality control mechanism of the immune system that ensures the specificity of the immune response by integrating signals from two independent cell types with different activation requirements (Calame 2001; Elgueta et al. 2009). Innate lymphoid cells (ILCs) serve as the innate counterparts to T cells. Within the three groups of ILCs, ILC2s, despite lacking an antigen receptor and CD40L (Robinette et al. 2015), have been observed to stimulate the division and thymus-independent (TI) antibody production of B1 cells - an innate subset of B cells - *in vitro* (Drake et al. 2016; Moro et al. 2010). But before evaluating further this innate pathway for antibody production, it is essential to introduce ILCs and B cells first.

1.2 Ontogeny of innate lymphoid cells

ILCs are tissues-resident immune cells that stimulate and interact with innate and adaptive immune cells and parenchymal cells, like epithelial, stromal and mesenchymal cells, contributing to the regulation of acute and chronic inflammation and preservation of tissue homeostasis (Klose and Artis 2016; Klose and Artis 2020). ILCs develop in the bone marrow from the common lymphoid progenitor (CLP), like B and T cells. The development from the CLP to the ILC progenitor necessitates the involvement of various critical transcription factors (TFs), including Tcf-1, Id2, Plzf, Nfil3, Tox, Gata-3, and IL-7 (Klose and Artis 2020; Schlenner et al. 2010). Then, due to different lineage-specific TFs

three ILC subsets develop from the ILC progenitor: ILC1, ILC2 and ILC3 (Klose and Artis 2020). They are the innate equivalents of T cells, as the three ILC subsets resemble the three major T helper cell subsets (Th1, Th2, Th17 cells) in effector functions, surface markers and transcription factor profiles. Yet, despite their lymphoid morphology, they do not possess rearranged antigen receptors, unlike T and B cells, and therefore they are not able to generate an adaptive immune response, but a rapid cytokine response to different stimuli to regulate following adaptive and innate immune reactions (Spits et al. 2013; Klose and Artis 2020).

1.3 Group 1 innate lymphoid cells

ILC1s are the innate counterparts of Th1 cells. They share similar effector cytokines, like IFN-y and TNF, for promoting type 1 immunity against intracellular microbes and some viruses (Annunziato et al. 2015). ILC1s and Th1 cells are both developmentally dependent on T-bet (Klose et al. 2014; Daussy et al. 2014; Robinette and Colonna 2016). ILC1s have been identified in various tissues, including liver, thymus, intestinal lamina propria and epithelium, adipose tissue, and uterus, revealing a tissue-specific heterogeneity that implies distinctions in their transcriptional programs (O'Sullivan et al. 2016; Doisne et al. 2015; Vosshenrich et al. 2006; Klose et al. 2014). Despite similarities in killer receptors with NK cells, such as NKp46, NKG2D, and NK1.1, ILC1 biology remain insufficiently explored (Almeida et al. 2018; Takeda et al. 2005). ILC1s rapidly exhibit immediate effector functions, upregulating pro-inflammatory cytokines like IFN-γ and TNF-α in response to viral infection. In the context of MCMV infection, liver ILC1s emerge as the earliest source of IFN-γ, crucial for limiting viral replication and enhancing host survival. They express higher levels of Granzyme A and C than NK cells, emphasizing their potent cytolytic activity, along with upregulation of TRAIL, enabling them to trigger

cell death in target cells (Krmpotic et al. 2003; Weizman et al. 2019; Robinette et al. 2015; Weizman et al. 2017; Sojka et al. 2014; Taggenbrock and van Gisbergen 2023). Additionally, they are involved in various inflammatory conditions, including chronic inflammation during colitis, multiple sclerosis, and sepsis (Klose et al. 2014; Daussy et al. 2014; Fuchs et al. 2013; Kwong et al. 2017; Quatrini et al. 2017). Long-lasting phenotypic changes, indicative of immunological memory, occur in ILC1s after infection with MCMV. These memory-like ILC1s, particularly in the liver, exhibit an enhanced ability to produce IFN-γ upon secondary infection, contributing to early antiviral immunity (Weizman et al. 2019; O'Leary et al. 2006; Taggenbrock and van Gisbergen 2023). Their protective effects extend to anti-tumor immunity, although they appear less effective in countering tumor growth in established tumors compared to NK cells. Notably, ILC1s contribute to early elimination of tumor cells, particularly in preventing metastasis, while NK cells sustain antitumor immune responses in established tumors (Nixon et al. 2022; Ducimetière et al. 2021; Taggenbrock and van Gisbergen 2023).

1.4 Group 2 innate lymphoid cells

1.4.1 Similarities and interaction with Th2 cells

Involved in the lineage-specific development of ILC2s are Gata-3, Bcl11b, RORα (Hoyler et al. 2012; Wong et al. 2012; Spooner et al. 2013; Walker et al. 2015), and Gfi1. ILC2s are the innate counterparts of Th2 cells, they share the TF Gata-3 (Zheng and Flavell 1997; Zhang et al. 1997) and both participate in regulating pathways of type 2 immunity, including defence against helminths and parasites, tissue repair and remodelling, but also contribute to atopic diseases like allergic asthma, dermatitis, and rhinitis (Spits et al. 2013; Klose et al. 2017; Klose and Artis 2020). Both, ILC2s and Th2 cells, produce type 2 key cytokines like IL-13, IL-5, and AREG (Lawrence et al. 1996; Else and Grencis 1991;

Abbas et al. 1996; Mosmann and Coffman 1989; Fallon et al. 2006; Voehringer et al. 2006). Investigating the regulatory role of these two cell types in type 2 immunity, mouse models deficient in ILC2s, T cells or both unveiled distinctive contributions. ILC2-deficient and T/Th2 cell-deficient mice exhibited a delayed expulsion of worms (Gurram et al. 2023; Jarick 2022; Zaiss et al. 2023). Remarkably, mice lacking T cells and ILC2s remained infected with *N. brasiliensis* even 200 days post infection, indicating a collaborative role of ILC2s and Th2 cells in efficient parasite expulsion (Gurram et al. 2023). This collaborative mechanism involves processes such as interactions via MHC class II-TCR, where ILC2s activate Th2 cells, leading to the secretion of IL-2 by Th2 cells. This IL-2 release enhances the activation of ILC2s, further amplifying the promotion of a type 2 immune response (Oliphant et al. 2014; Schwartz et al. 2017; Mirchandani et al. 2014; Zaiss et al. 2023). Additionally, an interaction mediated by PD-L1–PD-1 between ILC2s and Th2 cells was discovered, resulting in an increased production of type 2 cytokine IL-13 by Th2 cells. (Schwartz et al. 2017). In allergic disease models, distinct responses are reported depending on the nature of the challenge. In acute papain challenges, it was noted that only ILC2s were activated, whereas in chronic disease models, both, ILC2s and T cells, were activated, with ILC2s exhibiting notable expansion alongside T cell activation. (Gurram et al. 2023). These findings underscore the bidirectional interactions between ILC2s and Th2 cells, jointly orchestrating an effective immune response. While ILC2s respond rapidly to stimuli, $CD4^+$ T cells undergo several days of differentiation and proliferation upon antigen-specific activation to transform into Th2 cells. Although their immune response is initially delayed, Th2 cells ultimately establish a consistent, local cytokine production (Minutti et al. 2017; Zaiss et al. 2015; Zaiss et al. 2023). Moreover, T cells release IL-4 to facilitate the production of IgE which contributes to worm expulsion

by amplifying mast cell degranulation (Katona et al. 1991; Finkelman et al. 2004; Zaiss et al. 2023).

1.4.2 ILC2 effector cytokines

As mentioned above ILC2s secrete type 2 cytokines such as IL-13, IL-5, IL-9, and AREG. In the context of *N. brasiliensis* infection, IL-13 is particularly crucial for the weep-and-sweep reaction, characterized by fluid accumulation in the intestine and increased smooth muscle contraction to facilitate expulsion rather than elimination (McKenzie et al. 1999; Bancroft et al. 1998; Madden et al. 2002). Furthermore, IL-13 promotes differentiation of goblet and tuft cell progenitors, resulting in tuft and goblet cell hyperplasia, triggers the secretion of IL-25 by tuft cells and shedding of the epithelial layer, all contributing to the effective clearance of worms (Tsou et al. 2022; Zaiss et al. 2006; Cliffe et al. 2005). The type 2 cytokine AREG shares similar functions with IL-13 in tissue remodelling during worm infection (Zaiss et al. 2006; Tsou et al. 2022; Cliffe et al. 2005). IL-9 functions as an autocrine amplifier of ILC2s by enhancing type 2 cytokine production and supporting tissue restoration in the lung during worm infection (Turner et al. 2013; Wilhelm et al. 2011). IL-5 mediates recruitment, activation and survival of eosinophils, thereby promoting type 2 inflammation and sustain metabolic homeostasis (Molofsky et al. 2013; Jarick 2022). In the absence of IL-5, mice displayed a notable deficiency in eosinophilia during parasite and worm infection (Kopf et al. 1996). Additionally, IL-5-deficient mice showed prevention of lung damage and airway hyperreactivity following challenges with aeroallergen-induced lung damage (Foster et al. 1996). ILC2s were identified as the primary producers of IL-5 (Nussbaum et al. 2013), and they were shown to release IL-5 spontaneously (Molofsky et al. 2013). Mice lacking ILC2s exhibited similar effects on eosinophils as observed in IL-5-deficient mice. They experienced reduced eosinophil counts under normal conditions and failed to attract

eosinophils to the airways in allergic asthma models. Correspondingly, ILC2-deficient mice displayed diminished IL-5 levels in baseline conditions and lacked the ability to release IL-5 upon stimulation (Jarick 2022). This underscores the crucial role of ILC2s for maintaining IL-5-dependent eosinophil homeostasis.

1.4.3 Upstream regulation of ILC2s

Activation of ILC2s occurs at mucosal barriers, driven by diverse factors like alarmins, inflammatory mediators, neuropeptides, androgens, and metabolites. These soluble mediators predominantly derive from non-immune cells, including epithelial cells, stromal cells, and neurons (Klose and Artis 2020; Laffont et al. 2017). IL-25 is released by tuft cells (chemosensory cells of the intestine) when they sense succinate – a substance produced by worms and protozoans (Schneider et al. 2019; Moltke et al. 2016). Additionally, ILC2s promote proliferation of tuft cells during helminth infection which is significantly compromised in ILC2-deficient mice (Jarick 2022; Moltke et al. 2016; Miller et al. 2020; Varela et al. 2022). Another essential element for ILC2 activation is IL-33, as documented in various references (Neill et al. 2010; Moro et al. 2010; Hung et al. 2013). Several studies pinpoint stromal cells as a primary source of IL-33 in the intestine. IL-33 is indispensable for expelling different kind of helminths (Hung et al. 2013; Topczewska et al. 2023; Yasuda et al. 2012; Meiners et al. 2020; Zaiss et al. 2023). TSLP is vital for ILC2 activation in papain-induced allergic asthma models, but mice deficient in TSLP effectively handle worm infections, such as *N. brasiliensis* and *H. polygyrus*, suggesting compensatory factors for worm expulsion (Kabata et al. 2020; Massacand et al. 2009; Zaiss et al. 2023). IL-25 and IL-33 pathways collaborate, as evidenced by mice lacking receptors for both, which is displayed by a delayed expulsion of *N. brasiliensis* (Neill et al. 2010; Zaiss et al. 2023). The cooperative actions of IL-25 and IL-33 support distinct ILC2 subsets during worm infection. Under normal conditions, ILC2s in the lung, colon

and mesenteric lymph nodes express high levels of the IL-33 receptor (ST2), whereas expression of the IL-25 receptor (IL17RB) is low in these cells. Vice versa is the expression of ST2 and IL17RB by ILC2s in the small intestine (Flamar et al. 2020; Huang et al. 2015; Zaiss et al. 2023). During helminth infection, a specific ILC2 subset known as 'inflammatory ILC2s' (iILC2s), strongly activated by IL-25 rather than IL-33, migrates from the intestine to emerge in the lung and mesenteric lymph nodes (Huang et al. 2018; Moro et al. 2016; Zaiss et al. 2023). IILC2s present a distinct phenotype compared to naturally occurring ILC2s (nILC2s) at steady state. While nILC2s express ST2 and CD127, iILC2s lack ST2 expression, exhibit low CD127 expression, and showcase heightened expression of IL17RB (Huang et al. 2018; Flamar et al. 2020; Huang and Paul 2016; Zaiss et al. 2023). During the tissue repair phase following infection clearance, ILC2s primarily originate from the lung and express ST2 (Ricardo-Gonzalez et al. 2020; Zaiss et al. 2023).

1.5 Group 3 innate lymphoid cells

ILC3s exhibit a heterogenous nature and actively engage in effective antimicrobial defence. ILC3s resemble Th17 cells, both cell types express the retinoid orphan receptor gamma t (RORγt) and produce IL-17 and IL-22, contributing to type 17 immune responses (Zenewicz et al. 2008; Zheng et al. 2008; Sonnenberg et al. 2012; Klose and Artis 2020). The different subsets of ILC3s include fetal lymphoid tissue inducer (LTi) cells which orchestrate lymphoid tissue development (Mebius et al. 1997), $CCR6^+$ ILC3s - LTi-like lymphocytes of adult mice (Klose et al. 2013; Sawa et al. 2011; Eberl et al. 2004) -, and $CCR6^-$ ILC3s. Increased expression of T-bet in $CCR6^-$ ILC3s confers characteristics akin to ILC1s, including the expression of natural killer-cell receptors and markers associated with type 1 immunity, like NK1.1 and IFN-γ (Klose et al. 2013; Rankin et al. 2013; Sciumé et al. 2012; Vonarbourg et al. 2010). IL-22 is the key effector cytokine of ILC3s, and its

receptor is predominantly found on non-immune cells, particularly epithelial cells (Wolk et al. 2004). Effects downstream of IL-22 include antimicrobial peptide secretion and the promotion of barrier functions, achieved through processes like glycosylation of the epithelium (Klose et al. 2013; Zheng et al. 2008; Sonnenberg et al. 2011; Hernández et al. 2015; Abt et al. 2015; Goto et al. 2014), to provide protection of intestinal stem cells against damage, such as that induced by irradiation (Gronke et al. 2019). Beyond the gut, ILC3s have been linked to immune responses against fungal pathogens like Candida albicans (Pavlova and Sharafutdinov 2020). Their role extends to lung health, where the IL-17/IL-22 axis becomes crucial for proper functioning. In influenza infections, ILC3-derived IL-22 protects host tissues, emphasizing the role of ILC3s in balancing immune responses and protecting against secondary infections (Ardain et al. 2019). ILC3s showcase a dual role in tumorigenesis, displaying both tumor-protective and tumor-promoting effects (Huber et al. 2012; Gronke et al. 2019). ILC3s express MHC class II and can influence immune responses. They play a role in T-cell selection through processes such as intestinal selection, where $CCR6^+$ ILC3s present peptides on MHC class II without co-stimulation to prevent inflammation and maintain immune balance at mucosal sites in the gut (Hepworth et al. 2015; Hepworth et al. 2013; Klose and Artis 2020). $CCR6^+$ ILC3s predominantly localize in cryptopatches, attracting B cells to facilitate further maturation in isolated lymphoid follicles (ILFs) of the intestine (Eberl et al. 2004). These ILFs create an environment for the interaction between ILC3s and B cells, leading to T cell-independent generation of IgA (Tsuji et al. 2008; Klose and Artis 2020). Furthermore, studies indicated that $CCR6^+$ ILC3s play a role in shaping the interaction between follicular T helper cells and B cells in the colon-draining lymph node, via MHC class II. This regulatory influence contributes to the control of T cell-dependent IgA production (Melo-Gonzalez et al. 2019; Klose and Artis 2020).

1.6 B1 cells

1.6.1 B1 cell origin and phenotype

B1 cells are an innate B cell population which produce natural, T cell-independent, low-affinity antibodies, to provide a first line of defence for the organism during the early stages of an infection and promote tissue homeostasis (Aziz et al. 2015; Baumgarth 2011; Baumgarth 2016; Ochsenbein et al. 1999; Boes et al. 1998; Martin et al. 2001; Smith and Baumgarth 2019). B1 cells derive in their earliest timepoint of ontogeny from the yolk sac and embryonic para-aortic splanchnopleure (Godin et al. 1993; Montecino-Rodriguez and Dorshkind 2012). It remains uncertain whether B1 cells arising from this source contribute to the adult B1 cell population (Montecino-Rodriguez and Dorshkind 2012). During later stages of the fetal period, B1 cells emerge from hematopoietic stem cells from the liver. Additionally, a limited number also originate from the bone marrow shortly before birth (Montecino-Rodriguez and Dorshkind 2012). After birth they migrate to the body cavities, primarily peritoneal and thoracic cavity (Hayakawa et al. 1985; Ansel et al. 2002; Baumgarth 2011), where they constitute the majority of the B cell subpopulations. Besides the body cavities they egress to the omentum (Ansel et al. 2002), mucosal and respiratory tissues (Kato et al. 2013) and spleen (Kraal 1992; Stall et al. 1996). They undergo continuous trafficking from and to the body cavities through the omentum, a process facilitated by CXC-chemokine ligand 13 (CXCL13), likely produced by resident macrophages (Ansel et al. 2002). B1 cells continuously and spontaneously produce mainly “natural” IgM (Baumgarth 2016; Baumgarth 2011; Savage et al. 2017; Martin and Kearney 2001; Berland and Wortis 2002; Hayakawa et al. 1985). In their fetal origin lies the reason why B1 cells produce natural antibodies with low affinity and a lower diversity compared to follicular B cells. By incorporating non-germline-encoded N nucleotides into

the junctions of the rearranging gene segments of BCRs, terminal deoxynucleotide transferase (TdT) increases the junctional diversity in the V(D)J rearrangement during B cell maturation (Wong et al. 2019; Benedict et al. 2000). Yet, the expression of this enzyme in fetal derived B cells – like B1 cells – is reduced, leading to a limited junctional diversity (Wong et al. 2019; Berland and Wortis 2002). B1 cells express surface markers such as CD19, IgM, and CD43, along with low levels of IgD and B220 (Baumgarth 2011; Haas 2015; Tung et al. 2004). In contrast to B1 cells, follicular B2 cells express CD23 and display high levels of IgD and B220 (Baumgarth 2011; Allman and Pillai 2008). This distinctive marker profile enables the identification and differentiation of B1 and B2 cell subsets. In body cavities B1 cells express the integrin CD11b but quickly lose it upon emigration to other organs (Baumgarth 2016). Upon encountering stimuli such as infections, B1 cells undergo rapid activation, prompting their migration to lymphatic tissues. The loss of CD11b and subsequent differentiation into antibody secreting cells complicates their differentiation from activated B2 cells within these tissues (Waffarn et al. 2015; Smith and Baumgarth 2019). B1 cells are distingushed by the presence or absence of CD5, with $CD5^+$ cells classified as B1a and $CD5^-$ cells classified as B1b (Baumgarth 2011; Baumgarth 2016). A hypothesis proposed that B1a cells secrete natural antibodies and B1b cells provide an acquired and long-lasting immune response to certain types of bacteria (Haas et al. 2005). However, this functional classification was deemed overly simplistic, particularly as other studies suggested that in B1a cells antibody production was induced following infection as well (Baumgarth 2016).

1.6.2 Effector functions of B1 cells

B1 cells predominantly produce IgM (Baumgarth 2016; Baumgarth 2011; Savage et al. 2017; Martin and Kearney 2001; Berland and Wortis 2002; Hayakawa et al. 1985), with additional capability to generate IgG3, IgA and IgE (Rastogi et al. 2022; Drake et al. 2016;

Martin et al. 2018; Baumgarth 2016). Their antibodies bind against phosphatidylcholine and phosphorylcholine (Briles et al. 1982; Lalor 1991; Hardy et al. 2004; Mercolino et al. 1988; Wang and Clarke 2004; Grönwall et al. 2012), found in membranes of apoptotic cells, and against specific membrane components of pathogens, like LPS (Cole et al. 2009; Koide et al. 2001; Moon et al. 2012; Reid et al. 1997; Yang et al. 2007) and bacterial capsular polysaccharides (Haas et al. 2005; Grönwall et al. 2012). Binding to these self- and foreign antigens triggers complement activation and opsonization, leading to a rapid clearance of them which prevents early pathogen replication (Baumgarth et al. 2000; Boes et al. 1998; Ochsenbein et al. 1999) and initiation of an exaggerated immune response (Aziz et al. 2015; Morris et al. 2019). Several investigations have been conducted into the immune responses of B1 cells to tissue injury and infection. For instance, B1 cells mount an initial defence against *Streptococcus pneumoniae (S. pneumoniae)* by secreting natural antibodies by B1a cells and anti-pneumococcal capsular polysaccharide 3 antibodies by B1b cells (Haas et al. 2005; Briles et al. 1982). *Francisella tularensis (FT)* LPS triggers an antibody response that is dominated by B1a cells. After clearance of infection, B1a plasma cells keep producing a lower level of antibodies against *FT* LPS for several weeks to protect from reinfection (Cole et al. 2009). In the context of influenza virus infection, B1a cells generate polyreactive, natural IgM antibodies as a first line of defence at the respiratory tract epithelium and in the regional lymph nodes (Baumgarth et al. 1999; Baumgarth et al. 2000; Baumgarth 2011; Choi and Baumgarth 2008). Other diverse stimuli, including different bacteria (Martin et al. 2001; Alugupalli et al. 2004; Cole et al. 2009; Ordoñez et al. 2018), viruses (Baumgarth et al. 2000; Baumgarth et al. 1999; Ochsenbein et al. 1999), fungi (Subramaniam et al. 2010; Jackson-Jones et al. 2016) and cytokines (Nisitani et al. 1995), trigger activation and migration of B1a and B1b cells from the peritoneal cavity to the spleen or mucosal tissues,

where they undergo maturation into cells which primarily produce IgM and IgA (Martin et al. 2001; Yang et al. 2007; Nisitani et al. 1995). In addition to antibodies, B1 cells are known to release cytokines such as IL-3, GM-CSF, and IL-10. IL-3 and GM-CSF are involved in fostering the proliferation of myeloid cells (Chousterman and Swirski 2015; Weber et al. 2015; Weber et al. 2014). Notably, IL-10 exhibits anti-inflammatory attributes. It is documented to impede macrophage functions, including the release of reactive species and cytokines (Fiorentino et al. 1991; Popi et al. 2004). In disease models, IL-10 has demonstrated efficacy in reducing disease severity, exemplified in a colitis model where it modulated neutrophil infiltration and $CD4^+$ T cell activation (Maseda et al. 2013), and mitigated inflammation in a mouse model of cutaneous hypersensitivity (Nakashima et al. 2010). Additionally, IL-10 has been observed to exert an autocrine growth factor effect on B1 cells, further contributing to their regulation (O'Garra et al. 1992).

1.6.3 Cytokine regulation of B1 cells

In addition to stimulation by antigens, B1 cells are regulated by cytokines such as IL-5, IL-6 and, in an autocrine manner, IL-10. IL-5 stimulation initiates diverse signalling cascades, including JAK2/STAT5 (Kouro et al. 1996; Ogata et al. 1998; Takaki et al. 1994) and Btk (Sato et al. 1994) pathways, which contribute to the upregulation of genes associated with cell growth and viability. IL-5 is reported as a critical survival factor for B1 cells. Several publications have demonstrated the impact of IL-5 on proliferation and functions of B1 cells by using mice deficient in IL-5 production or the receptor for IL-5, IL-5 Rα: A decrease in B1 cells (Kopf et al. 1996; Bao et al. 1998) and B1 cell-derived IgA (Bao et al. 1998) has been documented in *Il5*$^{-/-}$ mice. Similarly, *IL-5Rα*$^{-/-}$ mice exhibited reduced B1 cell populations and diminished production of B1 cell-derived IgM, IgG3 (Yoshida et al. 1996) and IgA (Hiroi et al. 1999; Moon et al. 2004). Notably, administration of anti-IL-5 monoclonal antibody in wild-type mice resulted in a reduction in absolute

numbers of B1 cells to a degree comparable to that observed in *IL-5Rα*$^{-/-}$ mice (Moon et al. 2004). Furthermore, IL-6 was reported to promote B1 cell survival via STAT3 signalling pathway (Chou et al. 2006; Thies et al. 2013). In the context of promoting B1 cell proliferation it has been demonstrated *in vitro* that peritoneal macrophages are the main source of IL-6 (Thies et al. 2013).

1.7 B2 cells

B2 cells undergo a complex and multifaceted developmental process that starts in the bone marrow where hematopoietic stem cells differentiate into progenitor B2 cells, initiating the process of B2 cell maturation (Hardy et al. 1991; Rolink and Melchers 1996; Löffert et al. 1994). One of the defining features of B2 cell development is the rearrangement of immunoglobulin (Ig) genes through V(D)J recombination (Tonegawa 1983; Rajewsky 1996; Nguyen et al. 2016), faciliated by the lymphoid-specific RAG-1 and RAG-2 endonucleases that initiate DNA cleavage at defined recombination signal sequences that flank the V, D and J gene segments (Nguyen et al. 2016). This process generates a remarkably diverse repertoire of BCRs, equipping them to recognize a vast spectrum of antigens (Tonegawa 1983; Rajewsky 1996). As they transition from immature to mature naïve B2 cells, they progress through various developmental stages characterized by distinct surface marker expression, functional properties, and selection events such as the deletion of autoreactive B2 cells. (Rolink et al. 2001; Osmond 1986). Transitional B2 cells, emerging from the bone marrow, migrate to the spleen where they undergo further differentiation and selection processes (Loder et al. 1999). Within these specialized microenvironments, mature B2 cells continue to develop into functionally distinct subsets, including follicular and marginal zone B cells (Loder et al. 1999; Kraal 1992; Oliver et al. 1997). Marginal zone B cells, localized in the marginal zone of the

spleen, serve as a first line of defence, rapidly initiating immune responses to provide early protection against invading pathogens (Martin et al. 2001; Kraal 1992; Cerutti et al. 2013; Oliver et al. 1997). Throughout follicular B cell development and activation, somatic hypermutation (SHM) and class-switch recombination (CSR) are responsible for genetic alterations in the Ig genes, resulting in the generation of high-affinity antibodies with enhanced effector functions (Di Noia and Neuberger 2007; Li et al. 2004; Chi et al. 2020). SHM, facilitated by activation-induced cytidine deaminase (AID), introduces point mutations in the variable regions of Ig genes, leading to the selection of B cell clones with improved antigen-binding affinity (Zan and Casali 2013; Hwang et al. 2015; Maul and Gearhart 2010; Methot and Di Noia 2017; Muramatsu et al. 2000; Pham et al. 2003; Yu 2022; Bergthorsdottir et al. 2001). CSR, on the other hand, allows B cells to switch the constant region of their Ig heavy chain, thereby altering the effector function of antibodies without changing their antigen specificity (Hwang et al. 2015; Methot and Di Noia 2017; Muramatsu et al. 2000). Upon encounter with cognate antigens, activated follicular B cells establish germinal centres, where they undergo clonal expansion and differentiation into plasma cells and memory B cells. Memory B cells exhibit a swift and vigorous immune reaction upon encountering a familiar antigen once again (Carsetti et al. 2004; NOSSAL 1962; Radbruch et al. 2006; Shlomchik and Weisel 2012; Slifka et al. 1998; Victora and Nussenzweig 2012; Manz et al. 1997; *Antibody Production in relation to the Development of Plasma Cells. In vivo and in vitro Experiments* 1948; Nutt et al. 2015).

1.8 Antibody production in adaptive and innate immunity

In the adaptive immune system follicular B cells generate antibodies against TD antigens, which generally consists of proteins, with the assistance of $CD4^+$ T helper cells (Murphy and Weaver). B cells utilize their BCR to bind to native antigens, while T cells employ

their TCR for recognition of antigens presented by APCs, including dendritic cells and B cells, via MHC class II (Murphy and Weaver). The engagement of CD28 on T cells with B7.1 and B7.2 on APCs promotes survival and activation of T cells, primarily through the increased release of IL-2 by T cells in an autocrine manner (Boise et al. 1995; Fraser et al. 1991; Howland et al. 2000; Jenkins et al. 1991; Jenkins and Schwartz 1987; Wells et al. 1997; Schweitzer and Sharpe 1998; Norton et al. 1992; Lindstein et al. 1989; Linsley et al. 1990; Harding et al. 1992). Furthermore, expression of CD40L is initiated on activated T cells (Bretscher 1999; Guo et al. 1996; Elgueta et al. 2009; Grewal and Flavell 1996). Through engaging of CD40L with CD40 on B cells, B cells become activated by triggering the non-canonical NFκB pathway (Elgueta et al. 2009; Murphy and Weaver), which promotes B cell survival by upregulating the expression of anti-apoptotic molecules, such as Bcl-2 (Murphy and Weaver). The activation of CD40 plays a critical role in facilitating the formation and advancement of germinal centres (GCs), promoting isotype switching and affinity maturation. These processes are essential for the generation of plasma cells capable of producing high-affinity antibodies (Foy et al. 1994a; Foy et al. 1994b; Foy et al. 1993; Danese et al. 2004; Xu et al. 1994). Deficiency in CD40L results in the absence of germinal centres and the complete elimination of the adaptive immune response (Banchereau et al. 1994; Kawabe et al. 1994; Xu et al. 1994). By integrating signals from two independent cell types with different activation requirements the interaction between T and B cells constitutes a quality control mechanism of the immune system that ensures to identify foreign antigens and create targeted immunological pathways to eliminate pathogens or infected cells (Marshall et al. 2018). An innate pathway of antibody response without T cell help is initiated by TI antigens. In this pathway B1 cells play a crucial role as well as marginal zone B cells in the spleen. Two types of TI antigens exist: TI-1 antigens and TI-2 antigens, both triggering the NFκB

pathway (Murphy and Weaver). Binding of TI-1 antigens initiates proliferation and differentiation of B lymphocytes without the dependence on the specificity of their BCRs. The activation of B cells by TI-1 antigens is facilitated through Toll-like receptors (TLRs), which are expressed on the surface of B lymphocytes upon BCR stimulation. Examples include LPS and bacterial DNA (Murphy and Weaver). TI-2 antigens consist of molecules with “highly repetitive structures” (Murphy and Weaver), like bacterial capsular polysaccharides. Activation of B cells by TI-2 antigens occurs through cross-linking of BCRs, triggering the accumulation and cross-activation of these receptors (Murphy and Weaver). TI-2 antigen responses are crucial against encapsulated bacteria (Lindberg 1999), such as *Haemophilus influenzae*, *S. pneumoniae* and *Neisseria meningitidis*, which can cause severe diseases, especially in vulnerable populations like young children, older adults and individuals with immunodeficiencies (Curcio et al. 2015; O'Brien et al. 2009; Butler and Myers 2018; Pace and Pollard 2012; Stephens et al. 2007). It has been demonstrated that capsular polysaccharides from *S. pneumoniae* induce a T cell independent immune response through the activation of B1b cells, and antibodies derived from B1b cells are effective in protecting against infection (Haas et al. 2005). Notably, purified pneumococcal polysaccharides are utilized as a vaccine to induce a TI antigen response in humans (Hascelik et al. 2023). Although the immune response can be enhanced through conjugation with an immunogenic protein to trigger a more effective, thymus-dependent (TD) antigen response, unconjugated polysaccharide vaccines remain important as they cover a broader range of *S. pneumoniae* serotypes (Hascelik et al. 2023). This shows that B1 cells not only contribute to maintaining tissue homeostasis but also appear to play an important role in preventing severe infections.

1.9 Investigating the role of ILC2 as a crucial regulator of B1 cells

While B2 cells receive T cell assistance via CD40L to initiate an antibody response (Calame 2001; Elgueta et al. 2009), it remains to be investigated if antibody production by B1 cells relies on another cellular partner. *In vitro* research suggested that ILC2s, despite not expressing CD40L and an antigen receptor (Robinette and Colonna 2016), could promote proliferation and antibody production of B1 cells through IL-5: The association between B1 cells and ILC2s was initially discovered by Moro et al. (Moro et al. 2010), who demonstrated that ILC2s induced a division of B1 cells *in vitro*. Her subsequent investigations revealed that recombinant IL-5 also induced proliferation of B1 cells. Furthermore, Drake et al. (Drake et al. 2016) reported a substantial increase of B1 cell populations when co-cultured with ILC2s, with LPS stimulation further enhancing the production of IgM and IgA in this system. Absence of IL-5 caused impairments in functions and viability of B1 cells (Kopf et al. 1996; Bao et al. 1998; Hiroi et al. 1999; Moon et al. 2004; Yoshida et al. 1996). Moreover, experimental investigations employing *in vitro* co-cultures of ILC2s – presumably the primary producers of IL-5 (Nussbaum et al. 2013) - and B1 cells revealed stimulatory effects of ILC2s on the proliferation and antibody production of B1 cells (Moro et al. 2010; Drake et al. 2016). These findings suggest that the regulatory control of B1 cells is exerted by ILC2s and mediated through the IL-5 signalling pathway. I investigated the dependency of B1 cells on ILC2s and elucidated the underlying pathways *in vivo* by employing a mouse model with targeted knockout of ILC2s (Jarick 2022). The findings indicated that mice lacking ILC2s exhibited diminished frequencies and absolute numbers of both B1a and B1b cells. Through single-cell RNA sequencing, it was further elucidated that IL-5rα^{+} B1 cells displayed impaired proliferation, accompanied by the downregulation of various IL-5-dependent target genes. Additionally, conditional deletion of *Il5* in ILC2s resulted in reduced populations of B1 cells, directly

implicating that ILC2-derived IL-5 shape the B1 cell phenotype. Consistent findings were observed in *Il33*$^{-/-}$ and *Nmur1*$^{iCre+}$ *St2*$^{flox/flox}$ mice, underscoring the indispensable role of IL-33 as a crucial upstream signal for IL-5 production in ILC2s. Moreover, reductions of B1 cells with phosphatidylcholine-specific B cell receptors were observed in the absence of ILC2s. Thus, the data unveiled an innate mechanism of antibody production by B1 cells that is strictly reliant on ILC2-derived IL-5 and cannot be compensated by other cell types.

2 Methods

2.1 Mouse strains

C57BL/6mice (Mus musculus) were purchased from Janvier. *Nmur1*$^{iCre\text{-}T2A\text{-}GFP}$ (Jarick 2022; Tsou et al. 2022), *Id2*$^{flox/flox}$ (Niola et al. 2012), *Gata3*$^{flox/flox,}$ (Grote et al. 2008), *Il5* $^{-/-}$ (Nussbaum et al. 2013), *Il33* $^{-/-}$ (Oboki et al. 2010)*, Il17rb* $^{-/-}$ (Neill et al. 2010), *Tslpr* $^{-/-}$ *(Al-Shami et al. 2004)* and *Il1rl1(St2)* $^{flox/flox}$ (Hemmers et al. 2021), *Areg*$^{flox/flox}$ (Arpaia et al. 2015), *Il4/13*$^{flox/flox}$ (Voehringer et al. 2006), *Il6* $^{flox/flox}$ (Sanchis et al. 2020) and *Rosa26*$^{Rfp/Rfp}$ (Luche et al. 2007) on a C57BL/6 background were bred locally at Charité. *Il5*$^{flox/flox}$ mice were generated by GemPharmatech on a C57BL/6 background. In brief, exon 1 and 2 of the *Il5* gene were flanked by two LoxP sites and validated by Sanger sequencing. Experiments utilized male and female animals matched for sex and age, typically ranging from 7 to 14 weeks, unless stated otherwise. Animals were not assigned to experimental groups through blinded or randomized procedures. Statistical methods were not employed to determine the sample size of experimental groups. All animal procedures were approved and conducted in adherence to the guidelines outlined by the local animal care committees (LAGeSo Berlin). The licences for the animal experiments are T 0032/19 (date of permission: 11.02.2019) and T-CH 0023/22 (date of permission: 20.09.2022).

2.2 Cell Isolation

2.2.1 Peritoneal and thoracic cavity

For cell isolation of peritoneal cavity, the skin was removed, and the peritoneum flushed with 10 ml of ice-cold PBS. PBS is an isotonic salt solution which maintains a stable pH

for the cells. Skin was removed for cell isolation of thoracic lavage and 3 ml of ice-cold PBS was flushed between visceral and parietal pleura. Peritoneal and thoracic cells were spun down at 1500rpm for 10 minutes and the supernatant was discarded. Remaining pellets were used for staining.

2.2.2 Omentum

The process of cell isolation from the omentum followed the protocol described in the study by Jarick et al. (Jarick 2022) for isolating cells from the spleen and mesenteric lymph nodes: Omentum was incubated in RPMI 1640 medium (Gibco) supplemented with 1% BSA (Sigma-Aldrich), collagenase II (1 mg/ml; Sigma-Aldrich) and DNaseI (100 µg/ml) for 20 minutes on a shaker at 37°C. Afterwards, cells were dissociated using a pipette, and filtered through a 70 µm cell strainer, washed with PBS, spun down at 1500 rpm for 10 minutes and supernatant was discarded. Remaining pellets were used for staining.

2.2.3. Spleen

Spleens were chopped, filtered through a 70 µm cell strainer, washed with PBS and spun down at 1500 rpm for 10 minutes and supernatant was discarded. Remaining pellets were used for staining.

2.2.4 Small intestine

The process of leukocyte isolation from the small intestine followed the protocol described in the study by Jarick et al. (Jarick 2022): “Small intestine was removed, cleaned from remaining fat tissue, and washed in ice-cold PBS. Peyer’s patches were eliminated, small intestine was opened longitudinally and washed in ice-cold PBS. Dissociation of epithelial cells was performed by incubation on a shaker at 37°C in HBSS (Sigma-Aldrich),

containing 10 mM Hepes (Gibco) and 5 mM EDTA (Roboklon), two times for 15 minutes. After each step, samples were vortexed, and the epithelial fraction was discarded. Afterwards, remaining tissue was chopped into small pieces and enzymatic digestion was performed on a shaker at 37°C in HBSS with calcium, magnesium, no phenol red (Gibco), dispase (0,5 U/ml; Corning), collagenase D (0,5 mg/ml; Roche), DNaseI (100 µg/ml; Sigma-Aldrich)". Cells were washed with PBS, spun down and the supernatant was discarded. Pellets were resuspended in 40% Percoll, and 80% Percoll was underlayed by using pasteur pipettes. 40% Percoll and 80% Percoll is a solution of Percoll (GE Healthcare), PBS 10x (Thermo Fisher) and DMEM (Thermo Fisher). 40% Percoll contains 60% DMEM and 40% PBS 1x – which is made of PBS 10x, diluted in Percoll to 1x. 80% Percoll contains 20% DMEM and 80% PBS 1x – which is made of PBS 10x, diluted in Percoll to 1x. Tubes were centrifuged with 2500 rpm percoll gradient. Middle cell layer was harvested, washed with PBS and spun down at 1500rpm for 10 minutes and supernatant was discarded. Remaining pellets of leukocytes were used for staining.

2.2.5 Lung

Leukocyte isolation from the lungs was conducted following the protocol outlined by Jarick et al. (Jarick 2022): "Lungs were chopped and incubated in the same enzyme cocktail used for digestion for small intestine for 40 minutes on a shaker at 37°C. Remaining tissues were mashed with a syringe plunger and single cell suspensions were filtered through a 70 µm cell strainer. Leukocytes were further enriched by Percoll gradient centrifugation". Middle cell layer was harvested, washed with PBS and spun down at 1500rpm for 10 minutes and supernatant was discarded. Remaining pellets of leukocytes were used for staining.

2.3 Flow cytometry and cell sorting

Cells were stained according to the lymphocyte staining protocol for flow cytometry by Jarick et al. (Jarick 2022). For surface staining of B1 cells single cell suspensions were incubated on ice with anti-CD16/CD32 antibody and the following conjugated antibodies in PBS (Ca2+ and $Mg2^{+}$-free (Sigma-Aldrich)): CD45 (clone 30-F11 (BioLegend) or 104 (eBioscience)), CD19 (clone 6D5 (BioLegend)), CD45R (clone RA3-6B2 (BioLegend)), CD23 (clone B3B4 (BioLegend)), CD43 (clone S11 (BioLegend)), CD11b (clone M1/70 (eBioscience)), IgM (clone eB121-15F9 (eBioscience) or clone X-54 (REAfinity)), IgD (clone 11-26c(11-26) (eBioscience)), CD5 (clone 53-7.3 (BD Horizon)), CD21/35 (clone 7G6 (BD Bioscience)). T cells were excluded by staining for TCRβ (clone H57-597 (BioLegend)). Dead cells were excluded with SYTOX Blue Dead Cell Stain (Thermo Fisher Scientific) for surface staining or Zombie Aqua Fixable Viability Dye (BioLegend) for intracellular staining. After 25 minutes of incubation, cells were washed with PBS and spun down at 1500 rpm for 5 minutes. As IgD (clone 11-26c(11-26) (eBioscience)) is a biotinylated antibody, a second staining step was performed with Streptavidin in BUV 737 (BD Horizon) for 15 minutes, afterwards the cells were washed with PBS, spun down at 1500 rpm for 5 minutes and resuspended in PBS. If intracellular staining was performed after the surface staining, cells were resuspended in fixation buffer of the Foxp3 transcription factor buffer set (Thermo Fisher Scientific). After 2 hours of incubation or overnight incubation cells were washed with 10x washing buffer of the Foxp3 transcription factor buffer set, diluted in Milli-Q water. For intracellular staining Ki-67 (B56 (BD Bioscience)) was used in 10x washing buffer, diluted in Milli-Q water. After 1 hour of incubation cells were washed with PBS, spun down at 1500 rpm for 5 min and were ready to acquire.

For surface staining of ILC2s, single cell suspensions were incubated on ice with anti-CD16/CD32 antibody and the following conjugated antibodies in PBS (Ca2+ and $Mg2^{+}$-free (Sigma-Aldrich)): KLRG1 (clone 2F1 (BioLegend)), CD127 (clone A7R34 (BioLegend)), CD90 (clone 53-2.1 (BD Bioscience)), NK 1.1 (clone PK136 (Thermo Fisher Scientific)), St2 (clone DIH9 (BioLegend)), CD45 (clone 30-F11 (BioLegend)). B cells, T cells, mast cells, neutrophils and eosinophils were excluded by the markers, CD5 (clone 53-7.3 (BD Horizon)), CD3ε (clone 145-2C11 (BioLegend)), CD19 (clone 1D3 (Thermo Fisher Scientific)), Ly6G (clone 1A8-Ly6g (Thermo Fisher Scientific)), FceR1α (clone MAR-1 (Thermo Fisher Scientific)). Dead cells were excluded with SYTOX Blue Dead Cell Stain (Thermo Fisher Scientific). Flow cytometry experiments were conducted utilizing a custom configuration Fortessa flow cytometer and the FACS Diva software (BD Biosciences), with subsequent analysis performed using FlowJo V9.9.3 or V10.6.2 software (TreeStar). Additionally, sort purification was accomplished employing a custom configuration FACSAria cell sorter (BD Biosciences).

2.4 Quantitative real-time PCR

2.4.1 RNA Isolation

Cells were stained according to the protocol of flow cytometry and cell sorting above. Sorted cells were homogenized in 500 µl Trizol (Thermo Fisher Scientific) and stored at -80°C (according to (Jarick 2022)). RNA isolation was performed in accordance with the protocol provided by the Immunological Genome Project (Immunological Genome Project): Samples were thawed at room temperature. After 100 µl Chloroform was added to each sample, they were shaken vigorously for 15 seconds and incubated for 3 minutes at room temperature. Then, they were centrifuged at 12000g at 4 °C for 15 minutes. Afterwards the aqueous phase was transferred to a fresh tube and 1 µl Glycoblue and

250 µl Isopropanol was added to each sample. After that, the samples were incubated at -80 °C for at least 1 hour. To further proceed, the samples were thawed on ice. After they were centrifuged at 12000g at 4°C for 20 minutes, the supernatant was removed, and the pellet washed with 75% ice-cold ethanol solution. They were further centrifuged at 7400g at 4 °C for 15 minutes. The supernatant was removed, and the RNA pellet was air dried for 30 minutes and then resuspended in 12 µl Nuclease-free water. The concentration of RNA was measured using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific).

2.4.2 cDNA

The total RNA underwent reverse transcription using the High-Capacity cDNA Reverse Transcription kit following the manufacturer's protocol (Thermo Fisher Scientific).

2.4.3 Quantitative real-time PCR reaction

The QuantStudio 5 Real-Time PCR system (Thermo Fisher Scientific) was employed to detect the reaction, utilizing the cDNA in conjunction with SYBR Green Master Mix and the following primers: *Il5* (forward: 5'-CTCTGTTGACAAGCAATGAGACG-3', reverse: 5'-TCTTCAGTATGTCTAGCCCCTG-3'), *Ccnd2* (forward: 5'-CCTGGATGCTAGAGGTCTGTG-3', reverse: 5'- GGCCTTAGTGTGATGGGGAA-3'), *Ass1* (forward: 5'- ACACCTCCTGCATCCTCGT-3', reverse: 5'-GCTCACATCCTCAATGAACACCT-3'), *Anxa2* (forward: 5'-ATGTCTACTGTCCACGAAATCCT-3', reverse: 5'- CGAAGTTGGTGTAGGGTTTGACT-3'). Gene expression was normalized to the housekeeping *Hprt1* (forward: 5'-GATACAGGCCAGACTTTGTTGG-3', reverse: 5'-CAACAGGACTCCTCGTATTTGC-3').

2.5 Single-cell RNA and BCR sequencing of B cells

2.5.1 Library preparation and sequencing

Single-cell sequencing was conducted according to the methodology delineated in the publication by Ferreira-Gomes et al. 2021 (Ferreira-Gomes et al. 2021). Protocol description was adopted from Jarick et al. (Jarick 2022): “[Flow sorted peritoneal cavity B cells of 5 *Nmur1*$^{iCre\text{-}T2A\text{-}GFP}$ *Id2*$^{flox/flox}$ and 5 *Id2*$^{flox/flox}$ mice were pooled], and applied to the 10X Genomics workflow for cell capturing and scRNA gene expression (GEX) library preparation using the Chromium Single Cell 5’ v2 Library & Gel Bead Kit (10x Genomics). Final GEX libraries were obtained after fragmentation, adapter ligation, and final Index PCR using the Single Index Kit TT Set A. Qubit HS DNA assay kit (Life Technologies) was used for library quantification and fragment sizes were determined using the Fragment Analyzer with the HS NGS Fragment Kit (1–6000 bp) (Agilent). Sequencing was performed on a NextSeq2000 device (Illumina) using a NextSeq 1000/2000 P2 reagent (200 cycles) with the recommended sequencing conditions for 5’ GEX libraries (read1: 26nt, read2: 90nt, index1: 10nt, index2: 10).”

2.5.2 Single-cell transcriptome

“Raw sequence reads were processed using cellranger (version 5.0.0), including the default detection of intact cells. Mkfastq and count were used in default parameter settings for demultiplexing and quantification of gene expression. Refdata-cellranger-mm10-1.2.0 was used as reference. The number of expected cells was set to 3000.

The cellranger output was analyzed in R (version 4.0.3) using the Seurat package (version 4.0.2). The single cell transcriptomic profiles for 12000 KO cells and 12000 WT cells were integrated, normalized, variable genes were detected, and a uniform manifold approximation and projection (UMAP) was performed in default parameter settings using

FindIntegrationAnchors, IntegrateData, FindVariableGenes, RunPCA and RunUMAP with 30 principle components. Expression values are represented as ln (10,000 * UMIsGene)/UMIsTotal + 1). Similar clusters were identified using shared nearest neighbor (SNN) modularity optimization, SNN resolutions ranging from 0.1 to 1.0 in 0.1 increments were computed, or gating was performed manually using the Loupe Browser (10X Genomics). Subsequently, clusters were annotated by projection of indicated genes on the UMAP to assign different cell types. Signature genes were identified using FindAllMarkers in default parameter settings. Heatmaps are based on z-transformed expression values for genes with significant differences to means in different clusters as judged by a Bonferroni corrected P-value (Wilcoxon rank sum Test) below 0.01 and a minimal absolute fold-change to the mean of log2(1.3)" (Jarick 2022).

2.5.3 Ig gene analysis

Full length Ig gene sequences obtained from cellranger (version 7.2.0) were analyzed using Change-O (version 1.3.0), filtered for productive Ig heavy and light chain, and Ig gene frequencies were calculated using the alakazam package (version 1.2.1) (Gupta et al. 2015). Prior to all comparisons cell numbers were equalized for each sample.

2.6 Statistical analysis

The data is presented as mean ± standard deviation. P values for datasets were calculated using unpaired two-tailed Student's or Welch's t-test, as well as ordinary one-way ANOVA with Tukey's multiple comparisons test, with a 95% confidence interval. It was assumed that the data followed a normal distribution. Statistical analyses were conducted using GraphPad Prism V9 software (GraphPad Software, Inc.). (*p <0.05; **p <0.01; ***p <0.001; ****p <0.0001 and ns, not significant).

3 Results

3.1 B1 do not develop properly in the absence of ILC2s

3.1.1 Reduction of B1 cell populations in *Nmur1*$^{iCre+}$ *Id2*$^{flox/flox}$ mice

It was recently shown that eosinophils do not develop in normal proportions in ILC2-deficient mice (Jarick 2022). Previous work has established an essential function of the IL-5–IL-5rα axis for eosinophil development (Kopf et al. 1996; Yoshida et al. 1996) and ILC2s have been identified as the primary producers of IL-5 (Nussbaum et al. 2013). Since *in vitro* work suggested that B1 cells may also be dependent on IL-5 signals (Kopf et al. 1996; Yoshida et al. 1996; Hiroi et al. 1999; Moon et al. 2004), I aimed to test whether ILC2s are required for B1 cell development and maintenance. The ILC2 knockout mouse model I worked with was designed with the Neuromedin U receptor 1 (*NMUR1*) to selectively target ILC2s. This involved the insertion of a P2A-iCre-T2A-eGFP cassette into exons 2 and 3 of the *Nmur1* gene, resulting in the generation of mice known as *Nmur1*$^{iCre\text{-}T2A\text{-}GFP}$ mice (Jarick 2022). ILC2-deficient mice were generated by crossing *Nmur1*$^{iCre\text{-}T2A\text{-}GFP}$ mice to *Id2*$^{flox/flox}$ animals that lack this essential transcriptional repressor for ILC development specifically in ILC2s (Jarick 2022; Hoyler et al. 2012; Klose et al. 2014; Moro et al. 2010). To test if ILC2 deficiency affects B1 cell development, cells from peritoneal cavity of *Nmur1*$^{iCre\text{-}T2A\text{-}GFP}$ *Id2*$^{flox/flox}$ mice (ILC2 KO) and littermate controls were isolated and flow cytometry was used to analyse the B1 cell population. I gated B1 cells as IgM^{+} / CD19^{+}, CD23^{-} cells (Baumgarth 2011) from CD45^{+} live cells and excluded T cells with TCRβ (Fig. 1a). The results clearly showed reduced proportions and absolute cell numbers of B1 cells in both subsets B1a and B1b cells in ILC2-deficient mice (Fig.1a). CD11b is an additional marker to identify B1 cells from body cavities (Baumgarth 2016).

Therefore, CD11b^{+} B1 cells were analysed, revealing a noticeable decrease in ILC2 KO mice. (Fig.1a). Moreover, B1 cells were diminished in *Nmur1*$^{iCre+}$ *Id2*$^{flox/flox}$ mice in all organs investigated, including omentum, thoracic cavity, lung, and spleen compared to *Id2*$^{flox/flox}$ littermate control mice (Fig. 1b). In the spleen I used CD21/CD35 to gate out marginal zone B cells. In the omentum I gated B1 cells as live CD45^{+}, TCRβ^{-}, IgD^{low}, IgM^{+} / CD19^{+} cells, staining of CD23 was impossible after digestion of omentum.

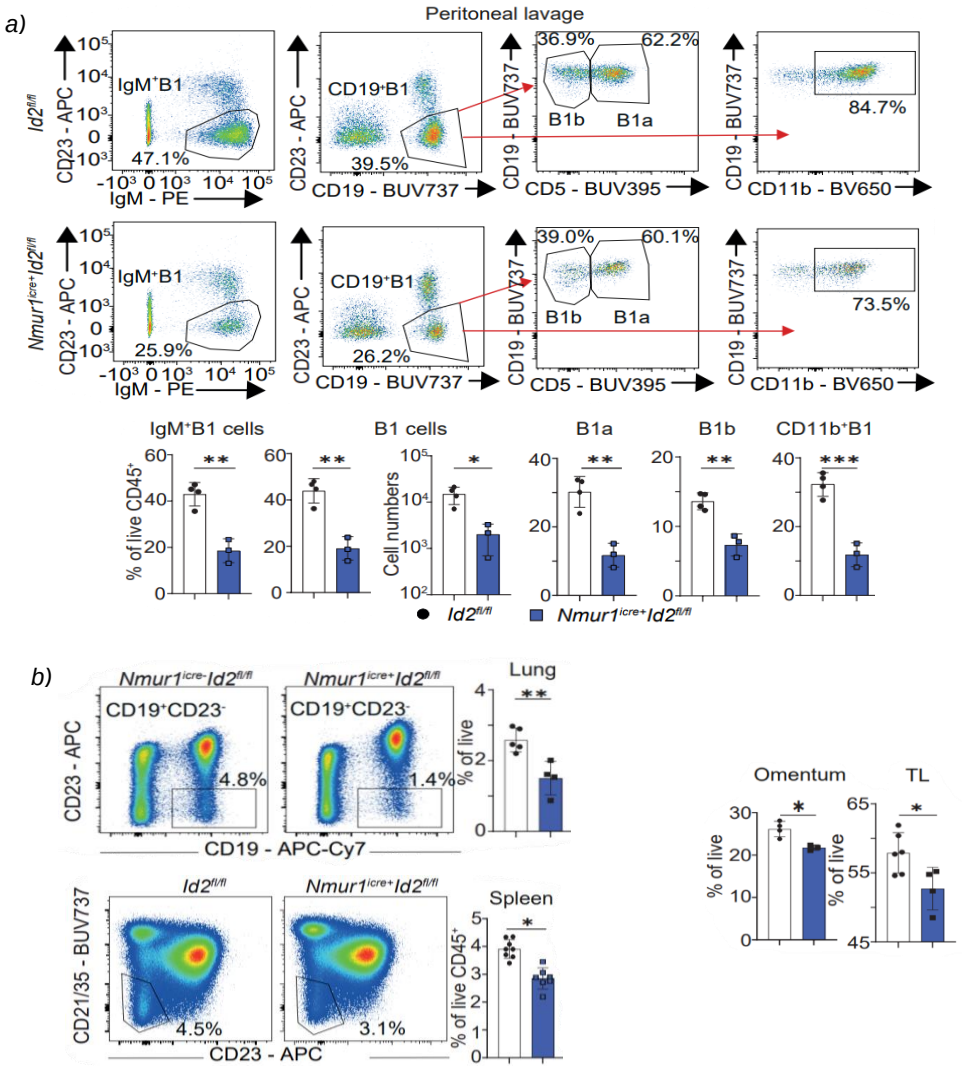

Figure 1: B1 cell populations are reduced in NMUR1^{iCre+} Id2$^{flox/flox}$ mice

a) *Flow cytometric plots and absolute and relative quantification of B1 cells (gated as live CD45^{+}, TCRβ^{-}, CD23^{-}, IgM^{+} / CD19^{+} cells) from peritoneal lavage of Nmur1^{iCre+} Id2$^{flox/flox}$ mice and Id2$^{flox/flox}$ littermate controls in steady state. B1 cells can be distinguished in B1a (CD5^{+}) and B1b (CD5^{-}) cells and can express the marker CD11b. b) Flow cytometric plots of lung and spleen and relative quantification of B1 cells from omentum, thoracic lavage (TL), lung, and spleen of Nmur1^{iCre+} Id2$^{flox/flox}$ and Id2$^{flox/flox}$ littermate controls in steady state. In the spleen identification of B1 cells requires the additional marker CD21/CD35 to exclude marginal zone B cells (CD23^{-}, CD21/35^{+}). Each symbol represents data from one mouse, data are representative of at least two experiments with three or more mice per group. Mean +/-SD, Student's t-test,** p<0.01, *** p<0.001, **** p<0.0001.*

With flow cytometry I compared different gating strategies for B1 cells (Baldan et al. 2014; Baumgarth 2011; Cuenca et al. 2016; Aziz et al. 2015) to show that the reduction observed in ILC2 KO mice is consistent across different gating strategies (Fig.2).

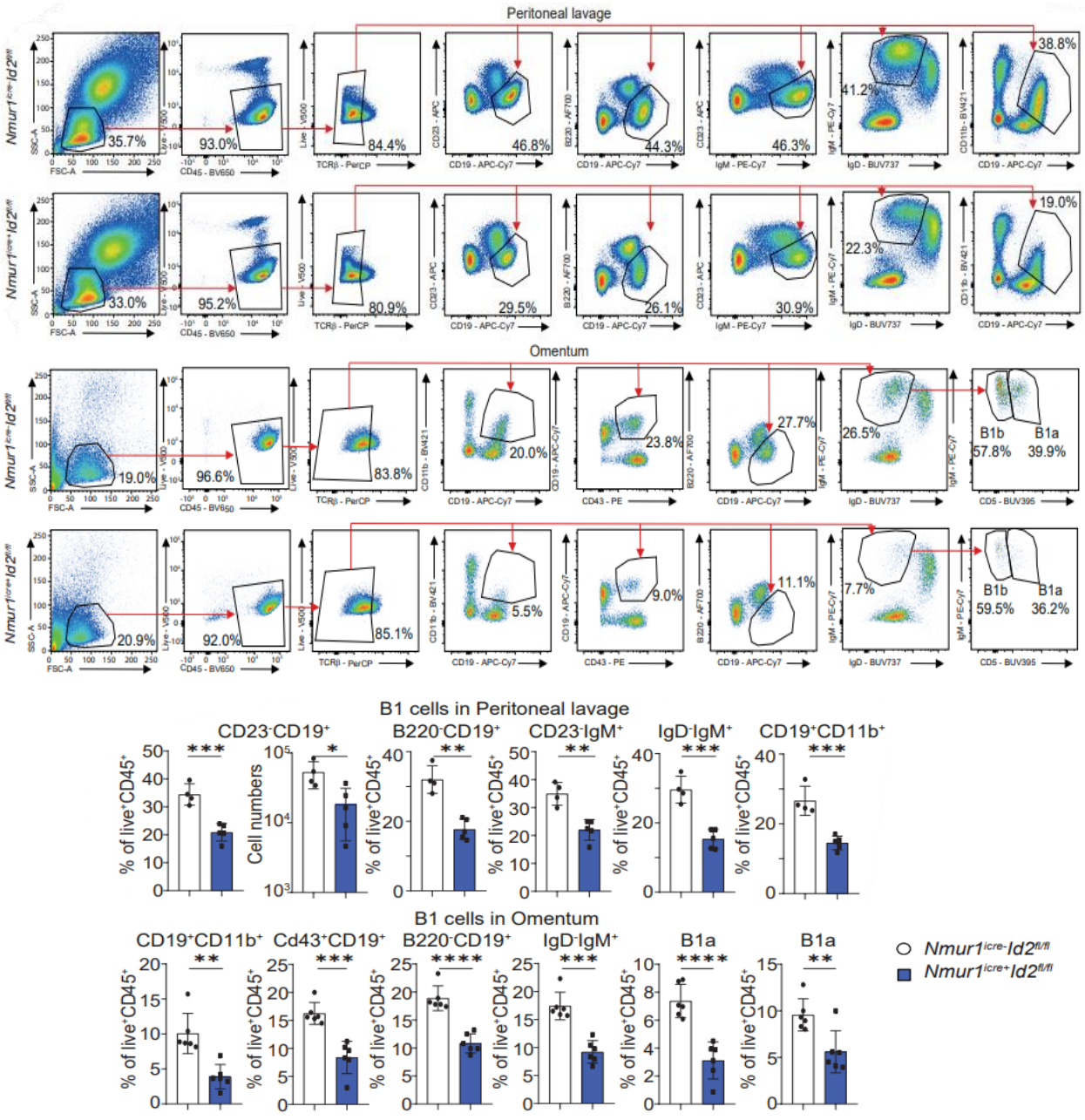

Figure 2: Different gating strategies show similar results

*Flow cytometric plots, relative and absolute quantification of different gating strategies of B1 cells from peritoneal lavage and omentum of Nmur1^{iCre+} Id2$^{flox/flox}$ and Nmur1^{iCre-} Id2$^{flox/flox}$ littermates in steady state. For pre-gating T cells were excluded from CD45^{+} live cells with TCRβ. B1 cells were gated as CD19^{+} and CD23^{-}, CD19^{+} and B220^{low}, IgM^{+} and CD23^{-}, IgM^{+} and IgD^{low} or CD19^{+} and CD11b^{+} in peritoneal lavage. Same markers were used in omentum, except of CD23, which is impossible to stain after digestion of omentum. Instead, gating with CD19 and CD43 and gating of B1a and B1b cells in omentum are shown. B1 cell gating strategies according to: (Baumgarth 2011; Baldan et al. 2014; Cuenca et al. 2016; Aziz et al. 2015). Each symbol represents data from one mouse, data are representative of at least two experiments with three or more mice per group. Mean +/-SD, Student's t-test,** p<0.01, *** p<0.001, **** p<0.0001.*

Next, I analysed B1 cells in relative and absolute cell numbers over time in the peritoneal cavity and observed reduced B1 cell frequency and counts at all time points analysed (Fig. 3). Thus, B1 cells were reduced at various time points suggesting that the reduction in B1 cells was not compensated over time.

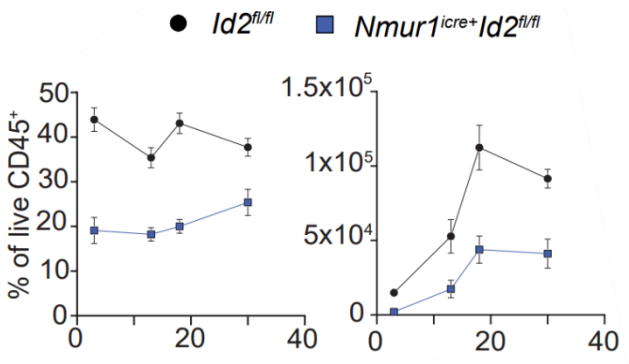

Figure 3: ILC2s are important for B1 cell survival across different ages
*Flow cytometric relative and absolute quantification of peritoneal cavity B1 cells of Nmur1^{iCre+} Id2$^{flox/flox}$ mice and Id2$^{flox/flox}$ littermate controls across different ages (3, 13, 18, 30 weeks old) in steady state. Each symbol represents data from one age group. Each age group included three or more mice per group. Data are representative of at least two experiments. Mean +/-SD, Student's t-test,** p<0.01, *** p<0.001, **** p<0.0001*

3.1.2 Reduction of B1 cell populations in *Nmur1*$^{iCre+}$ *Gata3*$^{flox/flox}$ mice

Nmur1^{iCre+} *Gata3*$^{flox/flox}$ mice (Jarick 2022) were generated by crossing *Nmur1*$^{iCre-T2A-GFP}$ mice to *Gata3*$^{flox/flox}$, a transcriptional differentiation factor for ILC2s (Hoyler et al. 2012). The purpose of using this alternative ILC2-deficient mouse strain was to confirm the previous results. I compared B1 cell populations from peritoneal and thoracic cavity of *Nmur1*$^{iCre+}$ *Gata3*$^{flox/flox}$ mice and *Gata3*$^{flox/flox}$ littermates by using flow cytometry. B1a and B1b cells were reduced in *Nmur1*$^{iCre+}$ *Gata3*$^{flox/flox}$ mice (Fig. 4), comparable to levels observed in *Nmur1*$^{iCre+}$ *Id2*$^{flox/flox}$ mice (Fig. 1).

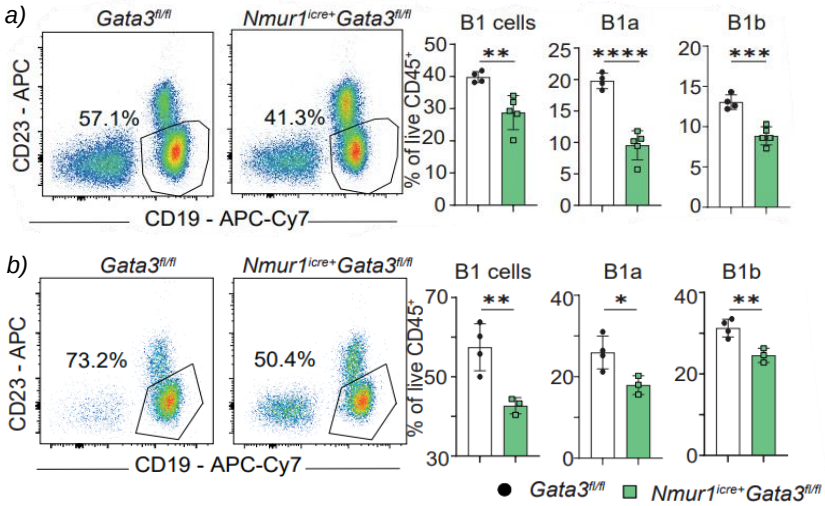

Figure 4: Reduction of B1 cells in Nmur1^{iCre+} Gata3$^{flox/flox}$ mice
*Flow cytometric plots and relative quantification of B1, B1a and B1b cells from a) peritoneal lavage and b) thoracic lavage of Nmur1^{iCre+} Gata3$^{flox/flox}$ mice and Gata3$^{flox/flox}$ littermates in steady state. B1 cells were gated as live CD45$^+$, TCRβ$^-$, CD23$^-$ and CD19$^+$ cells. Each symbol represents data from one mouse, data are representative of at least two experiments with three or more mice per group. Mean +/-SD, Student's t-test,** p<0.01, *** p<0.001, **** p<0.0001*

3.2 IL-5rα$^+$ B1 cells require ILC2 signals for survival and proliferation

3.2.1 Genes expressed by B1 cells are affected in the absence of ILC2s

To dissect the ILC2-mediated effects on different populations of B cells, peritoneal IgM$^+$ and IgD$^+$ cells from *Nmur1*$^{iCre+}$ *Id2*$^{flox/flox}$ and littermate *Id2*$^{flox/flox}$ mice were sorted, and single-cell RNA sequencing was performed. The plots in Fig. 5, illustrating the sorting strategy, revealed a visible reduction of B1 cells (IgM$^+$, IgD^{low}) in ILC2 KO mice, consistent with previous findings from the flow cytometry experiments.

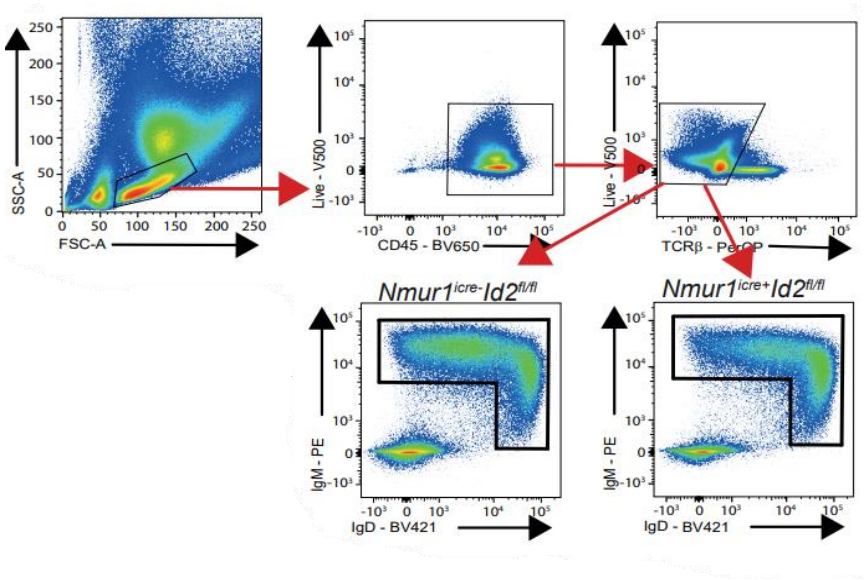

Figure 6: Sorting strategy for scRNA-seq
Sorting strategy of B cells from peritoneal cavity of Nmur1^{iCre+} Id2$^{flox/flox}$ mice and littermate Nmur1^{iCre-} Id2$^{flox/flox}$ mice in steady state for scRNA-seq. 5 mice per genotype were pooled for the sequencing. B cells were gated as live CD45^{+}, TCRβ^{-}, IgM^{+}, IgD^{+} cells.

In line with the flow cytometry results, markers expressed by B1 cells, such as CD11b and CD5 (Baumgarth 2016), were underrepresented in *Nmur1*$^{iCre+}$ *Id2*$^{fl/fl}$ mice, compared to controls, whereas CD19 and B220, which serve as general B cell markers (Baumgarth 2011; Cuenca et al. 2016), were equally detected in both groups (Fig. 6).

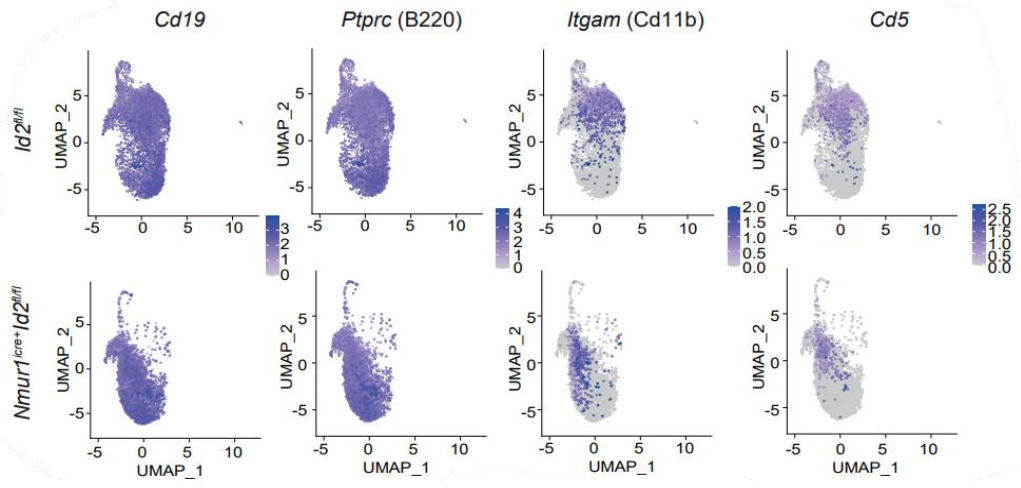

Figure 5: B1 cell markers are underrepresented in ILC2-deficient mice
UMAPs of general B cell surface markers (CD19, Ptprc = B220) and B1 cell surface markers (Itgam = CD11b and CD5) of flow-sorted and sequenced peritoneal cavity B cells of Nmur1^{iCre+} Id2$^{flox/flox}$ mice and Id2$^{flox/flox}$ littermate controls.

Unbiased clustering of detected single-cell transcriptomes revealed 5 clusters (Fig. 7a). Clusters 0-3 were identified as B1 cells and clusters 4-5 as B2 cells, defined by genes that identify B1 and B2 cells (Fig. 7b). Genes expressed by B1 cells are *Pepd*, *Itgb1*, *Tyrobp*, *IL-18*, *ASS1*, *Anxa2*, *CD9*, *CD5*, *Rassf4*, *Plac8*, *Pdlim1*, *S100a4*, *S100a6*, *Itgam*, *Zbtb32*, *Tubb6* (Luo et al. 2022; Mabbott and Gray 2014; Francés et al. 2007). To this plot *Ccnd2*, *Zcwpw1*, *Nme2*, *Lgals1*, *Uchl3*, *Dnajc7*, *Dok3*, *Sp140* were added. These genes are not yet related to B1 cells, but the sequencing data revealed that they are relatively high expressed in B1 cells compared to B2 cells. B2 cell clusters were defined by their high expression of *Fcer2a* (Best Cory G. 1995) and *Ptprc* (Baumgarth 2016). In addition, *Pgap1*, *CD69*, *Ccr7*, *Laptm5* and *Serpinb1a* showed a relatively high expression in B2 cells. B1 cell clusters showed a noticeable decrease in ILC2-deficient mice, especially clusters 0 and 2, whereas B2 cell clusters appeared to be unaffected (Fig. 7a). Cluster 0, defined by the expression of proliferation markers like *Mki67* (referred to UMAPs of Fig. 10), represented proliferative B1 cells. This cluster was barely present in ILC2-deficient mice, therefore, proliferation of B1 cells is obviously affected in the absence of ILC2s. Cluster 2 was defined by genes like *Zcwpw1*, *ASS1*, *S100a6*, *Ccnd2*, *Anxa2*, *Uchl3*. Except of cluster 0 representing the proliferating cells, differences between the other clusters, such as in function or development, could not be identified.

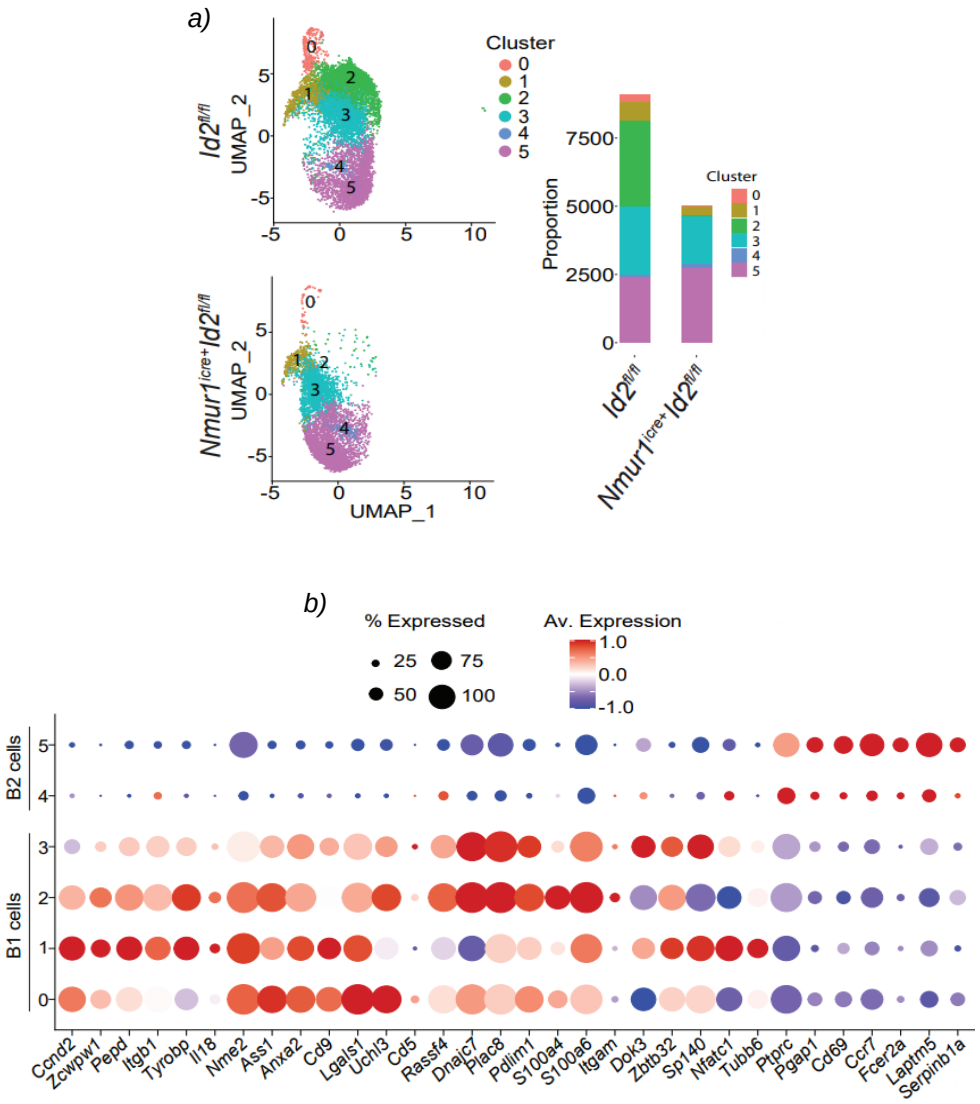

Figure 7: B1 cell clusters are underrepresented in ILC2-deficient mice
a) Clustering of flow-sorted and sequenced peritoneal cavity B cells of Nmur1^{iCre+} Id2$^{flox/flox}$ and Id2$^{flox/flox}$ mice in steady state at resolution of 0.3 and proportions of clusters. b) B1 and B2 cell specific genes (39, 41–44) in each cluster were plotted according to their expression and to the percentage of expression.

Differential expression of top 30 genes (Fig. 8a) in B1 cells revealed downregulation of several genes in ILC2-deficient mice, which play pivotal roles in B1 cell function and survival. E.g., *Ccnd2* is a cell cycle gene, *ASS1* is involved in protein metabolism, *Anxa2* in regulation of cellular growth and signal transduction pathways, *Itgb1* and *Lmo7* in cytoskeletal regulation, *S100a6* and *S100a4* in calcium regulation (Mabbott and Gray 2014; Luo et al. 2022; Francés et al. 2007). Among those genes top DEG *Zcwpw1*, *Ccnd2*, *ASS1* and *Anxa* 2 (Fig. 8b) were validated in peritoneal sort-purified B1 cells by

qPCR (Fig. 8c). The results revealed a notable decrease in their expression levels in ILC2-deficient mice.

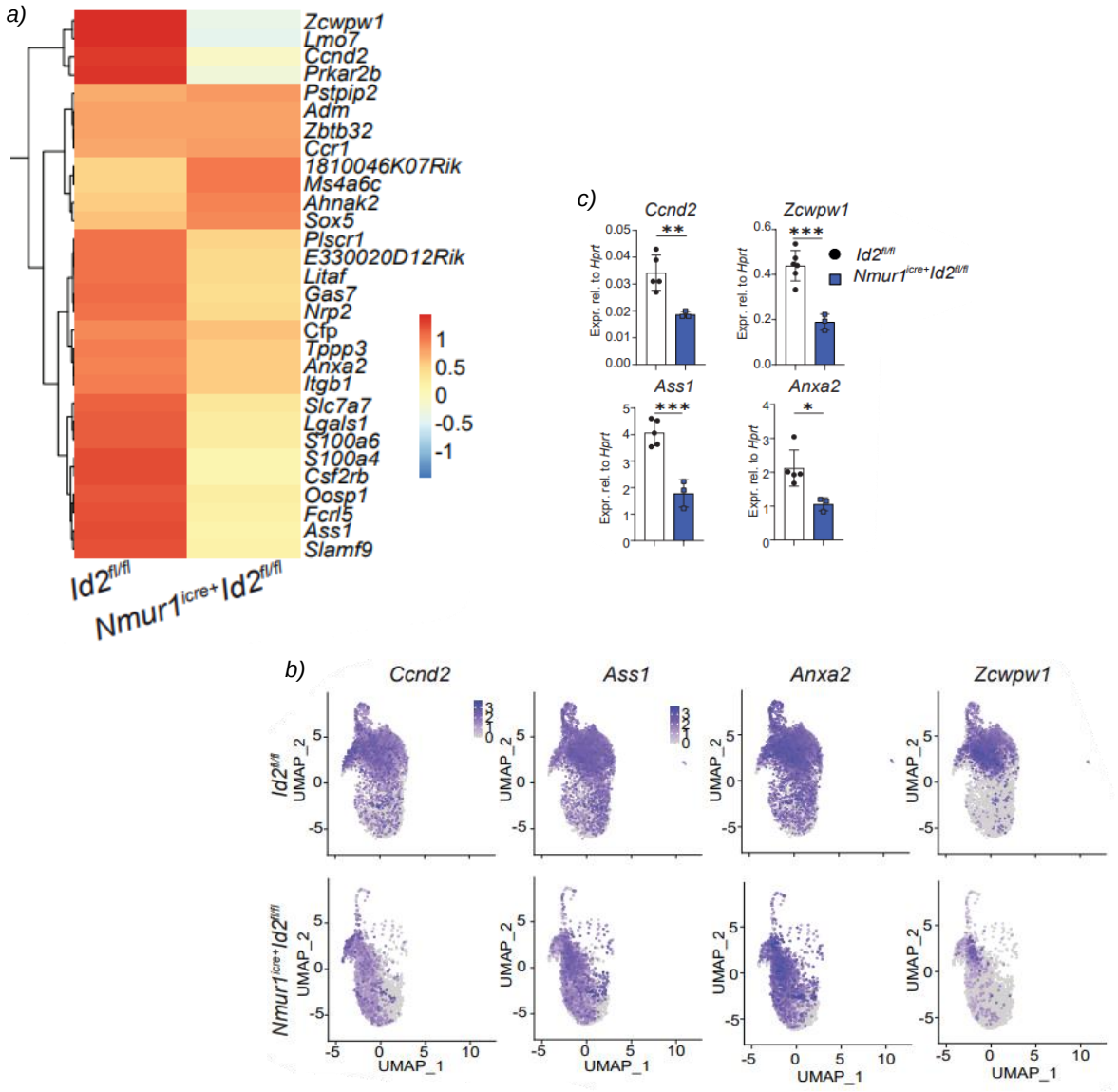

Figure 8: B1 cell index genes are downregulated in the absence of ILC2s

a) *Heatmap of top 30 differentially expressed genes in peritoneal cavity B1 cells of Nmur1^{iCre+} Id2$^{fl/fl}$ vs. littermate Id2$^{fl/fl}$ mice (data from scRNA-sequ.). b) UMAPs of Ccnd2, Ass1, Anxa2, Zcwpw1 of peritoneal cavity B cells of Nmur1^{iCre+} Id2$^{fl/fl}$ and littermate Id2$^{fl/fl}$ mice (data from scRNA-sequ.). c) Validation (by qPCR) of expression of Ccnd2, Ass1, Anxa2, Zcwpw1 in sort-purified peritoneal cavity B1 cells of Nmur1^{iCre+} Id2$^{fl/fl}$ vs. littermate Id2$^{fl/fl}$ mice. For c) applies: Each symbol represents data from one mouse, data are representative of at least two experiments with three or more mice per group. Mean +/-SD, Student's t-test, ** p<0.01, *** p<0.001, **** p<0.0001.*

3.2.2 B1 cells fail to proliferate in the absence of ILC2s

B1 cells in ILC2 KO mice exhibited impaired proliferation, as indicated by the underrepresentation of cluster 0, the proliferative B1 cell cluster (Fig. 7a). Essential pathways associated with cell division, including chromosome segregation, nuclear division, DNA replication, and progression through the mitotic cell cycle phases, were markedly downregulated in B1 cells of *Nmur1*$^{iCre+}$ *Id2*$^{fl/fl}$ mice compared to their *Id2*$^{fl/fl}$ littermate controls (Fig. 9). The limited proliferation of B1 cells in the absence of ILC2s is reflected in the diminished expression of *MKI67*, a widely recognized marker for proliferation (Fig. 10a). Through flow cytometry experiments, this outcome was not only verified in the peritoneal cavity but also across various examined organs. (Fig. 10b).

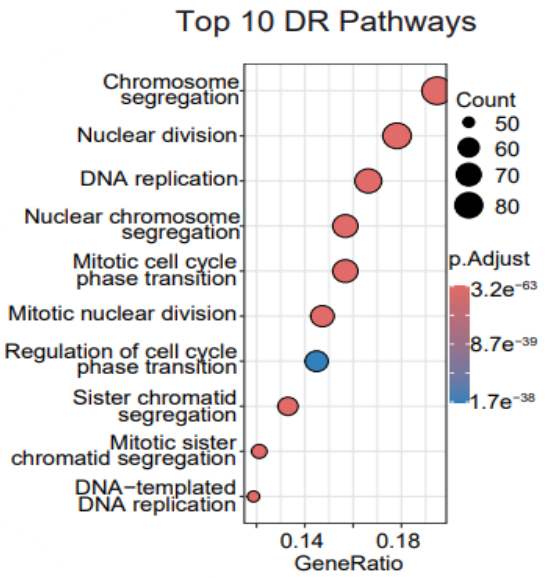

Figure 9: Proliferation pathways of B1 cells are affected in ILC2-deficient mice

Top 10 differentially regulated pathways in Cluster 0 (from Fig. 7a)

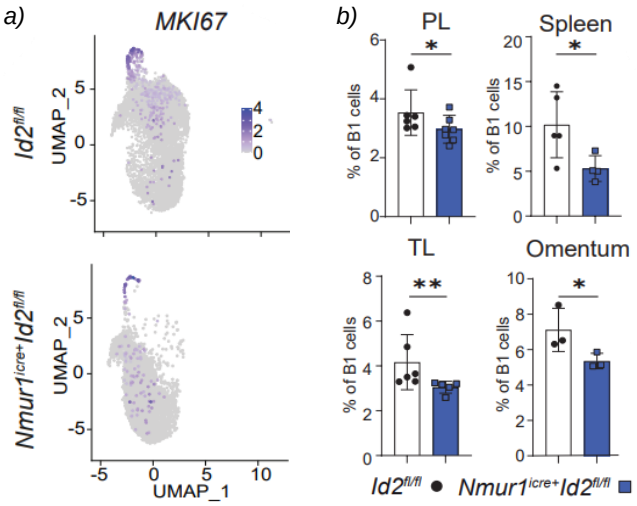

Figure 10: Proliferation is impaired in B1 cells without ILC2 signalling

*a) UMAPs of MKI67 of peritoneal cavity B cells of Nmur1^{iCre+} Id2$^{flox/flox}$ mice and Id2$^{flox/flox}$ littermate controls (data from scRNA-sequ.). b) Flow cytometric relative quantification of Ki-67^{+} B1 cells from peritoneal lavage (PL), thoracic lavage (TL), spleen, omentum of Nmur1^{iCre+} Id2$^{flox/flox}$ mice and Id2$^{flox/flox}$ littermate controls. For b) applies: Each symbol represents data from one mouse, data are representative of at least two experiments with three or more mice per group. Mean +/-SD, Student's t-test,** p<0.01, *** p<0.001, **** p<0.0001.*

3.2.3 Expression of IL-5 induced genes in IL-5rα^{+} B1 cells are reduced in the absence of ILC2s

To delineate the mechanisms of the responsible cytokine-cytokine-receptor signalling cascade, the expression of cytokine-receptors in B1 and B2 cells were identified. B1 cells expressed several receptors of the innate immune system, like Il5rα, Il6st, Il4rα (Fig. 11).

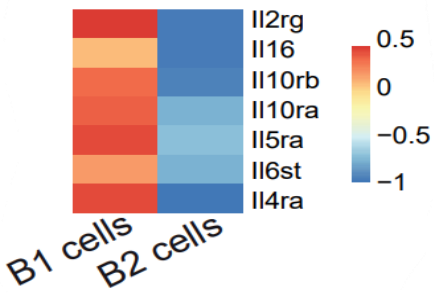

Figure 11: B1 cells express receptors of innate immune system

Expression of cytokine-receptors on B1 and B2 cells from peritoneal cavity of ILC2-sufficient mice (data from scRNA-sequ.).

Studies have suggested that B1 cells rely on IL-5 for their survival, with B1 cell populations being diminished in *Il5*$^{-/-}$ (Kopf et al. 1996) and *Il5rα*$^{-/-}$ mice (Yoshida et al. 1996; Moon et al. 2004; Hiroi et al. 1999). Given the significance of IL5rα-signals and the decrease in IL5rα^{+} B1 cells in ILC2-deficient mice (Figure 12a), an analysis of published IL-5-dependent gene sets (Horikawa and Takatsu 2006) using the single-cell sequencing data was conducted (Fig. 12b). Consistently, with IL-5 acting as a crucial downstream signal, most known IL-5-dependent genes were found to be downregulated in B1 cells of *Nmur1*$^{iCre+}$ *Id2*$^{fl/fl}$ mice, with no expression detected in B2 cells (Fig. 12b).

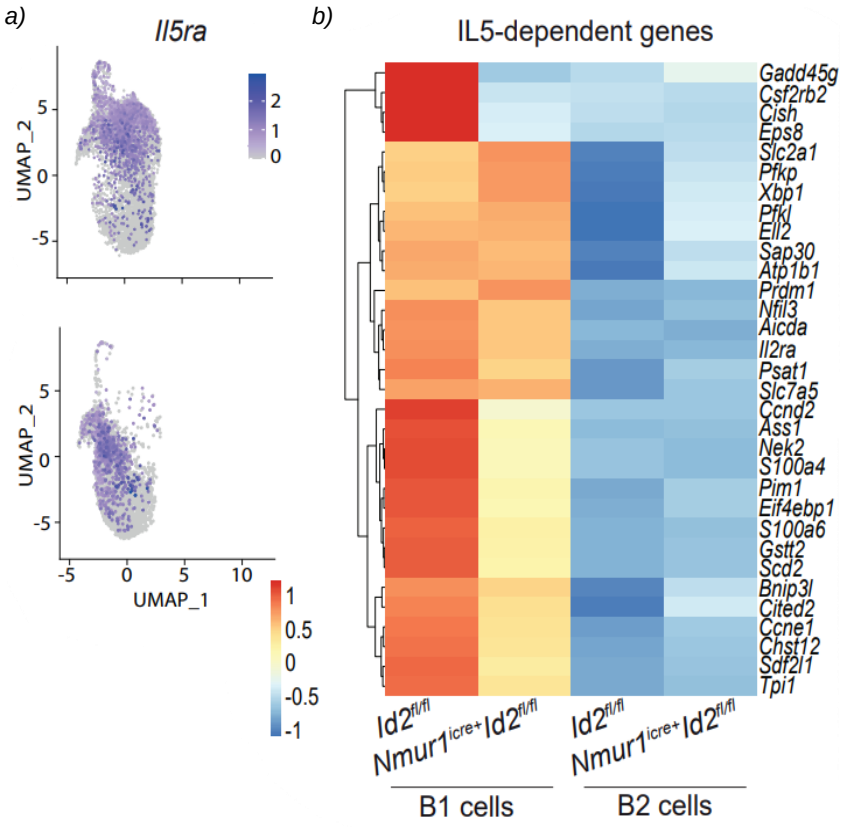

Figure 12: IL-5 dependent genes are downregulated in B1 cells in ILC2 KO mice

a) UMAPs of IL5rα^{+} peritoneal cavity B cells of Nmur1^{iCre+}Id2$^{fl/fl}$ and littermate Id2$^{flox/flox}$ mice in steady state (data from scRNA-sequ.). b) Differentially regulated gene expression of reported IL5 induced genes (Horikawa and Takatsu 2006) in B1 and B2 cells from peritoneal cavity of Nmur1^{iCre+}Id2$^{fl/fl}$ and littermate Id2$^{flox/flox}$ mice at steady state (data from scRNA-sequ.).

While some IL-5-induced genes could be linked to B1 cells and B1 cell functions, such as *ASS1*, *Anxa2*, *S100a6*, and *S100a4* (Luo et al. 2022; Mabbott and Gray 2014), others remained uncharacterized within B1 cells, but are known to encompass diverse processes essential for proper cell function: cell cycle (*Ccnd2*, *Ccne1*, *Gadd45g*), metabolism (*CHST12*, *GSTT2*, *PFKL*, *PFKP*, *PSAT1*, *SCD2*, *TPI1*, *ASS1*,*TPI1*), signal transduction (*S100a6*, *Cish*, *Eps8*, *Nek2*, *PIM1*, *S100a4*, *Rras2*, *Pim1*,*Pkib*), transcription activation (*Cited2*, *ELL2*, *PRDM1*, *XBP1*, *Nfil3*, *Sap30*, *Xbp1*), translation (*Eif4ebp1*) (Horikawa and Takatsu 2006). It was possible to replicate the decrease in expression of key genes like *Ass1*, *Anxa2*, *Ccnd2,* and *Zcwpw1* through qPCR in sort-purified B1 cells from peritoneal cavity of *Il5*$^{-/-}$ mice (Fig. 13), with comparable levels observed in *Nmur1*$^{iCre+}$ *Id2*$^{fl/fl}$ mice (Fig. 8c), indicating a common downstream signalling pathway in ILC2-deficient and *Il5*$^{-/-}$ mice.

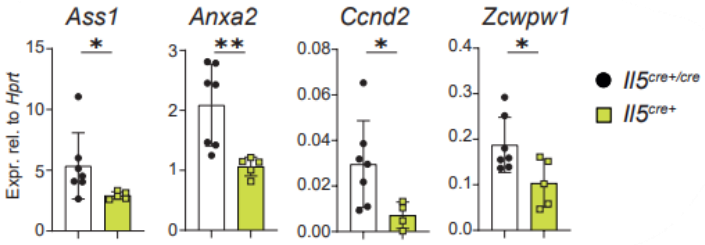

Figure 13: Validation of downregulated IL-5 dependent genes in Il5^{cre+} mice

*Peritoneal cavity B1 cells from Il5^{cre+} and littermate Il5$^{cre+/-}$ mice were sort-purified and qPCR was performed for indicated genes (Ass1, Anxa2, Ccnd2 and Zcwpw1). Each symbol represents data from one mouse, data are representative of at least two experiments with three or more mice per group. Mean +/-SD, Student's t-test, ** p<0.01, *** p<0.001, **** p<0.0001.*

Given that *Zcwpw1* emerged as the most significant differentially regulated gene in ILC2-deficient mice and was also affected in *Il5*$^{-/-}$ mice, the next step involved phenotyping *Zcwpw1*$^{-/-}$ mice, lacking gene expression of *Zcwpw1*, for B1 cells. However, no differences in relative and absolute cell numbers were observed when compared to

littermate control mice (Fig. 14). In summary, these findings suggest that B1 cells crucially rely on signals for survival and proliferation from ILC2s, likely mediated through the IL-5rα pathway.

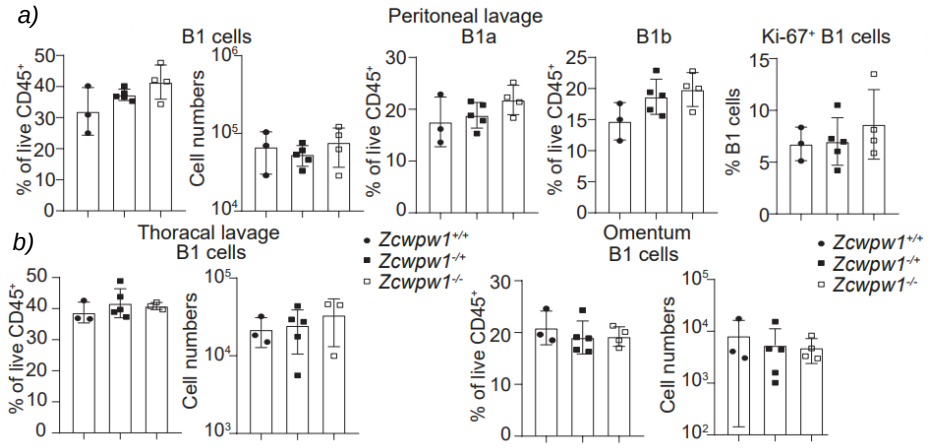

Figure 14: Survival of B1 cells is not dependent on ZCWPW1
a) *Flow cytometric relative and absolute quantification of B1, B1a, B1b cells and ki-67+ B1 cells from peritoneal lavage of littermate Zcwpw1-/-, Zcwpw1+/- and Zcwpw1+/+ mice at steady state. b) Flow cytometric relative and absolute quantification of B1 cells from thoracal lavage and omentum of littermate Zcwpw1-/-, Zcwpw1+/- and Zcwpw1+/+ mice at steady state. Each symbol represents data from one mouse, data are representative of at least two experiments with three or more mice per group. Mean +/-SD, Student's t-test, ** p<0.01, *** p<0.001, **** p<0.0001.*

3.3 B1 cells are dependent on ILC2-derived IL-5

3.3.1 B1 cells are reduced in the absence of ILC2-derived IL-5

The reduction of IL-5rα+ B1 cells and IL-5 target genes in B1 cells of ILC2-deficient mice (Fig. 12) strongly implies that ILC2s serve as an essential source of IL-5. To investigate further, *Il5*$^{-/-}$ mice were utilized and flow cytometry experiments were conducted to evaluate whether IL-5 influences the B1 cell phenotype. Indeed, in the absence of IL-5, B1 cells exhibited reductions in both relative and absolute cell numbers (Fig. 15) comparable to those observed in *Nmur1*$^{iCre+}$ *Id2*$^{flox/flox}$ and *Nmur1*$^{iCre+}$ *Gata3*$^{flox/flox}$ mice, thus confirming previous studies (Kopf et al. 1996; Bao et al. 1998; Hiroi et al. 1999; Moon et al. 2004; Yoshida et al. 1996).

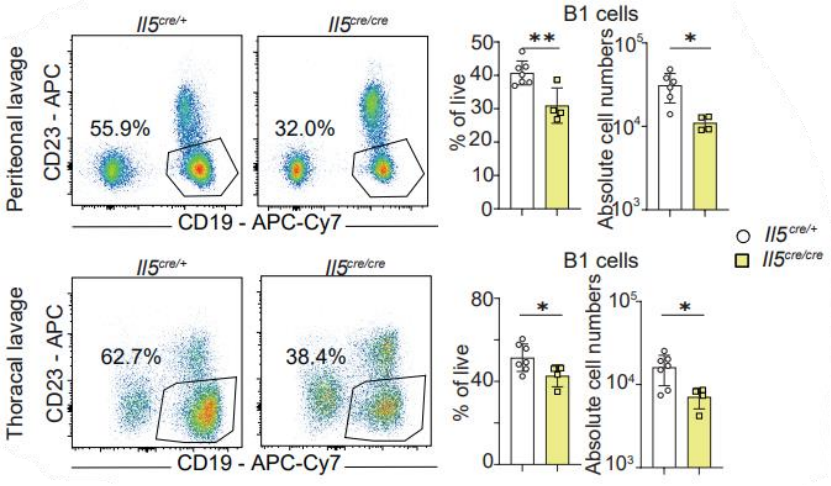

Figure 15: B1 cells are reduced in Il5$^{-/-}$ mice
*Flow cytometric plots and relative and absolute quantification of B1 cells of Il5^{cre+} mice and IL5$^{cre+/cre-}$ littermates in peritoneal and thoracal lavage. B1 cells were gated as live CD45^{+}, TCRβ^{-}, CD23^{-} and CD19^{+} cells. Each symbol represents data from one mouse, data are representative of at least two experiments with three or more mice per group. Mean +/-SD, Student's t-test,** p<0.01, *** p<0.001, **** p<0.0001.*

To directly assess the non-redundant role of ILC2s as a source of IL-5, my laboratory eventually managed to generate *Nmur1*$^{iCre+}$ *Il5*$^{fl/fl}$ mice to selectively delete *Il5* in ILC2s. Subsequently, B1 cells in *Nmur1*$^{iCre+}$ *Il5*$^{fl/fl}$ mice were examined using comparable flow cytometry readouts as in previous experiments. The analysis revealed reduced B1 cell populations in both relative and absolute cell numbers across all investigated organs: peritoneal and thoracic cavity and omentum (not shown) (Fig. 16). These findings aligned with the results obtained in ILC2-deficient (Fig. 1,2,3,4) and *Il5*$^{-/-}$ mice (Fig. 15). Therefore, these data from the conditional deletion of *Il5* in ILC2 support the model that ILC2s are essential producers of IL-5 for B1 cell survival, a function that cannot be compensated by other cell types.

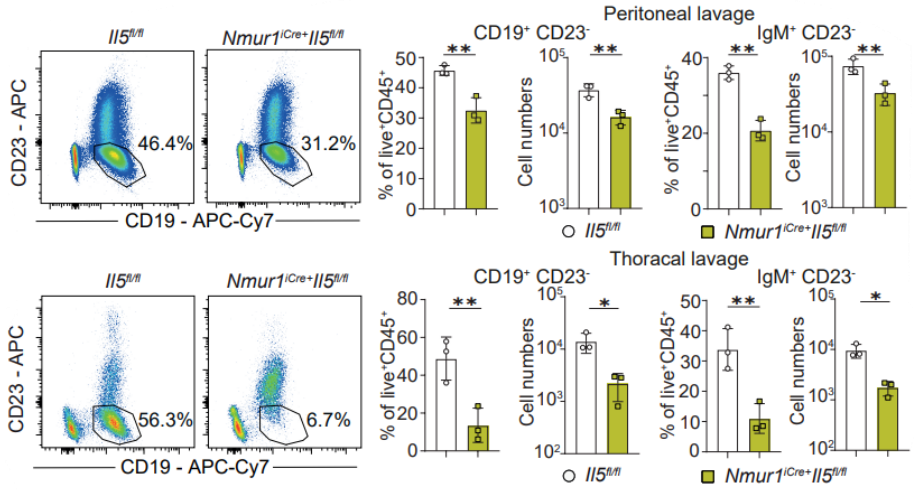

Figure 16: Conditional deletion of Il5 in ILC2s results in reduction of B1 cells

*Flow cytometric plots and relative and absolute quantification of B1 cells from peritoneal and thoracal lavage of Nmur1^{iCre+} Il5$^{flox/flox}$ mice and Il5$^{flox/flox}$ littermates. B1 cells were gated as live CD45^{+}, TCRβ^{-}, CD23^{-} and CD19^{+}/IgM^{+} cells. Each symbol represents data from one mouse, data are representative of at least two experiments with three or more mice per group. Mean +/-SD, Student's t-test,** p<0.01, *** p<0.001, **** p<0.0001.*

3.3.2 Exclusion of other ILC2 effector cytokines for B1 cell regulation

The sequencing data revealed the expression of receptors for IL-4 and IL-6 by B1 cells. Like IL-5, they are also ILC2 effector cytokines. Moreover, studies have suggested that the viability of B1 cells are reliant on IL-6 (Otero et al. 2006; Thies et al. 2013). Therefore, to assess whether the regulatory mechanisms governing B1 cells are solely reliant on IL-5 derived from ILC2s, independent of other ILC2-derived cytokines, various mouse models were employed with conditional deletions targeting key effector cytokines of ILC2. My laboratory achieved to specifically knock out in ILC2s the production of IL-4/IL-13 through *Nmur1*$^{iCre+}$*Il4/Il13*$^{fl/fl}$, AREG through *Nmur1*$^{iCre+}$*AREG*$^{fl/f}$ and IL-6 through *Nmur1*$^{iCre+}$*Il6*$^{fl/fl}$. No decrease in B1 cells was observed in peritoneal and thoracic lavage and omentum in these three mouse models (Fig. 17). These results strongly indicate that B1 cell viability relies exclusively on ILC2 derived IL-5, with no apparent involvement of other ILC2 effector cytokines.

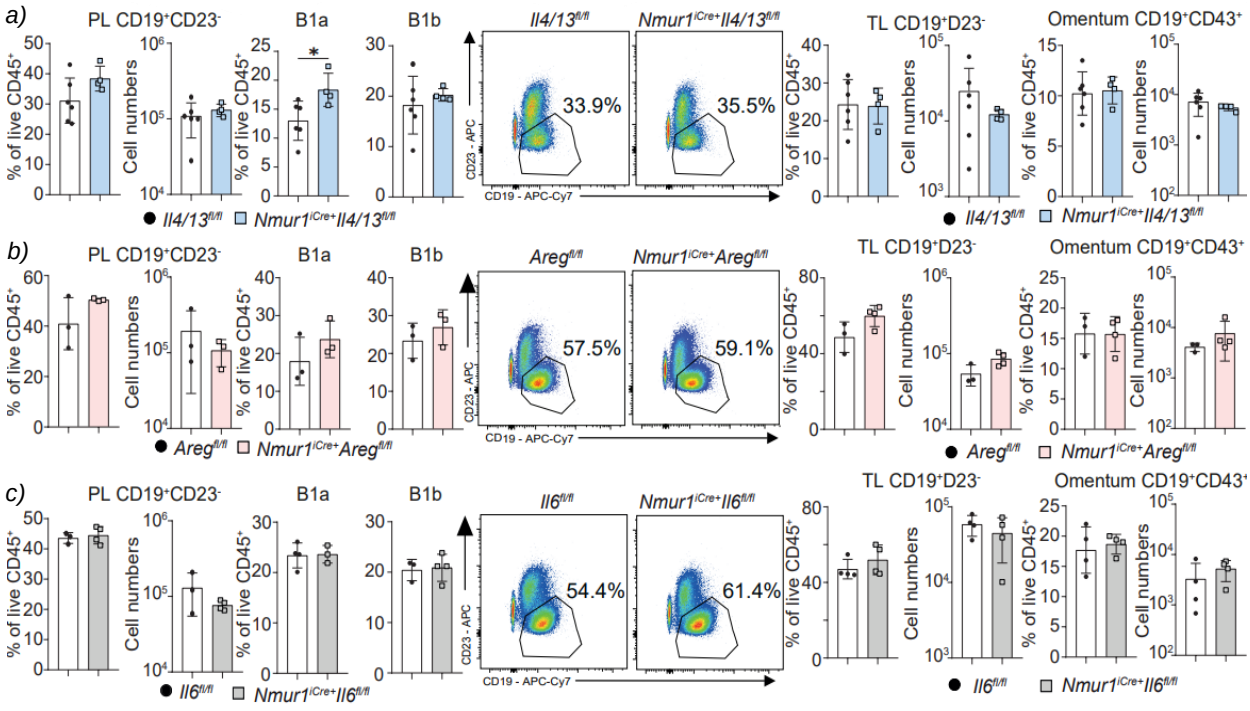

Figure 17: B1 cells are not dependent on ILC2-derived IL-13, AREG, and IL-6
*Flow cytometric plots of B1 cells (gated as live CD45$^+$, TCRβ$^-$, CD23$^-$, CD19$^+$ cells) from peritoneal lavage (PL) (middle plots) and flow cytometric relative and absolute quantification of B1 cells from peritoneal (PL), thoracic lavage (TL) and omentum and relative quantification of B1a and B1b cells from peritoneal cavity of a) Nmur1^{iCre+}Il4/Il13$^{fl/fl}$ mice and Il4/Il13$^{fl/fl}$ littermates, b) Nmur^{iCre+}AREG$^{fl/fl}$ mice and AREG$^{fl/fl}$ littermates, c) Nmur1^{iCre+}Il6$^{fl/fl}$ mice and Il6$^{fl/fl}$ littermates. Each symbol represents data from one mouse, data are representative of at least two experiments with three or more mice per group. Mean +/-SD, Student's t-test,** p<0.01, *** p<0.001, **** p<0.0001.*

3.4 B1 cells with PtC-specific BCRs are reduced in the absence of ILC2s

3.4.1 Validation of second single-cell RNA sequencing

B1 cell antibodies, targeting phosphatidylcholine (PtC), serve as a frontline defence against bacterial infections and bind to apoptotic cells. In adult mice, B1 cells predominantly express PtC-binding BCRs with specific heavy chain genes - *Ighv 11-2* and *Ighv 12-3* - and corresponding light chain genes - *Igkv 14-126* and *Igkv 4-91* (Luo et al. 2022; Worth et al. 2022). The nomenclature is based on the ImMunoGeneTics [IMGT] database (*IMGT Repertoire (IG and TR)*). The investigation aimed to understand how the BCR repertoire alters in the absence of ILC2s, particularly focusing on anti-PtC antibodies. To achieve this, a second round of single-cell RNA sequencing was

conducted, combined with BCR sequencing of peritoneal cavity B cells. The results displayed similar clustering and gene enrichment patterns (Fig. 18) compared to the previous sequencing experiment (Fig. 7). Genes such as *S100a4*, *Itgam*, *CD5*, *Ahnak2* (Mabbott and Gray 2014; Baumgarth 2016) identified clusters 0-3 as B1 cells, and *Fcer2a* (Best Cory G. 1995) distinguished clusters 4 and 5 as B2 cells (Fig. 18b). Cluster 0, characterized by the expression of proliferation-associated genes like *MKI67* and *Birc5*, was identified as the proliferating B1 cell cluster (Fig. 18b). Consistent with earlier findings, B1 cell clusters were notably underrepresented in ILC2 KO mice, especially clusters 0 and 2 (Fig. 18a).

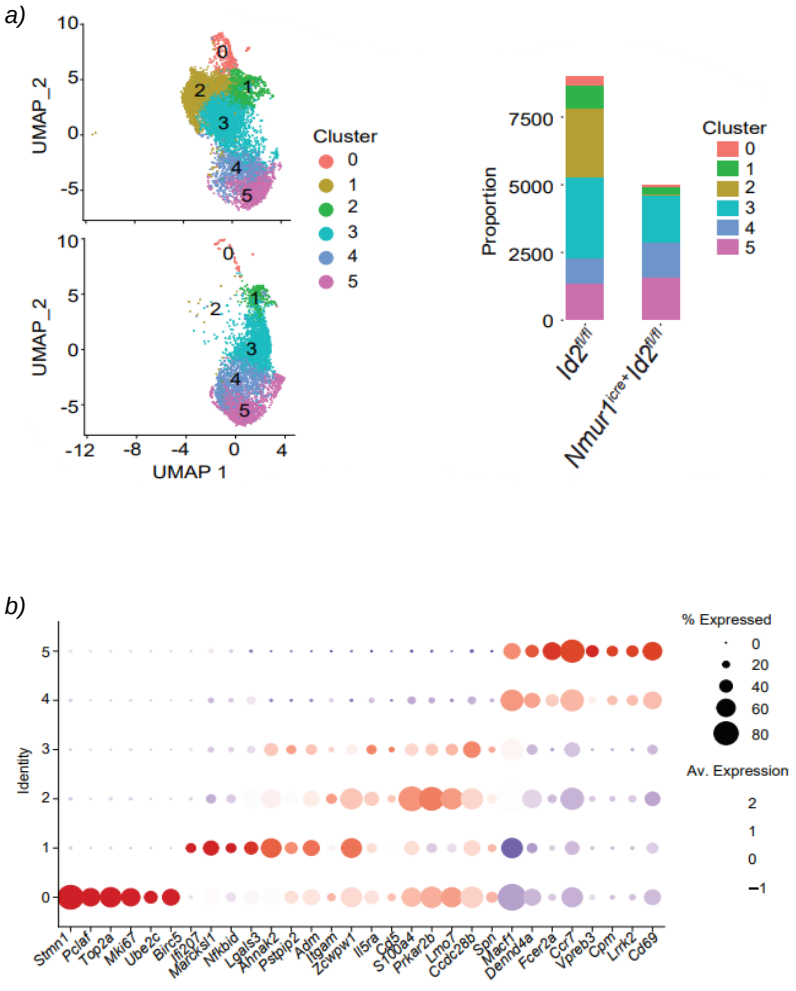

Figure 18: B1 cell clusters are underrepresented in ILC2 KO mice
a) Clustering of flow-sorted and sequenced peritoneal cavity B cells of Nmur1^{iCre+} Id2$^{flox/flox}$ mice and Id2$^{flox/flox}$ littermate controls in steady state (5 mice were pooled per genotype) at resolution of 0.3 and proportions of B1 cell clusters (0-3) and B2 cell clusters (4-5). b) Expression of B1 and B2 cell index genes of the 5 different clusters. Data from scRNA-sequ.

Furthermore, IL-5rα^{+} B1 cells were diminished (Fig. 19a), and the expression of *Il5*-induced genes (*Ccnd2*, *S100a4*, *S100a6*, *ASS1*, …) was reduced in B1 cells from *Nmur1*$^{iCre+}$ *Id2*$^{fl/fl}$ mice (Fig. 19b).

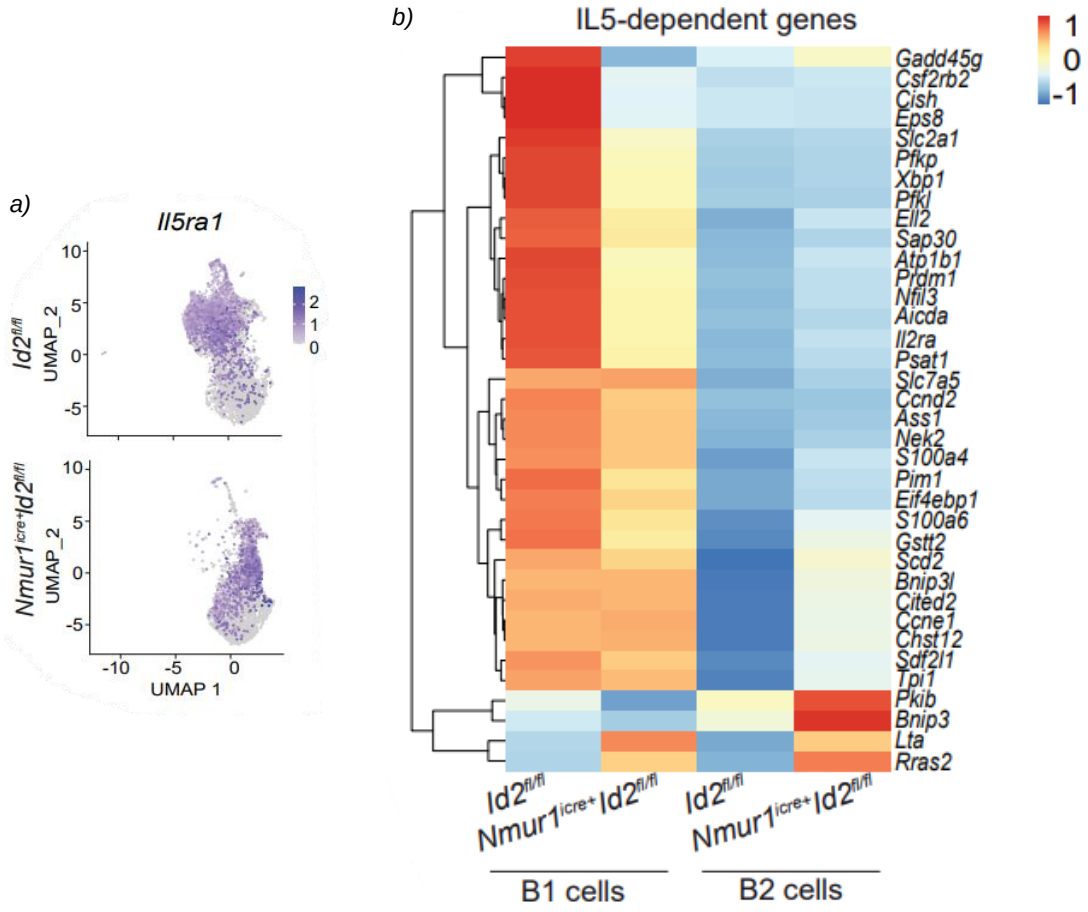

Figure 19: IL-5 induced genes in B1 cells are reduced in ILC2-deficient mice
a) UMAPs of IL5rα^{+} peritoneal cavity B cells of Nmur1^{iCre+}Id2$^{fl/fl}$ and littermate Id2$^{flox/flox}$ mice. b) Differentially regulated gene expression of reported IL5 induced genes (Horikawa and Takatsu 2006) in B1 and B2 cells from peritoneal cavity of Nmur1^{iCre+}Id2$^{fl/fl}$ and littermate Id2$^{flox/flox}$ mice. Data from scRNA-sequ.

3.4.2 ILC2s support the expansion of PtC-specific B1 cells

As expected, the expression of *Ighm* (IgM) was detected in all B cells, whereas *Ighd* (IgD) was restricted to B2 cells (Fig. 20a). In addition, B1 cell clusters were confirmed by their clear enrichment for V_H11-2 and V_H12-3 usage (Fig. 20b).

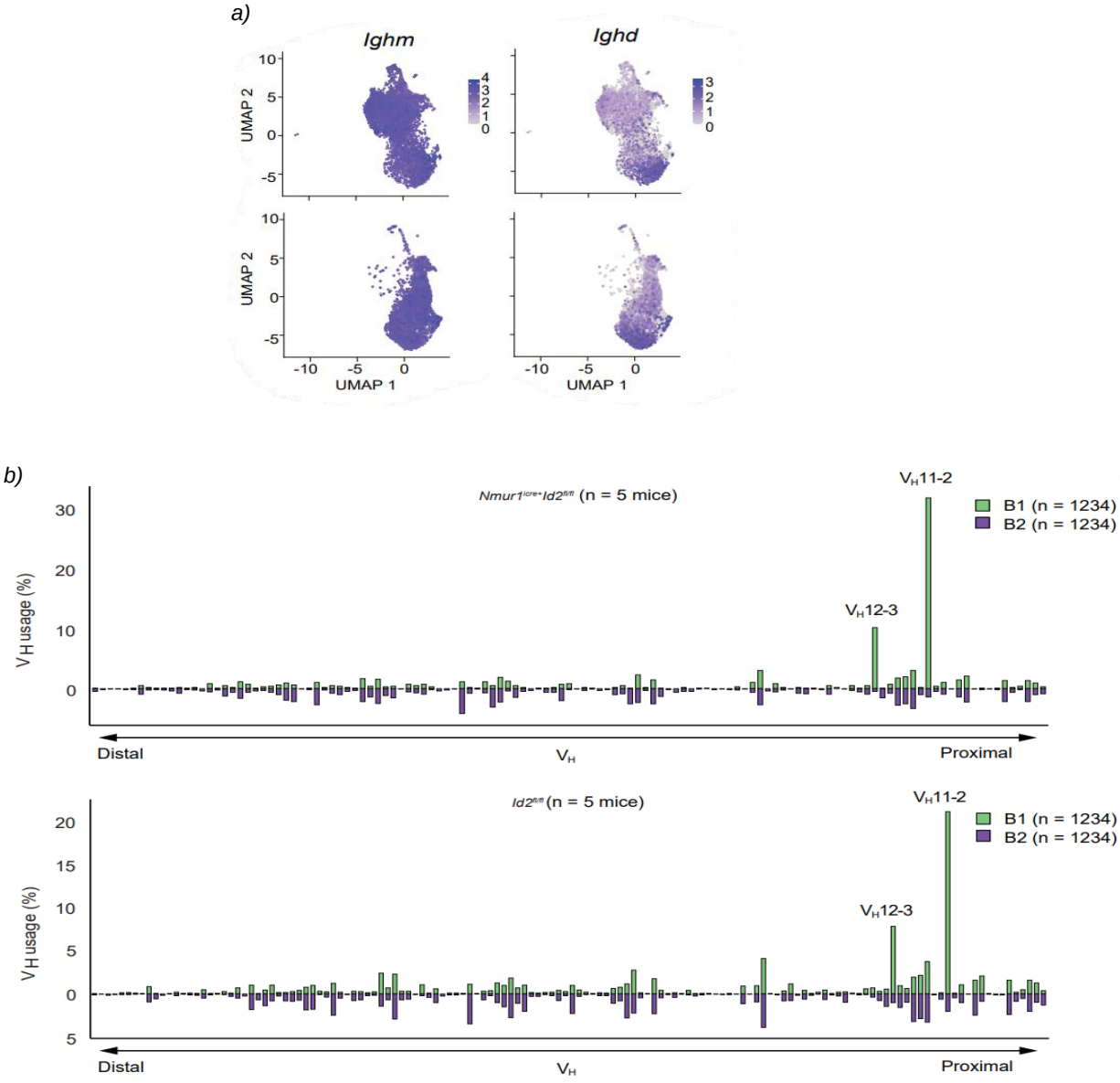

Figure 20: B1 cells mainly express IgM with enrichment for V_H11-2 and V_H12-3

a) UMAPs of heavy chains of IgM (Ighm) and IgD (Ighd) of peritoneal cavity B cells of Nmur1^{iCre+} Id2$^{flox/flox}$ mice and Id2$^{flox/flox}$ mice. b) Ighv usage of peritoneal cavity B1 and B2 cells of Nmur1^{iCre+} Id2$^{flox/flox}$ mice (upper panel) and littermate Id2$^{flox/flox}$ mice (lower panel). Ighv gene segments are ordered by their relative proximity to the D segments. Number of cells is depicted in the figure legend. Data from BCR-sequ.

Cells expressing *Ighv 11-2* / *Igkv 14-126* - and *Ighv 12-3* / *Igkv 4-91* - BCR combinations were notably reduced in B1 cells of *Nmur1*$^{iCre+}$ *Id2*$^{fl/fl}$ compared to control mice (Fig. 21a, b). Subsequently, the question arose whether this decrease in PtC-Ig gene transcripts stemmed from a reduction in PtC-reactive antibodies within the BCR repertoire or from the diminished number of B1 cells in ILC2-deficient mice. To address this, the cell quantities from both genotypes were aligned and their Ighv usage was compared.

Interestingly, no significant differences or shifts within the antibody repertoire was observed (Fig. 21c), when comparing *Nmur1*$^{iCre+}$ *Id2*$^{fl/fl}$ and control mice. Therefore, the data strongly support a non-redundant role for ILC2 in the development and maintenance of the B1 cell compartment and the overall levels of anti-Ptc antibodies, without impacting the specificity of the BCRs of B1 cells.

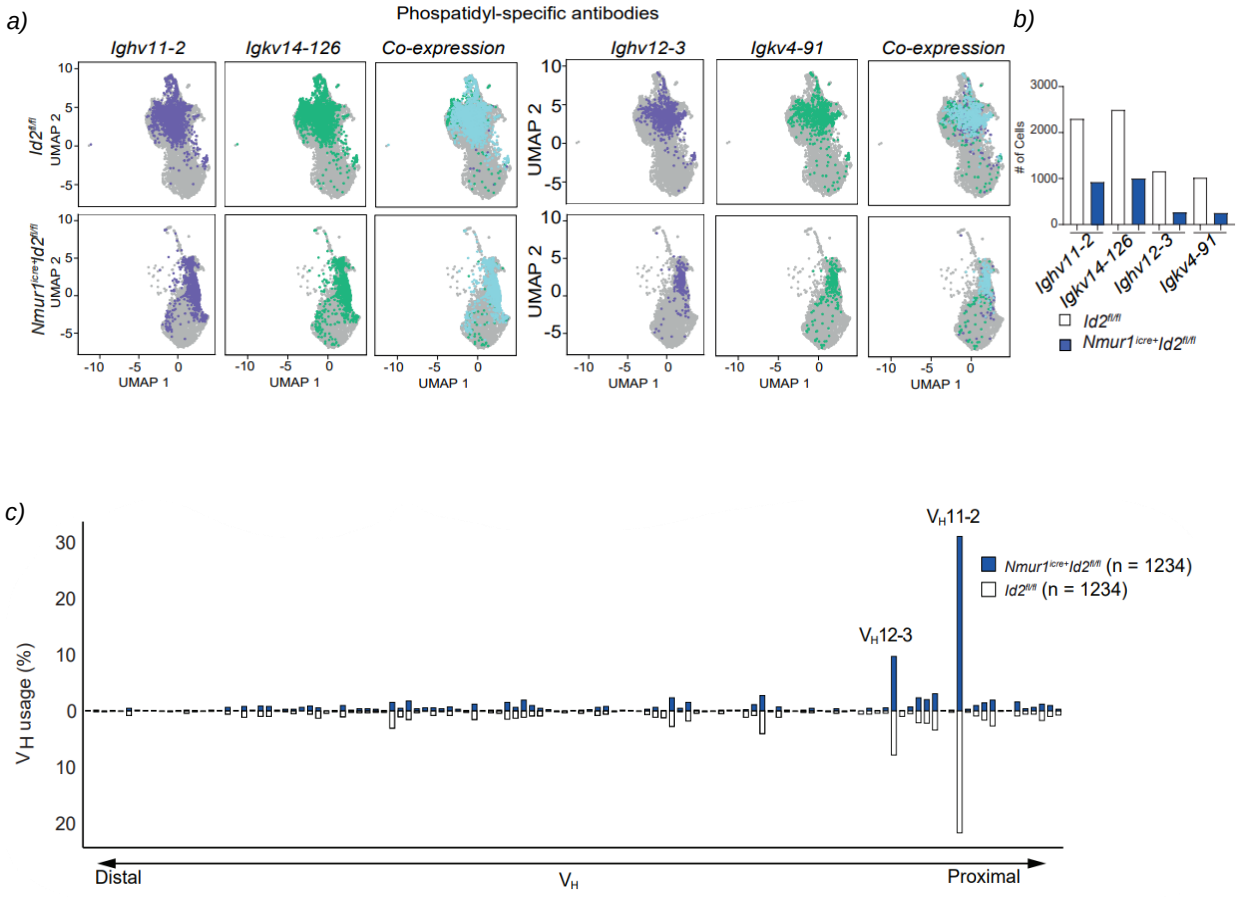

Figure 21: PtC-specific B1 cells are reduced in the absence of ILC2s
a) *UMAPs and b) quantification of PtC-specific heavy and corresponding light chains (Ighv 11-2 / Igkv 14-126 & Ighv 12-3 / Igkv 4-91) of peritoneal cavity B cells of Nmur1*$^{iCre+}$ *Id2*$^{flox/flox}$ *mice and littermate Id2*$^{flox/flox}$ *mice. c) Ighv usage in single B1 cells from Nmur1*$^{iCre+}$ *Id2*$^{flox/flox}$ *and littermate Id2*$^{flox/flox}$ *mice. Ighv gene segments are ordered by their relative proximity to the D segments. Number of cells is depicted in the figure legend. Data from BCR-sequ.*

3.5. IL-33 activates IL-5 production in ILC2s to support B1 cell survival

3.5.1 TSLP and IL-25 do not shape the B1 cell pool

Ultimately, the focus shifted towards understanding the upstream regulation of ILC2s, aiming to elucidate the significance of a particular ILC2-activating alarmin in relation to

B1 cell function. Numerous studies have indicated that alarmins such as IL-33, IL-25, and TSLP are not essential for the development of ILC2s but play a crucial role in their cytokine production. Consequently, diminished cytokine production has been observed in various strains and combinations of alarmin-deficient mice (Kabata et al. 2020; Fort et al. 2001; Klein Wolterink et al. 2012; Neill et al. 2010). To identify the upstream regulators necessary for stimulating IL-5 production in ILC2s to support B1 cells, knockout models targeting TSLP, IL-25, and IL-33 were employed. In papain-induced airway inflammation models, reductions of ILC2-derived IL-13 and IL-5 were observed in *Tslpr*$^{-/-}$ mice, deficient in the receptor for TSLP, indicating a significant role for TSLP in atopic diseases (Kabata et al. 2020; Klein Wolterink et al. 2012). However, *Tslpr*$^{-/-}$ mice clear *N. brasiliensis* and *H. polygyrus* infections as effectively as wild type mice, indicating compensatory mechanisms other than TSLP in orchestrating ILC2 response in worm infections (Massacand et al. 2009). The role of TSLP as a crucial activator of ILC2s to promote B1 cell survival has not been reported so far. In my flow cytometry experiments with *Tslpr*$^{-/-}$ mice no discernible difference was observed in the frequency and total numbers of B1 cells compared to *Tslpr*$^{+/+}$ controls across various organs (Fig. 22a). These findings suggest that TSLP does not play a significant role as an activating alarmin for ILC2s in regulating B1 cells. IL-25 and IL-33 were shown to be potent inducers of type 2 cytokines in models involving worm infections (Neill et al. 2010) and allergic diseases (Fort et al. 2001; Klein Wolterink et al. 2012). However, understanding of the IL-25/IL-33-ILC2-IL-5-B1 cell axis remained limited. Barbosa et al. (Barbosa et al. 2021) demonstrated that IL-25 induced secretion of IL-5 by ILC2s, promoting B1 cells to produce antibodies against *Francisella* LPS. To investigate whether IL-25 serves as the crucial activating alarmin for ILC2s to promote B1 cell survival, *Il17rb*$^{-/-}$ mice were used. This mouse strain lacks IL17RB, the receptor for IL-25. In steady state, no difference in

numbers and frequencies of B1, B1a and B1b cells was observed between *Il17rb*$^{-/-}$ and *Il17rb*$^{+/+}$ mice in various organs in flow cytometry experiments (Fig. 22b). This suggests that, at least in steady state conditions, ILC2s are not activated via IL-25 to promote survival of B1 cells.

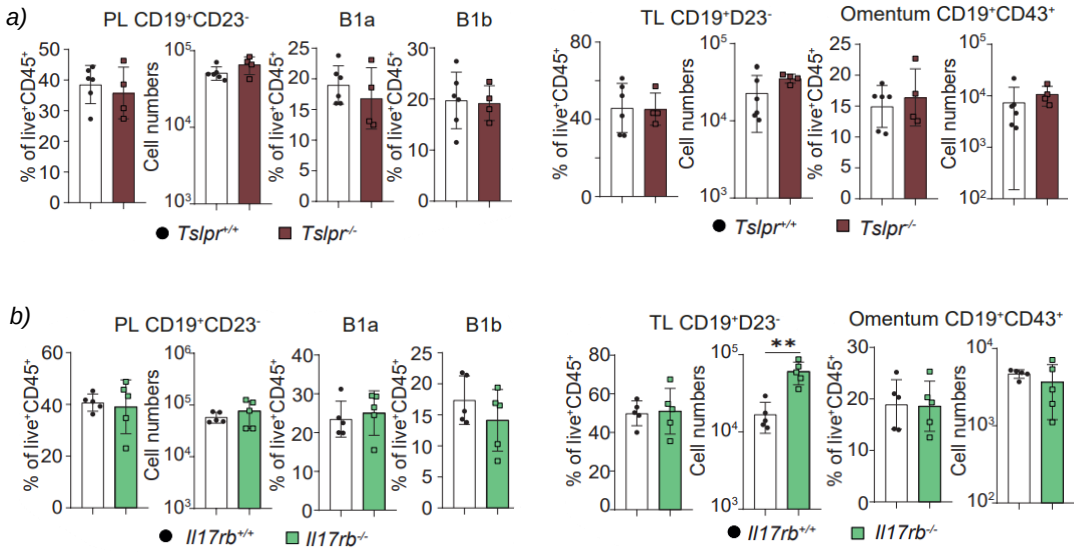

Figure 22: B1 cells are not reduced in the absence of TSLP and IL-25
*Flow-cytometric absolute and relative quantification of B1 cells, relative quantification of B1a and B1b cells from peritoneal lavage (PL), and absolute and relative quantification B1 cells from thoracic lavage (TL) and omentum of a) Tslpr $^{-/-}$ and Tslpr $^{+/+}$ mice and b) Il17rb $^{-/-}$ and Il17rb $^{+/+}$ mice in steady state. Each symbol represents data from one mouse, data are representative of at least two experiments with three or more mice per group. Mean +/-SD, Student's t-test,** p<0.01, *** p<0.001, **** p<0.0001.*

3.5.2 B1 cell survival promoted by ILC2s is dependent on IL-33

A reduction was observed in B1 cells in *Il33*$^{-/-}$ mice, deficient in IL-33 production, across various organs (Fig. 23a), suggesting that IL-33 serves as the predominant alarmin controlling IL-5 production in ILC2s under steady state conditions. Moreover, decreased expression of ki-67 was detected through flow cytometry in *Il33*$^{-/-}$ mice (Fig. 23b), mirroring similar findings in ILC2-deficient mice, indicating that IL-33 regulates the size of the B1 cell pool. However, considering that the IL-33 receptor is expressed by multiple cell types and direct effects of IL-33 on B1 cells independent of ILC2s have been reported previously (Komai-Koma et al. 2011), *Nmur1*$^{iCre\text{-}T2A\text{-}GFP}$ *St2*$^{flox/flox}$ mice, lacking ST2

exclusively on ILC2s (Topczewska et al. 2023), were used to investigate whether the observed B1 cell phenotype resulted from the absence of ILC2 activation by IL-33. Consistent with a cell-intrinsic requirement of ST2 in ILC2s, B1 cells were reduced in *Nmur1*$^{iCre\text{-}T2A\text{-}GFP}$ *St2*$^{flox/flox}$ mice (Fig. 23c). To assess the downstream effector cytokine response, ILC2s from small intestine of *Il33*$^{-/-}$ and *Il17rb*$^{-/-}$ mice were sort-purified and qPCR was performed. In line with the role of IL-33 in triggering IL-5 production in ILC2s, only ILC2s from *Il33*$^{-/-}$ mice, but not from *Il17rb*$^{-/-}$ mice, exhibited reduced *Il5* expression (Fig. 23d). From these findings, I concluded that ILC2s sense IL-33 directly to produce IL-5, thereby supporting B1 cell maintenance and survival.

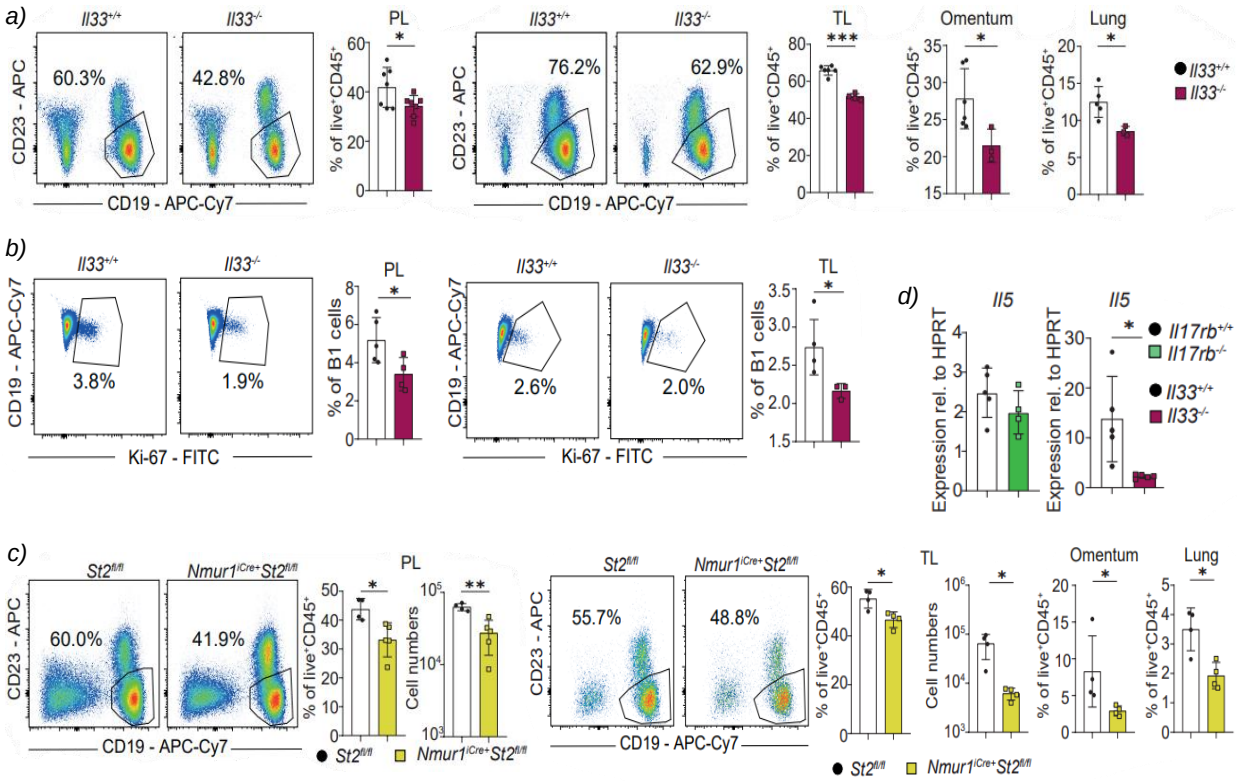

Figure 23: ST2-mediated activation of ILC2s is necessary for B1 cell survival

*a) Flow cytometric plots of B1 cells from peritoneal lavage (PL) and thoracic lavage (TL) and relative quantification of B1 cells from PL, TL, omentum, lung of Il33 $^{-/-}$ mice and Il33$^{+/+}$ controls in steady state. b) Flow cytometric plots and relative quantification of ki-67^{+} B1 cells from PL, TL of Il33 $^{-/-}$ mice and Il33$^{+/+}$ controls in steady state. c) Flow cytometric plots of B1 cells from PL and TL and relative and absolute quantification of B1 cells from PL, TL, and relative quantification of B1 cells from omentum and lung of Nmur1^{iCre+} St2$^{flox/flox}$ mice and St2$^{flox/flox}$ controls in steady state. d) qPCR of IL-5 of sort-purified small intestine ILC2s of Il33 $^{-/-}$ mice and Il33 $^{+/+}$ controls and Il17rb $^{-/-}$ mice and Il17rb$^{+/+}$ controls in steady state. Each symbol represents data from one mouse, data are representative of at least two experiments with three or more mice per group. Mean +/-SD, Student's t-test,** p<0.01, *** p<0.001, **** p<0.0001.*

4 Discussion

4.1 Summary of the results

B1 cells in *Nmur1*$^{iCre+}$ *Id2*$^{flox/flox}$ and *Nmur1*$^{iCre+}$ *Il5*$^{flox/flox}$ mice were drastically reduced, indicating that the survival and maintenance of B1 cells is dependent on ILC2-derived IL-5. This hypothesis is supported by the low expression of B1 cell identifying genes and IL-5 induced genes in B1 cells of ILC2-deficient mice which are pivotal for cell cycle and cell growth and are involved in metabolism, signalling, and maintaining the cell structure. In the absence of ILC2 signalling B1 cells are further affected in their function as PtC-specific antibody producer. Ultimately, IL-33 could be identified as the upstream regulator of IL-5 production by ILC2s, thereby promoting B1 cell proliferation.

4.2 Interpretation of the results

4.2.1 ILC2s are a non-redundant source of IL-5 for B1 cells

The extent to which the overlap in effector molecules among ILC2s and other immune cell populations determines their complementary and redundant functions constitutes a pivotal inquiry within contemporary immunological research. Challenges associated with precise genetic manipulation have hindered the empirical exploration of ILC redundancy. Despite the shared secretion of a spectrum of cytokines between ILCs and T cells, regulation, and kinetics of T cell and ILC responses may vary. Recent literature has provided compelling evidence underscoring the essential role of ILC2s in orchestrating type 2 immune responses across various genetic models (Gurram et al. 2023; Jarick 2022; Tsou et al. 2022). Investigations utilizing the ILC2-deficient mouse model *NMUR1*$^{iCre+}$ *Id2*$^{fl/fl}$ have revealed profound reductions in serum IL-5 levels and

eosinophils, a myeloid immune cell population which is reported to be reliant on IL-5 (Kopf et al. 1996; Nussbaum et al. 2013). Similar findings in *Il5*$^{-/-}$ mice (Jarick 2022; Foster et al. 1996; Kopf et al. 1996) corroborate the pivotal role of ILC2 as a major source for IL-5. My study delineates a novel facet of ILC2-mediated immune regulation by highlighting the indispensable role of ILC2s for facilitating the proper development and maintenance of B1 cells. While previous investigations have underscored the stimulatory effects of IL-5 and ILC2s on B1 cells *in vitro* (Moro et al. 2016; Drake et al. 2016), *in vivo* reliance of B1 cells on ILC2-derived IL-5 remained a subject to debate. By employing targeted deletion strategies, by utilizing *NMUR1*$^{iCre+}$ *Id2*$^{fl/fl}$ mice to specifically delete ILC2s and *NMUR1*$^{iCre+}$ *Il5*$^{fl/fl}$ mice to abrogate IL-5 production within ILC2s, this study conclusively demonstrated the non-redundant requirement of ILC2s as the primary source of IL-5 for B1 cell survival and function. Furthermore, the findings herein are reinforced by observations of T cell-independent homeostatic proliferation of B1 cells in *Rag2*$^{-/-}$ mice, which lack mature B2 and T cells, affirming the substantial involvement of non-T cell sources in IL-5 production to sustain B1 cell populations and antibody production (Moon et al. 2004). Nevertheless, diminished B1 cell populations persisted even in the absence of ILC2s and IL-5, suggesting compensation mechanisms by other stimuli than IL-5. Potential candidates include IL-6 (Thies et al. 2013; Chou et al. 2006) and / or IL-10 (O'Garra et al. 1992) which were reported to play pivotal roles in B1 cell activation and proliferation. These cytokines might partially substitute for the missing IL-5 influence.

4.2.2 IL-33 functions as a danger signal and induces a pathway of innate antibody production

The release of IL-33 and following induction of IL-5 secretion by ILC2s to activate B1 cells highlights an innate pathway for T cell-independent antibody production. It is well

established that T-cell dependent antibody production in follicles requires CD40L signalling which is crucial for B2 cell activation, survival, formation of germinal centres, isotype switching and affinity maturation (Foy et al. 1994a; Foy et al. 1994b; Foy et al. 1993; Danese et al. 2004; Xu et al. 1994; Banchereau et al. 1994; Kawabe et al. 1994). CD40L is transiently upregulated on activated $CD4^+$ T cells upon binding with their TCR to antigen peptides presented by APCs and following stimulation by APCs through co-stimulatory molecules and cytokine release (Armitage et al. 1992; Clark and Ledbetter 1994; Grewal and Flavell 1996; Grewal and Flavell 1997; Elgueta et al. 2009; Noelle et al. 1992). Initially, APCs bind to antigens through pattern recognition receptors (PRRs), which specifically recognize pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). Among the prominent families of PRRs are the TLRs (Suresh and Mosser 2013; Joffre et al. 2009; Broeke et al. 2013; Gaudino and Kumar 2019; Janeway and Medzhitov 2002; Matzinger 1994). Remarkably, PAMPs are danger signals similar to IL-33, and many TLRs (Creagh and O'Neill 2006; Verstak et al. 2009; Wicherska-Pawłowska et al. 2021; Suresh and Mosser 2013; Wesche et al. 1997; Muzio et al. 1997; Medzhitov et al. 1998) as well as the IL-33 receptor (Faas et al. 2021; Kroeger et al. 2009; Scalfone et al. 2013; Schmitz et al. 2005; Xu et al. 2018; Liew et al. 2016) utilize the signalling adapter Myd88 for signal transduction. Therefore, these findings provide further evidence for the concept that IL-33 functions as a danger-associated molecular pattern, coordinating an innate pathway to enhance antibody production through IL-5 activation in innate lymphocytes. This alternative innate pathway of antibody production assumes particular significance during infancy, when the adaptive immune system is still nascent and B2 cells are characterized by low-level expression of co-receptors, such as CD40, and therefore exhibit limited capacity for antibody affinity maturation. Consequently, they are not able to generate effective immune responses to

antigens (Simon et al. 2015; Kaur et al. 2007; Ridings et al. 1998). To compensate for the developing adaptive immune system, newborns rely on innate antibody production to defend against pathogens during this critical period.

4.2.3 Regulation of B1 cell antibody response by ILC2s in type 2 immunity

Regarding antibody production in type 2 immunity, Th2 cells drive the activation of B2 cells and subsequent IgE production via IL-4 (Finkelman et al. 1988; Pène et al. 1988; Corry and Kheradmand 1999). However, the involvement of ILC2s in innate antibody production by B1 cells in type 2 immune responses warrants further investigations. Drake et al. (Drake et al. 2016) demonstrated *in vitro* that ILC2s exerted a regulatory influence on IgE generation by B1 and B2 cells. Furthermore, in a recent symposium, Kazuyo Moro presented unpublished findings indicating that ILC2s regulate IgE secretion by B1 cells through IL-4 signalling (Moro 2019). Another study by Martin et al. (Martin et al. 2018) reported significant IgE production by B1 cells under steady state conditions and following helminth infection, in the absence of B2 cells. Notably, this study proposed a T cell-dependent mechanism involving CD40L and IL-4 for IgE production by B1 cells, with IL-5 further augmenting IgE production and B1 cell proliferation. Moreover, IL-25 treatment notably augmented IgE synthesis by B1 cells from *N. brasiliensis*-infected mice, highlighting the importance of this alarmin in promoting cytokine production by ILC2s and Th2 cells during helminth infection (Moltke et al. 2016). In contrast, IL-33 injection did not elicit a similar increase in IgE production by B1 cells (Martin et al. 2018). Additionally, a study investigating lung inflammation revealed that ILC2s induced IgM secretion by B1 and B2 cells in the thoracic cavity in response to damage signals triggered by fungal allergens and nematodes. It was shown that this process was dependent on IL-33 receptor signalling and ILC2-derived IL-5 (Jackson-Jones et al. 2016). Overall, the precise regulation and role of B1 cells in type 2 immunity alongside B2 cells necessitates

further elucidation. While IL-5 appears to enhance B1 cell-derived IgM production, both IL-4 and IL-5 emerge as potential key cytokines in promoting IgE release by B1 cells. Martin et al. proposed a T cell-dependent mechanism for IgE production by B1 cells (Martin et al. 2018). Indeed, Oliveira et al. showed that IgM and IgG3 were produced by B1 cells in the absence of T cells whereas generation of IgE by B1 cells could be both, T cell-dependent or -independent (Oliveira et al. 2005). However, a separate study showed that B1 cells exhibited limited proliferation and antibody response to T cell stimulation via CD40L (Erickson et al. 2001). To elucidate the contributions of ILC2s, Th2 cells and specific type 2 cytokines to promoting B1 cell antibody generation in type 2 immunity, particularly IgE production, further investigations are necessary.

5 Conclusions

This thesis provides a valuable insight in the non-redundant role of ILC2s in regulating B1 cells. The study contributes to future research exploring potential therapeutic interventions that target the ILC2 - B1 cell axis to maintain tissue homeostasis and regulate type 2 inflammation.

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Eidesstattliche Versicherung

„Ich, Karoline Florentine Troch, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Aufdeckung einer entscheidenden Rolle von ILC2s in der Regulierung von B1 Zellen durch IL-5“ / „Unveilling a crucial role for lLC2s in B1 cell regulation via IL-5“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren/innen beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) werden von mir verantwortet.

Ich versichere ferner, dass ich die in Zusammenarbeit mit anderen Personen generierten Daten, Datenauswertungen und Schlussfolgerungen korrekt gekennzeichnet und meinen eigenen Beitrag sowie die Beiträge anderer Personen korrekt kenntlich gemacht habe (siehe Anteilserklärung). Texte oder Textteile, die gemeinsam mit anderen erstellt oder verwendet wurden, habe ich korrekt kenntlich gemacht.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Erstbetreuer/in, angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.og) zur Autorenschaft eingehalten. Ich erkläre ferner, dass ich mich zur Einhaltung der Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis verpflichte.

Weiterhin versichere ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits an einer anderen Fakultät eingereicht habe.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§§156, 161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

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Datum Unterschrift

Lebenslauf

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

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Bescheinigung des akkreditierten Statistikers

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Bescheinigung

Hiermit bescheinige ich, dass Frau Karoline Troch innerhalb der Service Unit Biometrie des Instituts für Biometrie und klinische Epidemiologie (iBikE) bei mir eine statistische Beratung zu einem Promotionsvorhaben wahrgenommen hat. Folgende Beratungstermine wurden wahrgenommen:

- *Termin 1: 16.01.2024*

Folgende wesentliche Ratschläge hinsichtlich einer sinnvollen Auswertung und Interpretation der Daten wurden während der Beratung erteilt:

- *Angabe der Zellhäufigkeiten als absolute Häufigkeiten und Anteile in Prozent.*
- *Darstellung als Boxplots (mit Median und Quartilen)*
- *Gruppenvergleich mit Mann-Whitney Tests und T-Test.*

Diese Bescheinigung garantiert nicht die richtige Umsetzung der in der Beratung gemachten Vorschläge, die korrekte Durchführung der empfohlenen statistischen Verfahren und die richtige Darstellung und Interpretation der Ergebnisse. Die Verantwortung hierfür obliegt allein der Promovierenden. Das Institut für Biometrie und klinische Epidemiologie übernimmt hierfür keine Haftung.

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