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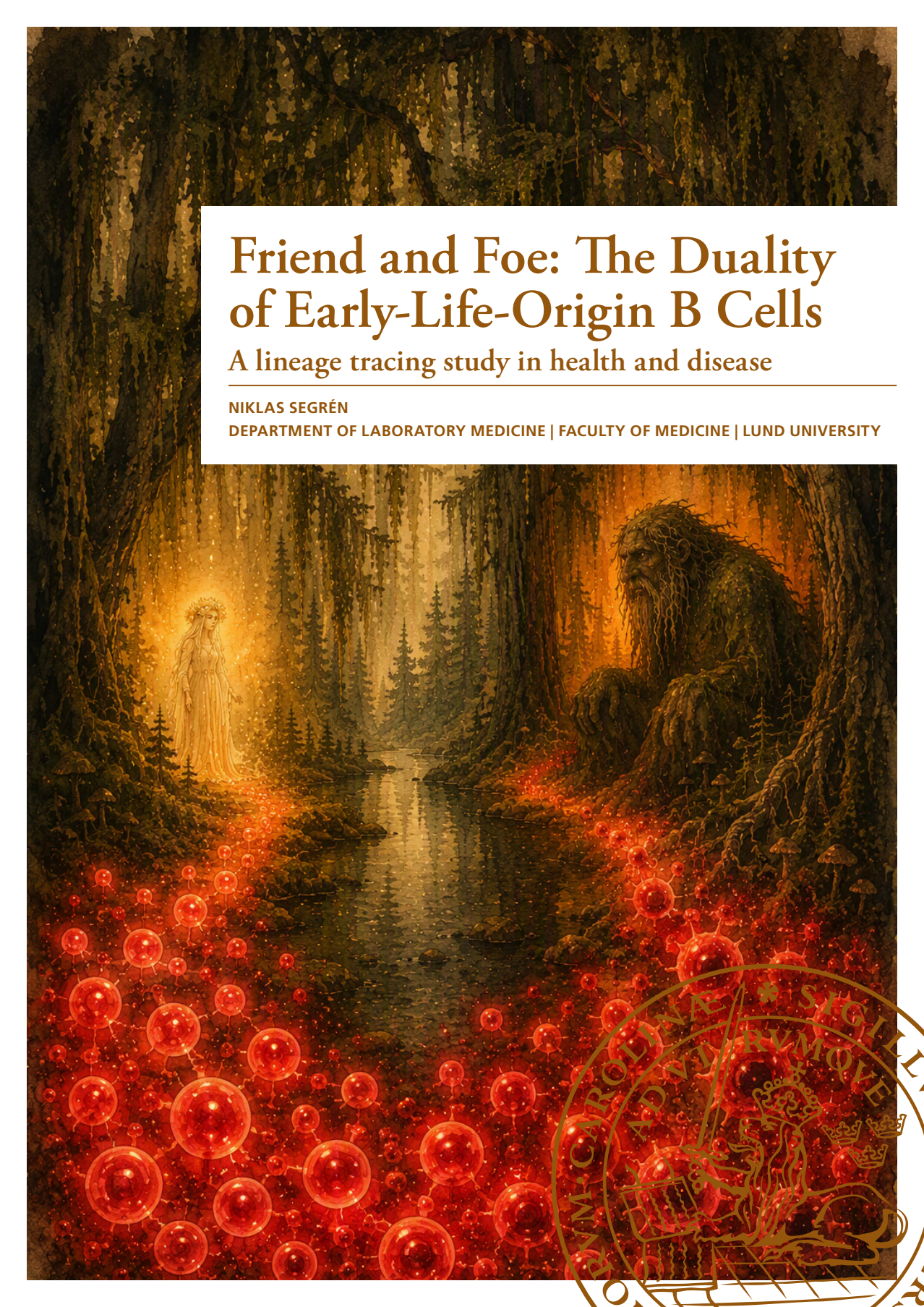
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Friend and Foe: The Duality of Early-Life-Origin B Cells

A lineage tracing study in health and disease

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DEPARTMENT OF LABORATORY MEDICINE | FACULTY OF MEDICINE | LUND UNIVERSITY

Friend and Foe: The Duality of Early-Life-Origin B Cells

Friend and Foe: The Duality of Early-Life-Origin B Cells

A lineage tracing study in health and disease

Niklas Segrén



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Abstract:

The adult immune system is composed of a developmentally heterogeneous mix of B cells. Over time, it is seeded by distinct ontogenetic waves of B cells. Short-lived B cells are continuously replenished by hematopoietic stem and progenitor cells (HSPCs), whereas B cells with self-renewal capacity remain in the organism long-term. The functional consequences of such a mixed developmental history for immune homeostasis and dysregulation have remained poorly understood, in part due to the lack of tools capable of resolving ontogenetic B cell waves *in situ*.

Here we established a genetic lineage tracing approach to permanently label, or “time-stamp”, B cells at defined developmental stages and study their contribution to the adult immune system in an unperturbed setting. This approach enabled us to uncover a previously unknown diversity of early-life-origin (ELO) B cells across adult B cell subsets. We identified a precise postnatal developmental window for the emergence of B-1 cells, a prototypical early-life restricted subset. We demonstrated that ELO B cells constitute a natural layer of immune memory, shaped by early-life antigen encounter and microbiota exposure (Paper I).

Molecular analysis revealed that ELO B cells share a conserved transcriptional signature characterized by an antigen-activated, memory-like status. ELO B-1 cells in particular, were enriched for surface expression of the memory markers CD80 and PD-L2, underscoring their role as a reservoir of neonatal immune experience. B cell chronic lymphocytic leukemia (CLL) is closely associated with an antigen-experienced, memory-like phenotype. Using a CLL mouse model, we demonstrated that leukemia-initiating cells emerge prior to postnatal day 10 and that disease progression is driven by ELO B-1 cells exhibiting a pronounced memory-like profile. Notably, we identified similarities between the transcriptional signatures of mouse ELO B-1 cells and human CLL samples, suggesting a comparable contribution of ELO B cells to disease development in humans (Paper II).

The developmental master regulator LIN28B instructs early-life B cell development and imprints fetal-specific gene-expression programs onto ELO B cells. These early-life cues leave ELO B cells more vulnerable to leukemic transformation later in life. We showed that transient ectopic LIN28B expression in adult HSPCs recapitulates certain aspects of fetal lymphopoiesis, generating B cells that are more susceptible to oncogenic transformation (Paper III).

Finally, we investigated the developmental origin of interleukin (IL)-10-competent immunoregulatory B cells. We showed that ELO B-1 cells represent a major source of IL-10 production and linked their immunoregulatory capacity to LIN28B-dependent early-life programming. Additionally, we uncovered that ELO B cells play a crucial role in regulating inflammation in an experimental intestinal colitis model (Paper IV).

Collectively, the work presented in this thesis highlights the unique early-life developmental window that gives rise to B cells with distinctive and enduring properties. The ELO B cell layer forms a natural archive of early-life immune exposure, contributing critically to immune protection and regulation throughout life. At the same time, the long-lived nature of these cells renders them susceptible to dysregulation with age, creating a pool of cells that can give rise to leukemia, thereby illustrating the dual protective and pathogenic potential of ELO B cells.

Key words: Early-Life-Origin B cells, B-1 cells, Lineage tracing, CLL, LIN28B, PD-L2, CD80, IL-10

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Friend and Foe: The Duality of Early-Life-Origin B Cells

A lineage tracing study in health and disease

Niklas Segrén



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Cover Image: Artistic interpretation of *The Duality of Early-Life-Origin B Cells* by Niklas Segrén.

The cover artwork was created in the style of traditional Swedish folklore artist John Bauer. Early-life-origin (ELO) B cells, depicted in red, embody both protective and pathological potential. Their beneficial functions are represented by the mystical forest nymph standing on one side of the pond, symbolizing immune homeostasis and health. In contrast, their capacity for age-associated immune dysregulation and leukemic transformation is represented by the ominous troll on the opposite shore. Together, these figures illustrate the dual nature of ELO B cells across the lifespan. The cover image was generated using M365 Copilot.

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MADE IN SWEDEN 

To my family and friends

"Aber erstmal lachen..."

"But first, let's laugh..."

My good friend Lukas, whenever something didn't go as planned.

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Paper I

A self-sustaining layer of early-life-origin B cells drives steady-state IgA responses in the adult gut

Immunity, 2022; Volume 55, Issue 10, DOI: 10.1016/j.immuni.2022.08.018

Stefano Vergani, Konjit Getachew Muleta, Clément Da Silva, Alexander Doyle, Trine Ahn Kristianssen, Selene Sodini, **Niklas Krausse**, Giorgia Montano, Knut Kotarsky, Joy Nakawesi, Hugo Åkerstrand, Stijn Vanhee, Sneha Lata Gupta, David Bryder, William Winston Agace, Katharina Lahl, and Joan Yuan

Paper II

Early-life B cell memory is archived in the mouse B-1 cell compartment and drives chronic lymphocytic leukemia-like disease

Cell Reports, 2026; Volume 48, Issue 4, DOI: 10.1016/j.celrep.2026.117202

Niklas Segrén, Stefano Vergani, Joan Escrivà-Font, Giorgia Montano, Elena Boldrin, Selene Sodini, Konjit Getachew Muleta, Shamit Soneji, Christopher D. Scharer, Camila Rosat Consiglio, Joan Yuan

Paper III

Impact of early-life-specific protein LIN28B on chronic lymphocytic leukemia-like disease in mice

Manuscript, 2026

Niklas Segrén, Elena Boldrin, Stefano Vergani, Giorgia Montano, Joan Yuan

Paper IV

Early-life programming endows B-1 cells with regulatory potential to protect against gut inflammation

Manuscript, 2026

Selene Sodini, Konjit Getachew Muleta, Knut Kotarsky, **Niklas Segrén**, Bin Yang, Giorgia Montano, Marta Ramos, Iben Lundgaard, William Agace, Joan Yuan

List of papers not included in this thesis

Developmental cues license megakaryocyte priming in murine hematopoietic stem cells

Blood Advances, 2022; Volume 6, Issue 24,

DOI: 10.1182/bloodadvances.2021006861

Trine A. Kristiansen, Qinyu Zhang, Stefano Vergani, Elena Boldrin, **Niklas Krausse**, Oscar André, Pontus Nordenfelt, Mikael Sigvardsson, David Bryder, Jonas Ungerback, Joan Yuan

Enhanced protein synthesis is a defining requirement for neonatal B cell development

Frontiers in Immunology, 2023; Volume 14, DOI: 10.3389/fimmu.2023.1130930

Hugo Åkerstrand, Elena Boldrin, Giorgia Montano, Stijn Vanhee, Karin Olsson, **Niklas Krausse**, Stefano Vergani, Maciej Cieřla, Christian Bellodi, Joan Yuan

Lineage tracing of stem cell-derived dopamine grafts in a Parkinson's model reveals shared origin of all graft-derived cells

Science Advances, 2024; Volume 10, Issue 42, DOI: 10.1126/sciadv.adn3057

Petter Storm, Yu Zhang, Fredrik Nilsson, Alessandro Fiorenzano, **Niklas Krausse**, Malin Åkerblom, Marcus Davidsson, Joan Yuan, Agnete Kirkeby, Tomas Björklund, Malin Parmar

Author's contribution to papers included in this thesis

In accordance with the guidelines of the Lund University Faculty of Medicine, the contributions of the author to each of the papers included in this thesis are detailed below.

Paper I

This project was initiated by the first author, Stefano Vergani, and my supervisor, Joan Yuan, and was already underway when I began my PhD studies. Throughout the project, I actively contributed to its development and refinement through continuous discussions and close collaboration with the first author.

I worked closely with the first author and performed or supported numerous experiments. My primary individual contribution was related to the B cell receptor sequencing (VDJseq) experiments. I was actively involved in sample preparation, sequencing library construction, sequencing, data analysis, and data interpretation for a substantial proportion of the experiments presented in Figures 3 and 4.

I frequently presented and discussed data from this study at scientific meetings, seminars, and conferences, gathering critical feedback that contributed to the advancement of the project. In addition, I provided comments and feedback throughout the manuscript preparation process.

Paper II

This project constituted the major part of my PhD studies and was conceived by me together with my supervisor, Joan Yuan. Under her supervision, I planned, conducted, and interpreted the majority of the experiments presented in this study. I was also responsible for the maintenance and breeding of several mouse strains used throughout the project and independently developed, validated, and applied bioinformatic tools and analytical pipelines.

The scRNA-seq experiment presented in Figure 1 was performed together with Stefano Vergani, with essential support from Giorgia Montano and Elena Boldrin. I conducted the majority of the downstream scRNA-seq analyses. Furthermore, I developed bioinformatic pipelines and analysed and interpreted the sequencing data, with critical support from Joan Escrivà-Font and Camila Rosat Consiglio.

For the multicolour flow cytometry experiments presented in Figure 2, I carried out sample preparation, data analysis, and data interpretation, with support from Elena Boldrin in panel design.

I planned and coordinated all long-term CLL development studies shown in Figure 3. This included designing and coordinating mouse breeding strategies and tamoxifen-induced time-stamping schedules, performing all longitudinal peripheral blood analyses, and analysing and interpreting the resulting data. In addition, I

conducted all endpoint analyses and designed and performed the associated FACS-sorting experiments.

I planned and performed all VDJseq experiments presented in Figure 4. This included all FACS-sorting experiments required for downstream analyses, sequencing library preparation, and in-house sequencing together with Giorgia Montano. I also carried out all downstream bioinformatic analyses using custom-developed tools, publicly available packages and pipelines.

For Figure 5, I performed all flow cytometry experiments as well as the FACS-sorting experiments required for bulk RNAseq analyses. I conducted the downstream bioinformatic analyses of the bulk RNAseq data with critical support from Shamit Soneji.

As this was my principal PhD project, I regularly presented and discussed the data at scientific meetings, seminars, and conferences, incorporating feedback that helped guide and strengthen the study.

The manuscript was conceived, planned, and written jointly by me and my supervisor. Communication with the journal, including responding to reviewer comments, preparing the accepted manuscript for production, and finalizing the published version, was also carried out jointly by me and my supervisor.

Paper III

This project was designed by me and my supervisor, Joan Yuan. I performed all experiments presented in the study, with support from the co-authors where required. I generated and maintained the TCL-1tg $\times$ tet-LIN28B mouse model and validated it as presented in Figure 1.

I planned and executed the transplantation experiments shown in Figure 2. Specifically, I performed FACS-sorting of HSPCs, carried out intravenous transplantations into irradiated recipient mice, administered doxycycline induction, and monitored disease progression through longitudinal blood analyses. I also planned and performed all endpoint experiments, with support from Elena Boldrin.

I planned and conducted all VDJseq experiments presented in Figure 3. This included all FACS-sorting procedures required for sample collection, sequencing library preparation, and in-house sequencing together with Giorgia Montano. I performed all downstream bioinformatic analyses using custom-developed bioinformatic tools, publicly available packages and pipelines.

Throughout the project, I frequently presented and discussed the findings at scientific meetings, seminars, and conferences, obtaining valuable feedback that contributed to the progression of the study.

I conceived, planned, and wrote the manuscript, with critical feedback provided by my supervisor and Elena Boldrin.

Paper IV

This project was initiated by the first author, Selene Sodini, together with my supervisor, Joan Yuan. I contributed to the development and refinement of the project through scientific discussions during group meetings and supported several experiments throughout the study.

My primary individual contribution was to the scRNAseq analyses presented in Supplementary Figure 1. Additionally, I provided feedback and comments at multiple stages of manuscript preparation.

Abstract

The adult immune system is composed of a developmentally heterogeneous mix of B cells. Over time, it is seeded by distinct ontogenetic waves of B cells. Short-lived B cells are continuously replenished by hematopoietic stem and progenitor cells (HSPCs), whereas B cells with self-renewal capacity remain in the organism long-term. The functional consequences of such a mixed developmental history for immune homeostasis and dysregulation have remained poorly understood, in part due to the lack of tools capable of resolving ontogenetic B cell waves *in situ*.

Here we established a genetic lineage tracing approach to permanently label, or “time-stamp”, B cells at defined developmental stages and study their contribution to the adult immune system in an unperturbed setting. This approach enabled us to uncover a previously unknown diversity of early-life-origin (ELO) B cells across adult B cell subsets. We identified a precise postnatal developmental window for the emergence of B-1 cells, a prototypical early-life restricted subset. We demonstrated that ELO B cells constitute a natural layer of immune memory, shaped by early-life antigen encounter and microbiota exposure (Paper I).

Molecular analysis revealed that ELO B cells share a conserved transcriptional signature characterized by an antigen-activated, memory-like status. ELO B-1 cells in particular, were enriched for surface expression of the memory markers CD80 and PD-L2, underscoring their role as a reservoir of neonatal immune experience. B cell chronic lymphocytic leukemia (CLL) is closely associated with an antigen-experienced, memory-like phenotype. Using a CLL mouse model, we demonstrated that leukemia-initiating cells emerge prior to postnatal day 10 and that disease progression is driven by ELO B-1 cells exhibiting a pronounced memory-like profile. Notably, we identified similarities between the transcriptional signatures of mouse ELO B-1 cells and human CLL samples, suggesting a comparable contribution of ELO B cells to disease development in humans (Paper II).

The developmental master regulator LIN28B instructs early-life B cell development and imprints fetal-specific gene-expression programs onto ELO B cells. These early-life cues leave ELO B cells more vulnerable to leukemic transformation later in life. We showed that transient ectopic LIN28B expression in adult HSPCs recapitulates certain aspects of fetal lymphopoiesis, generating B cells that are more susceptible to oncogenic transformation (Paper III).

Finally, we investigated the developmental origin of interleukin (IL)-10-competent immunoregulatory B cells. We showed that ELO B-1 cells represent a major source of IL-10 production and linked their immunoregulatory capacity to LIN28B-dependent early-life programming. Additionally, we uncovered that ELO B cells play a crucial role in regulating inflammation in an experimental intestinal colitis model (Paper IV).

Collectively, the work presented in this thesis highlights the unique early-life developmental window that gives rise to B cells with distinctive and enduring properties. The ELO B cell layer forms a natural archive of early-life immune exposure, contributing critically to immune protection and regulation throughout life. At the same time, the long-lived nature of these cells renders them susceptible to dysregulation with age, creating a pool of cells that can give rise to leukemia, thereby illustrating the dual protective and pathogenic potential of ELO B cells.

Abbreviations

AID	Activation-induced cytidine deaminase
BCR	B cell receptor
BM	Bone marrow
CDR3	Complementarity-determining region 3
CLL	Chronic lymphocytic leukemia
CLP	Common lymphoid progenitor
CSR	Class-switch recombination
D	Diversity
DSS	Dextran sulfate sodium
DT	Diphtheria toxin
DTR	Diphtheria toxin receptor
E	Embryonic day
ELO	Early-life-origin
FACS	Fluorescence-activated cell sorting
FL	Fetal liver
GAS	Group A <i>Streptococcus</i>
GC	Germinal center
H	Heavy chain
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem and progenitor cell
IG	Immunoglobulin
IL	Interleukin
iNKT	Invariant natural killer T
J	Joining
L	Light chain
M-CLL	Mutated chronic lymphocytic leukemia
MAIT	Mucosal-associated invariant T
MBC	Memory B cell

MHC II	Major histocompatibility complex class II
MZ	Marginal zone
N	Non-templated
NAb	Natural antibody
PAMP	Pathogen-associated molecular pattern
PC	Plasma cell
PerC	Peritoneal cavity
PP	Peyer's patch
PRR	Pattern-recognition receptor
PtC	Phosphatidyl choline
RV	Rotavirus
scRNAseq	Single-cell RNA sequencing
SHM	Somatic hypermutation
SI-LP	Small intestine lamina propria
SPF	Specific-pathogen-free
TCL-1	T cell leukemia/lymphoma 1
TCL-1tg	TCL-1 transgenic
TD	T cell-dependent
TdT	Terminal deoxynucleotidyl transferase
Tfh	T follicular helper
TH1	T helper cell type 1
TI	T cell-independent
TLR	Toll-like receptor
Tom	TdTomato
Treg	Regulatory T cell
U-CLL	Unmutated chronic lymphocytic leukemia
V	Variable

Popular Science Summary

The immune system is a remarkable biological system that protects us against harmful microbes while maintaining a peaceful relationship with our own tissues and beneficial microorganisms. To achieve this balance, the immune system must constantly distinguish between friend and foe. B cells are an important part of this process. They produce antibodies that recognize specific targets and help eliminate infections, while also providing long-term protection against diseases we have encountered before.

Most studies of B cells focus on adults. However, much less is known about whether the stage of life at which a B cell is first produced influences how it behaves later on. Furthermore, many discoveries in immunology have relied on experimental systems that alter normal development, making it difficult to understand how immune cells arise and function under natural conditions.

The work presented in this thesis investigates how B cells generated during early life contribute to the adult immune system. To do this, we developed a novel mouse model that allows B cells to be permanently labelled, or “time-stamped,” at specific stages of development. This label is inherited by the cells and their descendants, enabling us to follow them throughout the lifetime of the animal without disturbing the immune system.

Using this approach, we discovered that the adult immune system contains a previously unrecognized population of early-life-origin (ELO) B cells. Rather than disappearing as the animal grows, many of these cells survive long-term and contribute to several B cell compartments in adulthood. We also identified a precise developmental window shortly after birth during which B-1 cells, a specialized B cell subset, are generated. Importantly, we found that ELO B cells carry evidence of encounters with microbes early in life, demonstrating that the immune experiences of infancy can leave a lasting imprint that persists into adulthood (Paper I).

We next examined the molecular characteristics of ELO B cells. These analyses revealed that ELO B cells display features typically associated with activated and memory B cells. In particular, ELO B-1 cells showed signs of previous antigen exposure and expressed proteins commonly associated with immune memory. Together, these findings suggest that ELO B cells act as a natural archive of early-life immune experiences, preserving information about microbial encounters long after they have occurred (Paper II).

The long-lived nature of ELO B cells led us to ask whether they might also contribute to disease. We focused on chronic lymphocytic leukemia (CLL), the most common leukemia in adults. Although CLL is usually diagnosed late in life, we found that the cells capable of initiating leukemia arise during early development,

persist for many months, and eventually transform into cancerous cells with age. Remarkably, we identified molecular similarities between mouse ELO B cells and human CLL samples, suggesting that developmental events occurring early in life may influence disease risk decades later (Paper II).

To understand why ELO B cells are particularly susceptible to leukemic transformation, we investigated LIN28B, a molecular regulator that is active during fetal and neonatal life but normally switched off in adulthood. By experimentally re-activating LIN28B in adult blood-forming stem cells, we were able to recreate important aspects of early-life B cell development. This generated B cells that were more prone to transformation and leukemia formation, demonstrating that developmental programs established early in life can have long-lasting consequences (Paper III).

Finally, we investigated regulatory B cells, which help prevent excessive inflammation and maintain immune balance. We found that ELO B-1 cells are a major source of the anti-inflammatory molecule interleukin-10. We further showed that this regulatory function is linked to LIN28B-dependent developmental programming and that ELO B cells play an important role in controlling inflammation in an experimental model of intestinal disease (Paper IV).

Taken together, the studies in this thesis reveal that early life represents a critical developmental window that generates a distinct layer of long-lived B cells. These ELO B cells retain memories of early-life immune experiences, help regulate inflammation, and contribute to immune protection throughout life. At the same time, their longevity makes them vulnerable to age-related dysregulation, creating a population of cells that can evolve into leukemia. These findings demonstrate how events occurring during the earliest stages of life can shape immune function, health, and disease many years later.

Populärvetenskaplig Sammanfattning

Immunsystemet är ett enastående biologiskt system som skyddar oss mot skadliga mikroorganismer samtidigt som det fredligt samexisterar med kroppens egna vävnader och nyttiga mikroorganismer. För att upprätthålla denna balans måste immunsystemet ständigt skilja mellan ”vän” och ”fiende”. B-celler spelar en central roll i denna process. De producerar antikroppar som känner igen specifika mål, bekämpar infektioner och ger ett långvarigt skydd mot sjukdomsframkallande mikroorganismer som kroppen tidigare har stött på.

De flesta studier av B-celler fokuserar på vuxna individer. Däremot vet man betydligt mindre om huruvida den tidpunkt i livet då en B-cell först bildas påverkar hur den fungerar senare. Dessutom bygger många immunologiska upptäckter på experimentella modeller som påverkar och förändrar den normala utvecklingen av cellen. Därför är det ofta svårt att förstå hur immunceller uppstår och fungerar under naturliga förhållanden.

Arbetet i denna avhandling undersöker vilken roll B-celler som bildas tidigt i livet har för det vuxna immunsystemet. För detta ändamål utvecklade vi en ny musmodell som gör det möjligt att permanent märka, eller ”tidsstämpla”, B-celler vid bestämda utvecklingsstadier. Märkningen förs vidare till alla dotterceller, vilket gör det möjligt att följa dem under hela djurets livstid utan att påverka immunsystemet.

Med hjälp av denna metod kunde vi visa att det vuxna immunsystemet innehåller en tidigare okänd population av B-celler med ursprung i den tidiga livsfasen (early-life-origin, ELO). I stället för att försvinna under uppväxten finns många av dessa celler kvar under hela livet och återfinns i flera olika B-cellspopulationer hos vuxna individer. Vi identifierade också ett tydligt avgränsat utvecklingsfönster strax efter födseln då B-1-celler, en specialiserad undergrupp av B-celler, bildas. Särskilt viktigt var observationen att ELO B-celler bär spår av mikrobiella kontakter från den tidiga livsfasen. Detta visar att immunologiska erfarenheter tidigt i livet kan lämna bestående avtryck som kvarstår in i vuxen ålder (Paper I).

Därefter undersökte vi de molekylära egenskaperna hos ELO B-celler. Analyserna visade att dessa celler uppvisar kännetecknen som vanligtvis förknippas med aktiverade minnes-B-celler. Särskilt ELO B-1-celler uppvisade tecken på tidigare antigenexponering och uttryckte ytproteiner som är karakteristiska för immunologiskt minne. Tillsammans tyder dessa resultat på att ELO B-celler fungerar som ett naturligt arkiv över tidiga immunologiska erfarenheter och lagrar information om mikrobiella kontakter från den tidiga livsfasen under lång tid (Paper II).

Den långa livslängden hos ELO B-celler väckte frågan om de även kan bidra till sjukdomsutveckling. Därför fokuserade vi på kronisk lymfatisk leukemi (KLL), den vanligaste formen av leukemi hos vuxna. Trots att sjukdomen oftast diagnostiseras

sent i livet kunde vi visa att de celler som kan ge upphov till leukemi uppstår redan under den tidiga utvecklingen, kvarstår under lång tid och först senare i livet omvandlas till cancerceller. Anmärkningsvärt nog fann vi molekylära likheter mellan ELO B-celler hos möss och mänskliga KLL-prover. Detta tyder på att utvecklingsprocesser tidigt i livet kan påverka risken för sjukdom flera decennier senare (Paper II).

För att förstå varför ELO B-celler är särskilt mottagliga för leukemisk omvandling studerade vi LIN28B, en molekylär regulator som är aktiv under fosterlivet och det neonatala utvecklingsstadiet men normalt stängs av i vuxen ålder. Genom att experimentellt återaktivera LIN28B i vuxna blodbildande stamceller kunde vi återskapa viktiga aspekter av den tidiga B-cellsutvecklingen. Detta ledde till bildandet av B-celler som var mer benägna att genomgå malign omvandling och utveckla leukemi. Resultaten visar att utvecklingsprogram som är aktiva tidigt i livet kan få långvariga konsekvenser (Paper III).

Slutligen undersökte vi regulatoriska B-celler, som hjälper till att förhindra överdrivna inflammatoriska reaktioner och upprätthålla immunsystemets balans. Vi kunde visa att ELO B-1-celler utgör en viktig källa till den antiinflammatoriska signalmolekylen interleukin-10. Vidare visade vi att denna regulatoriska funktion är kopplad till LIN28B-beroende programmering under den tidiga utvecklingen. ELO B-celler spelar dessutom en viktig roll i kontrollen av inflammation i en experimentell modell för kronisk tarminflammation (Paper IV).

Sammantaget visar arbetena i denna avhandling att den tidiga livsfasen utgör ett avgörande utvecklingsfönster då en särskild population av långlivade B-celler bildas. Dessa ELO B-celler bevarar minnen av tidiga immunologiska erfarenheter, bidrar till regleringen av inflammation och stödjer immunförsvaret genom hela livet. Samtidigt gör deras långa livslängd dem mottagliga för åldersrelaterad felreglering, vilket innebär att de kan utgöra den cellpopulation ur vilken leukemier uppstår. Resultaten belyser hur händelser under de allra tidigaste stadierna av livet kan påverka immunsystemets funktion samt hälsa och sjukdom långt senare i livet.

Populärwissenschaftliche Zusammenfassung

Das Immunsystem ist ein bemerkenswertes biologisches System, das uns vor schädlichen Mikroorganismen schützt und gleichzeitig in friedlicher Koexistenz mit den körpereigenen Geweben sowie nützlichen Mikroorganismen lebt. Um dieses Gleichgewicht aufrechtzuerhalten, muss das Immunsystem ständig zwischen „Freund“ und „Feind“ unterscheiden. B-Zellen spielen dabei eine zentrale Rolle. Sie produzieren Antikörper, die bestimmte Zielstrukturen erkennen, Infektionen bekämpfen und einen langfristigen Schutz gegen Krankheitserreger ermöglichen, mit denen der Organismus bereits in Kontakt gekommen ist.

Die meisten Studien zu B-Zellen konzentrieren sich auf ihre Funktion im Erwachsenenalter. Deutlich weniger ist jedoch darüber bekannt, ob der Zeitpunkt im Leben, an dem eine B-Zelle erstmals gebildet wird, ihr späteres Verhalten beeinflusst. Darüber hinaus beruhen viele Erkenntnisse der Immunologie auf experimentellen Modellen, die die normale Entwicklung beeinflussen und verändern. Dadurch ist es oft schwierig zu verstehen, wie Immunzellen unter natürlichen Bedingungen entstehen und funktionieren.

Die in dieser Dissertation vorgestellte Arbeit untersucht, welchen Beitrag B-Zellen leisten, die während der frühen Lebensphase entstehen. Hierzu entwickelten wir ein neuartiges Mausmodell, mit dem B-Zellen zu definierten Zeitpunkten der Entwicklung dauerhaft markiert oder „zeitgestempelt“ werden können. Diese Markierung wird an die Zellen und ihre Nachkommen weitergegeben, sodass sie während der gesamten Lebensspanne des Tieres verfolgt werden können, ohne das Immunsystem zu beeinflussen.

Mit diesem Ansatz konnten wir zeigen, dass das erwachsene Immunsystem eine bisher unbekannte Population von B-Zellen mit Ursprung aus der frühesten Lebensphase (early-life-origin, ELO) enthält. Anstatt während des Heranwachsens zu verschwinden, bleiben viele dieser Zellen lebenslang erhalten und finden sich in unterschiedlichen B-Zell-Populationen des erwachsenen Immunsystems wieder. Zudem identifizierten wir ein genau definiertes Entwicklungsfenster kurz nach der Geburt, in dem B-1-Zellen, eine spezialisierte Untergruppe von B-Zellen, entstehen. Besonders wichtig war die Beobachtung, dass ELO B-Zellen Spuren von Mikrobenkontakten aus der frühen Lebensphase tragen. Dies zeigt, dass immunologische Erfahrungen der frühen Entwicklungsphase einen bleibenden Einfluss haben können, der bis ins Erwachsenenalter erhalten bleibt (Paper I).

Anschließend untersuchten wir die molekularen Eigenschaften von ELO B-Zellen. Dabei zeigte sich, dass diese Zellen Merkmale aufweisen, die typischerweise mit aktivierten Gedächtnis-B-Zellen assoziiert werden. Insbesondere ELO B-1-Zellen zeigten Hinweise auf frühere Antigenkontakte und exprimierten Oberflächenproteine, die charakteristisch für immunologisches Gedächtnis sind. Gemeinsam deuten diese Ergebnisse darauf hin, dass ELO B-Zellen als eine Art

natürliches Archiv früher Immunerfahrungen fungieren und Informationen über mikrobielle Kontakte der frühen Lebensphase langfristig speichern (Paper II).

Die außergewöhnlich lange Lebensdauer von ELO B-Zellen führte zu der Frage, ob sie auch zur Entstehung von Krankheiten beitragen können. Deshalb untersuchten wir die chronische lymphatische Leukämie (CLL), die häufigste Leukämieform bei Erwachsenen. Obwohl die Erkrankung meist erst im höheren Alter diagnostiziert wird, konnten wir zeigen, dass die Zellen, die eine Leukämie auslösen können, bereits während der frühen Entwicklung entstehen, über lange Zeiträume bestehen bleiben und sich erst im Alter zu Krebszellen entwickeln. Bemerkenswerterweise fanden wir molekulare Ähnlichkeiten zwischen ELO B-Zellen von Mäusen und menschlichen CLL-Proben. Dies deutet darauf hin, dass Entwicklungsprozesse der frühen Lebensphase das Krankheitsrisiko noch Jahrzehnte später beeinflussen können (Paper II).

Um zu verstehen, warum ELO B-Zellen besonders anfällig für eine leukämische Transformation sind, untersuchten wir LIN28B, einen molekularen Regulator, der während der fetalen und neonatalen Entwicklung aktiv ist, im Erwachsenenalter jedoch abgeschaltet wird. Durch die experimentelle Reaktivierung von LIN28B in erwachsenen blutbildenden Stammzellen konnten wir wichtige Aspekte der frühen B-Zell-Entwicklung nachbilden. Dadurch entstanden B-Zellen, die anfälliger für eine bösartige Transformation und die Entwicklung einer Leukämie waren. Diese Ergebnisse zeigen, dass Entwicklungsprogramme, die früh im Leben aktiv sind, langfristige Folgen haben können (Paper III).

Abschließend untersuchten wir regulatorische B-Zellen, die überschießende Entzündungsreaktionen verhindern und zur Aufrechterhaltung des immunologischen Gleichgewichts beitragen. Wir konnten zeigen, dass ELO B-1-Zellen eine wichtige Quelle des entzündungshemmenden Botenstoffs Interleukin-10 darstellen. Darüber hinaus zeigten wir, dass diese regulatorische Funktion mit einer LIN28B-abhängigen Programmierung während der frühen Entwicklung verknüpft ist. ELO B-Zellen spielen zudem eine wichtige Rolle bei der Kontrolle von Entzündungen in einem experimentellen Modell chronischer Darmentzündung (Paper IV).

Insgesamt zeigen die Arbeiten dieser Dissertation, dass die frühe Lebensphase ein entscheidendes Entwicklungsfenster darstellt, in dem eine besondere Population langlebiger B-Zellen entsteht. Diese ELO B-Zellen bewahren Erinnerungen an frühe Immunerfahrungen, tragen zur Regulation von Entzündungen bei und unterstützen den lebenslangen Immunschutz. Gleichzeitig macht ihre lange Lebensdauer sie anfällig für Fehlregulationen im Alter, wodurch sie zu einer Zellpopulation werden können, aus der Leukämien entstehen. Die Ergebnisse verdeutlichen, wie Ereignisse in den frühesten Lebensabschnitten die Funktion des Immunsystems sowie Gesundheit und Krankheit noch viele Jahre später beeinflussen können.

Introduction

B cell development and differentiation

The immune system protects the body from internal and external threats through a multitude of specialized white blood cells collectively known as leukocytes. Among these, B lymphocytes represent a major lineage and are central players in adaptive immunity. B cells are continuously generated from hematopoietic stem and progenitor cells (HSPCs) and undergo a tightly regulated developmental process that ensures the generation of a functional and diverse antigen receptor repertoire. During this process, progenitor cells progress through a series of increasingly committed stages, ultimately giving rise to mature B cells [1]. The ordered assembly, expression and quality-control of the B cell receptor (BCR) are integral components of B cell development and fundamental in the generation of a highly diverse antibody repertoire capable to recognize a plethora of foreign and self-antigens [2, 3].

Adult B cell development and germinal center responses

In adults, B cell development begins in the bone marrow (BM), where hematopoietic stem cells (HSCs) differentiate into common lymphoid progenitors (CLPs) and subsequently into B lineage committed progenitors [4]. Early stages of B lymphopoiesis are dependent on stromal-cell-derived interleukin (IL)-7, which promotes survival and proliferation [5]. At the pro-B cell stage immunoglobulin (IG) heavy chain (H) locus rearrangement is initiated through recombination of variable (V_H), diversity (D_H) and joining (J_H) gene segments. This process, termed V(D)J-recombination, generates extensive BCR diversity through semi-random fusion of V_H , D_H and J_H elements [2, 6]. Junctional diversity is further enhanced through the incorporation of non-templated (N)-nucleotides mediated by Terminal deoxynucleotidyl transferase (TdT) [7]. Together V(D)J-recombination and N-nucleotide addition are two important mechanisms to achieve the highly diverse BCR repertoire.

Successful rearrangement and expression of a functional μ IGH marks the transition to the pre-B cell stage, where the μ IGH pairs with the surrogate light chain (L) components V_{preB} and $\lambda 5$ to form the pre-BCR complex [8-11]. Pre-BCR

signalling terminates further IGH recombination and, together with IL-7 signalling, promotes proliferation of large pre-B cells [12, 13]. This checkpoint ensures that only cells expressing a functional IGH progress further. After a proliferative expansion phase, cells transition to the small pre-B stage, where IGL recombination is initiated [14]. IGL assembly occurs through semi-random recombination of V_L and J_L gene segments, analogous to IGH recombination [6, 15]. The newly generated IGL pairs with the μ IGH to form a mature BCR, which is expressed on the cell surface, marking the transition to the immature B cell stage [14].

At this point, B cells undergo central tolerance selection, a critical checkpoint that eliminates or silences self-reactive clones. Strong BCR signalling at this stage indicates self-reactivity and triggers receptor editing, clonal deletion or anergy [3]. The central tolerance checkpoint is essential for preventing the emergence of autoreactive B cells, which are implicated in autoimmune disease [16]. Immature B cells that pass the tolerance checkpoint exit the BM as transitional B cells and complete their maturation in the spleen [14], where tonic BCR signalling supports their survival and differentiation into mature naïve B cells [17].

Upon encountering antigen in secondary lymphoid organs such as lymph nodes or the spleen, mature naïve B cells become activated through antigen binding to the BCR and, in the case of T cell-dependent (TD) responses, through cognate interaction with CD4⁺ T follicular helper (Tfh) cells. This interaction provides essential co-stimulatory signals, including CD40-CD40L engagement and cytokine support, which drive B cell proliferation and differentiation [18]. Activated B cells can initially differentiate extrafollicularly into short-lived plasmablasts. However, a substantial fraction enters germinal centers (GCs), specialized microanatomical structures that form in response to TD antigens within secondary lymphoid tissues.

Within GCs, B cells undergo iterative cycles of rapid proliferation, somatic hypermutation (SHM), class-switch recombination (CSR) and selection. SHM and CSR are both mediated by activation-induced cytidine deaminase (AID), which introduces point mutations into the variable regions of IG genes and promotes recombination of the IG constant region, enabling the switch from IgM to downstream isotypes such as IgG, IgA or IgE. While SHM diversifies the BCR repertoire and generates clones with a range of antigen affinities, CSR alters antibody effector function without changing antibody specificity. These mutated clones are then subject to stringent selection processes dependent on antigen capture and Tfh cell help. As a result, B cells expressing high-affinity receptors preferentially survive and expand, a process termed affinity maturation [19-21].

Following the GC reaction, selected B cells differentiate into long-lived plasma cells (PCs), which secrete high-affinity, class-switched antibodies, or into memory B cells (MBCs), which provide rapid and robust responses upon antigen re-encounter. Together, this TD pathway ensures the generation of a highly specific durable humoral immune response, forming the basis of long-term protective immunity [20, 22].

Fetal and neonatal B cell development

In contrast to adult B lymphopoiesis, early-life B cell development is characterized by distinct selection mechanisms and a functionally specialized B cell output. Rather than prioritizing maximal receptor diversity and stringent negative selection, fetal and neonatal B cell development favours the rapid generation of a pre-configured B cell compartment optimized for immediate immune protection.

A defining feature of this developmental phase is the preferential production of innate-like B cell populations, most notably B-1 cells and marginal zone (MZ) B cells, which are generated most efficiently during fetal and neonatal life [23, 24]. These populations are characterized by restricted BCR diversity, self-renewal capacity and the ability to mount antibody responses.

BCR repertoire formation during early life is fundamentally distinct from that in adult B cells. Reduced or absent expression of TdT during fetal development limits N-nucleotide addition, resulting in decreased junctional diversity and a repertoire that remains closer to the germline configuration [25, 26]. In parallel, central tolerance mechanisms are developmentally modulated. Compared to adult B lymphopoiesis, selection thresholds are relaxed, allowing the incorporation of self- and poly-reactive BCRs into the functional repertoire [27]. Additional developmental pathways further contribute to the incorporation of self-reactive specificities. Work from the Skok laboratory demonstrated that reduced IL-7R/STAT5 signalling in the fetal liver (FL) promotes early L recombination at the pro-B cell stage, effectively bypassing the pre-BCR checkpoint and enabling the generation of self-reactive B cells [28].

Collectively, these features define an early-life B cell developmental program that generates a restricted but functionally effective repertoire enriched for self- and poly-reactivity. While this strategy supports rapid immune protection and tissue homeostasis during early life, it also establishes a distinct B cell layer that persists into adulthood and may influence long-term immune functions and disease susceptibility. **Although substantial progress has been made in understanding early-life B cell development, important questions remain regarding the extent to which these cells contribute to adult immunity under steady-state conditions (Paper I) and during disease formation (Paper II).**

LIN28B as a central regulator of early-life development

LIN28B is an evolutionarily conserved RNA-binding protein that plays a fundamental role in regulating developmental timing, cellular metabolism and stemness across multiple tissues and species [29]. Originally identified as a heterochronic gene controlling developmental progression in *Caenorhabditis elegans* [30], LIN28B is now recognized as key post-transcriptional regulator that coordinates gene expression programs during early life. Its expression is tightly temporally controlled, with high levels during embryonic and neonatal stages followed by rapid downregulation after birth, reflecting its restriction to early developmental stages [31, 32].

At the molecular level, LIN28B primarily functions by inhibiting the biogenesis of the let-7 family of microRNAs. LIN28B binds to precursor let-7 transcripts and blocks their processing, thereby preventing the maturation of let-7 and resulting in the de-repression of a broad network of target genes [33]. In the hematopoietic system LIN28B thereby collectively promotes self-renewal capacity, metabolic activity and developmental plasticity in HSPCs, supporting the rapid expansion and differentiation that characterize fetal and neonatal hematopoiesis [34, 35]. Moreover, LIN28B contributes to lineage output by biasing fetal HSPCs toward lymphoid and erythroid differentiation programs [36].

Within the B cell lineage, LIN28B plays a central role in shaping early-life developmental checkpoints. Previous work from our group demonstrated that LIN28B lowers the selection threshold at the central tolerance checkpoint by amplifying the CD19/PI3K/c-Myc positive feedback loop, thereby facilitating the positive selection of self-reactive CD5⁺ immature B cells [27]. Consistent with this, Xu and colleagues showed that LIN28B enhances the selection and output of self-reactive B-1 cells during fetal and neonatal development and identified ARID3A and BHLHE41 as key transcriptional regulators downstream of this pathway [37]. In addition to its role in signalling and selection, LIN28B also regulates biosynthetic capacity in developing B cells early in life. Our laboratory has demonstrated that LIN28B directly interacts with ribosomal protein transcripts in pre-B and immature B cells, leading to increased protein synthesis and supporting the high metabolic demands of early-life B lymphopoiesis [38]. The central role of LIN28B in early-life B cell development is further highlighted by gain-of-function studies. Ectopic expression of LIN28 in adult BM progenitors is sufficient to reprogram B lymphopoiesis toward a fetal-like trajectory, restoring the generation of B-1 cells and other early-life-associated lymphocyte populations [32]. Similarly, overexpression of ARID3A, a downstream target of the LIN28B/let-7 axis, can partially recapitulate this program and promote the development of B-1a cells from adult progenitors [39].

Taken together, LIN28B acts as a critical mediator of fetal and neonatal hematopoiesis and a key molecular switch that defines early-life B cell identity by coordinating altered selection thresholds, enhanced biosynthetic activity, and the biased generation of innate-like B cell populations. **Despite these advances, important questions remain regarding how LIN28B-mediated early-life-programming influences susceptibility to leukemic transformation (Paper III) and shapes long-term immunoregulatory capacity (Paper IV).**

B-1 B cells

B-1 B cells represent a distinct subset of B lymphocytes that differ fundamentally from conventional B-2 B cells in their origin, maintenance and function. They emerge predominantly during a restricted developmental window in fetal and neonatal life and persist into adulthood through self-renewal rather than continuous replenishment from adult BM. B-1 cells perform a wide array of specialized functions in both early-life and adult immunity that diverge from classical B cell functions [40].

Discovery of B-1 cells

In 1983 the Herzenberg laboratory set out to find the cellular counterpart of human chronic lymphocytic leukemia (CLL) in mice. This effort led to the discovery of a B cell population expressing the Ly-1 surface marker, equivalent to the human Leu-1 marker, which is highly expressed on human CLL [41]. These markers were later designated CD5, a cell surface molecule primarily found on T cells but also on a subset of B cells. CD5 is a negative regulator of antigen-induced immune receptor signalling and plays an important role in attenuating immune responses [42]. The identified CD5⁺ B cell population was particularly abundant in young mice but declined with age [41].

Subsequent transplantation studies demonstrated that HSCs derived from fetal or neonatal sources efficiently reconstitute CD5⁺ B cells in recipient mice, whereas adult HSCs show a markedly reduced capacity to do so [43]. The predominant generation of CD5⁺ B cells during fetal and neonatal development sparked the concept of a layered immune system, in which distinct waves of immune cell populations arise at specific developmental stages [44]. Reflecting their developmental origin, CD5⁺ B cells were renamed B-1 B cells to distinguish them from the predominantly adult-derived conventional B-2 B cells [45]. The later identification of B cells with B-1 characteristics lacking CD5 expression further subdivided this population into B-1a (CD5⁺) and B-1b (CD5⁻) subsets [46].

B-1 cell ontogeny: Developmental waves of hematopoiesis

The developmental origin of B-1 B cells remains a subject of considerable debate. Current models propose three major waves of B-1 cell development alongside two waves of B-2 cell generation [47, 48]. The earliest wave originates from hemogenic endothelium-derived progenitors at embryonic day (E) 9, identified in the yolk sac and para-aortic splanchnopleura. This wave is restricted to B-1 cell production [24]. Importantly, these earliest B-1 cells arise independently of HSCs, as definitive HSCs emerge later, around E10.5 in mice [49, 50]. Consistent with this, a study with HSC-deficient mice has confirmed the existence of HSC-independent B-1 cell development [51].

A second developmental wave originates from FL and BM progenitors and produces predominantly B-1 cells, while B-2 cell generation has started at this stage at lower levels. Transplantation experiments have linked B-1 output during this phase to fetal HSCs [52, 53]. However, this interpretation has been challenged, as transplantation of highly purified FL HSCs failed to reconstitute the B-1a compartment [54]. Although, the strict immunophenotypic definition applied for HSCs in this study possibly excludes relevant progenitors. In contrast, cellular barcoding approaches by our group have demonstrated that fetal HSCs can give rise to both B-1a and B-2 cells, with B-1a output declining over time while B-2 production is maintained [55]. Supporting this view, a lineage tracing study has identified a transient, developmentally restricted HSC population with fetal characteristics that efficiently generates B-1a cells but does not persist into adulthood [56]. These findings align with a third wave of B cell development originating from postnatal BM HSCs, which predominantly generate B-2 output and exhibit markedly reduced B-1 potential [57]. Collectively, these observations support the concept that ontogenetically restricted waves of B-1 cell generation reflect developmental changes in HSC potential, accompanied by well-documented differences in proliferation, self-renewal capacity and gene expression between fetal and adult HSCs [34, 58-61].

Although these models have greatly advanced our understanding of B-1 cell ontogeny, much of the evidence is derived from transplantation experiments, which may not fully reflect steady-state hematopoiesis. Recent HSC lineage-tracing studies have provided valuable insights into multilineage contributions under physiological conditions [62-66]. **Nevertheless, the development of B cell-specific lineage tracing tools will be essential to fully resolve the ontogenetical composition of the adult B cell compartment and to define the relative contributions of distinct developmental waves to immune homeostasis and dysfunction (Paper I, II and IV).**

B-1 cell identity: Lineage versus selection models

Beyond questions of timing and anatomical origin, a central issue concerns the mechanisms that specify B-1 versus B-2 cell identity. Two major models have been proposed: the “lineage model”, which proposes distinct progenitors for B-1 and B-2 cells, and the “selection model”, which suggests that both subsets arise from a common progenitor and are specified by antigen-dependent BCR signalling [42, 67-69].

Early transplantation studies demonstrated that FL or fetal omentum efficiently reconstituted B-1 cells, whereas adult BM did not, supporting the existence of a developmentally restricted B-1 lineage [43, 44, 46, 70]. Subsequent work identified B-1-committed progenitors, further strengthening this model [53, 71, 72]. These progenitors were characterized as Lineage[−], CD45R[−], CD19⁺ cells, distinct from B-2-committed Lineage[−], CD45R⁺, CD19⁺ progenitors, and were most abundant during fetal and neonatal stages, declining postnatally. Moreover, single-cell culture experiments demonstrated that CLPs give rise exclusively to either B-1-restricted or B-2-restricted progenitors, suggesting that lineage commitment occurs prior to the CLP stage [53, 72].

In contrast, the selection model is supported by evidence that B cell identity is influenced by BCR signalling. Early studies showed that stimulation of conventional B-2 cells with T cell-independent (TI) type 2 antigens can induce a B-1-like phenotype [73, 74]. Further work demonstrated that autoreactive BCR signalling drives positive selection and maintenance of B-1 cells [75]. Notably, B cells expressing autoreactive BCRs specific for the self-antigen Thy-1 were positively selected rather than eliminated. In addition, experiments manipulating BCR signalling strength revealed that signalling intensity, rather than specificity alone, can determine B cell fate [76]. More recent studies showed that enforced expression of a B-1-specific BCR in mature B-2 cells can reprogram them toward a B-1 phenotype [77]. Consistent with this, genetic deletion of negative regulators of BCR signalling (e.g., CD5, Lyn, SHP1, Siglec-G) increases B-1 cell numbers [78-81]. In turn deletion of positive regulators (e.g., CD19, BTK, PI3K) decreases B-1 cell numbers [82-84]. Together, these data support an instructive role for BCR signalling in shaping B-1 versus B-2 cell identity.

General properties and tissue distribution

Besides the ongoing debate regarding their origin, B-1 cells are recognized as a functionally important B cell subset with unique immunological properties. Mouse B-1 cells are primarily localized in the pleural and peritoneal body cavities, with smaller populations residing in the spleen, BM, lymph nodes and mucosal tissues [85-87]. Phenotypically, they are defined as CD19⁺, B220^{low}, IgM^{high}, IgD^{low}, CD23[−], CD43⁺ and CD5^{+/-} [87, 88]. B-1 cells display characteristics of both innate and adaptive immune cells, positioning them as a bridge between these two arms of

immunity. Similar to tissue-resident immune populations, B-1 cells exhibit long-term persistence, self-renewal capacity, responsiveness to innate immune signals and tissue-specific transcriptional profiles that support local homeostasis [86].

B-1 BCR repertoire

BCR signalling is a central determinant of B-1 cell development, maintenance and function. The B-1 BCR repertoire is established predominantly during fetal and neonatal life and differs fundamentally from that of conventional B-2 cells. In contrast to the highly diverse repertoire of B-2 cells, B-1 cells arise under developmental conditions characterized by limited junctional diversity and biased V(D)J recombination, resulting in preferential usage of specific IGHV gene segments and the emergence of a restricted and partially conserved repertoire [85, 89, 90].

V_H-11 and V_H-12 gene segments are strongly enriched in B-1 cells [91, 92]. These gene segments encode BCRs with specificity for phosphatidyl choline (PtC), a major phospholipid component of eukaryotic cell membranes [93]. During cellular stress, apoptosis or membrane remodelling, disruption of membrane asymmetry leads to the exposure of phospholipids on the cell surface, where they can function as “eat-me” signals that promote recognition and clearance of damaged or dying cells [94, 95]. This externalization renders PtC a biologically relevant self-antigen, enabling its recognition by B-1 cell receptors and linking BCR specificity to the sensing of tissue damage and cellular turnover [96, 97].

A characteristic feature of the B-1 repertoire is its enrichment for self-reactive specificities, which are positively selected during development [75]. Early studies demonstrated that the B-1a repertoire is highly restricted and contains recurrent complementarity-determining region 3 (CDR3) sequences, reflecting strong selection for conserved antigen binding motifs [98, 99]. Over time, self-reactive clones undergo expansion, resulting in an oligoclonal repertoire dominated by a limited number of clones in adult animals. Importantly, this process occurs even in germ-free mice, indicating that self-antigen-driven selection, rather than microbial exposure, is the primary force shaping the mature B-1a BCR repertoire [99, 100].

Notably, although the B-1 repertoire has long been considered largely germline-encoded, recent evidence indicates that B-1 cells acquire SHMs over time, leading to progressive diversification of the repertoire in adult animals. Yang and colleagues demonstrated that B-1a cells undergo age-dependent accumulation of SHMs and even CSR, suggesting that their repertoire is not static but continues to evolve under antigenic selection [99]. This finding challenges the classical view of B-1 cells as strictly germline-like and instead supports a model in which an initially constrained, developmentally programmed repertoire is subsequently refined through antigen-driven diversification.

Regulation of B-1 cell self-renewal and homeostasis

A defining feature of B-1 cells is their ability to persist throughout adult life largely independently of continuous input from BM-derived HSPCs. In contrast to conventional follicular (B-2) B cells, which require ongoing generation from the BM and are continuously replaced in the periphery, B-1 cells are maintained through self-renewal and low-level homeostatic proliferation. Classical studies demonstrated that peripheral B-1 cells can persist for extended periods with minimal contribution from adult hematopoiesis, whereas follicular B cells are progressively renewed by newly generated BM-derived cells [101-104]. This capacity for long-term maintenance is particularly pronounced within the B-1a subset and represents a hallmark feature that distinguishes B-1 cells from the conventional adaptive B cell compartment.

At the molecular level, self-renewal is controlled by a specialized transcriptional program. The basic helix-loop-helix transcription factor BHLHE41, and to a lesser extent its paralogue BHLHE40, have emerged as central regulators of B-1a cell identity, governing the development, maintenance and self-renewal of this population while also shaping its characteristic BCR repertoire [105]. More recently, TCF1 and LEF1 have been identified as additional regulators required for B-1a cell homeostasis and long-term maintenance [106]. Other factors implicated in the self-renewal program include polycomb group protein BMI1 [107], which contributes to B-1a cell persistence through mechanisms shared with stem cell maintenance programs in hematopoietic and neural tissues [108, 109].

In addition to transcriptional control, B-1 cell survival relies on finely tuned BCR signalling pathways. Importantly, a distinction must be made between antigen-independent tonic BCR signalling and signalling induced by chronic recognition of endogenous or environmental antigens. Antigen-independent tonic BCR signalling provides basal survival signals through pathways such as PI3K-AKT and is required for the maintenance of mature B cells [110, 111]. In addition to these tonic survival signals, B-1 cells experience chronic antigen-dependent stimulation through recognition of self-antigens and microbial antigens. This persistent BCR engagement contributes to the unique activation state and functional properties of the B-1 compartment. The transcription factor Nur77 acts as a sensor of chronic antigen receptor engagement, helping to restrain excessive activation while preserving responsiveness to persistent stimulation [112]. In parallel, the RNA-binding protein HuR regulates post-transcriptional expression of components of the BCR signalling machinery and survival pathways, including BAFFR, thereby promoting B-1 cell fitness and longevity [113].

Given that the B-1 cell repertoire is enriched for both self-reactive and commensal-reactive specificities, precise control of BCR signalling strength is essential to prevent aberrant activation. In this context, inhibitory proteins of the sialic acid-binding immunoglobulin-like lectin (Siglec) family play a critical role. While CD22

(Siglec-2) primarily regulates BCR signalling in conventional B-2 cells, Siglec-G functions as the dominant inhibitory co-receptor in B-1 cells [114, 115]. Siglec-G directly associates with the BCR via sialic acid-dependent binding to surface IgM, enabling efficient suppression of BCR-induced signalling, including calcium mobilization [115, 116]. This interaction is particularly effective in B-1 cells, where the glycosylation pattern favours Siglec-G recruitment to the BCR complex [117]. Through this mechanism, Siglec-G dampens both basal BCR signalling and antigen-induced activation, thereby limiting B-1 cell activation, proliferation and survival. Consistent with this role, Siglec-G deficiency results in enhanced BCR signalling, increased calcium flux and expansion of the B-1a cell compartment [116, 118].

Importantly, dysregulation of this inhibitory pathway has direct implications for leukemogenesis. In the T cell leukemia/lymphoma 1 (TCL-1) transgenic (TCL-1tg) model of CLL, Siglec-G deficiency accelerates disease onset and increases disease severity, indicating that loss of inhibitory BCR control promotes malignant transformation and expansion of CD5+ B cells [119]. Conversely, increased Siglec-G expression protects against CLL development, highlighting its role as a critical checkpoint restraining pathogenic BCR signalling.

Together, these findings demonstrate that B-1 cell persistence is an actively regulated process requiring a finely balanced interplay between self-renewal programs, basal survival signalling, chronic antigenic stimulation and inhibitory receptor pathways. Transcription factors such as BHLHE41, TCF1 and LEF1 establish and maintain the B-1 cell state, while signalling regulators including Nur77, HuR and Siglec-G ensure that survival and activation signals remain appropriately calibrated. Through these mechanisms, B-1 cells achieve lifelong maintenance while minimizing the risk of pathological expansion and leukemic transformation.

Innate-like activation and effector functions

B-1 cells provide a rapid first line of defence against pathogens through innate-like activation pathways. They can be activated independently of BCR engagement via pattern-recognition receptors (PRRs), including Toll-like receptors (TLRs), or through cytokine receptors such as the IL-5 receptor. These signals trigger rapid differentiation into antibody-secreting PCs [120-122]. Through their ability to recognize conserved pathogen-associated molecular patterns (PAMPs), B-1 cells initiate immediate immune responses that help contain infection until more specific adaptive responses are established [123]. Their protective role has been demonstrated in infections with pathogens such as *Streptococcus pneumonia*, *Borrelia hermsii*, *Francisella tularensis* as well as influenza virus infection [124-127].

In addition B-1 cells have been found to function as antigen-presenting cells [128]. Notably, peritoneal B-1 cells have been shown to internalize bacteria via BCR-dependent phagocytosis [129], and to promote regulatory T cell (Treg) activation through antigen presentation [130-132]. These findings highlight their functional versatility across both innate and adaptive immunity.

IL-10 production and immune regulation

B-1 cells play a central role in immune regulation. They are a major source of the anti-inflammatory cytokine IL-10 [86, 133-135]. IL-10 exerts broad immunosuppressive effects, including inhibition of T helper cell type 1 (TH1) responses, downregulation of major histocompatibility complex class II (MHC II) expression and suppression of macrophage and monocyte activation [136, 137]. B cell-derived IL-10 is essential for maintaining immune tolerance and limiting immunopathology. Its protective role has been demonstrated in multiple disease models, including experimental autoimmune encephalomyelitis, collagen-induced arthritis and intestinal inflammation [138-141].

Regulatory B cells capable of producing IL-10 are collectively referred to as B10 cells [142]. Importantly, B10 cells do not constitute a distinct lineage defined by a specific surface phenotype but instead represent a functionally defined and heterogeneous group of B cells, capable of inducing IL-10 in response to innate and adaptive immune stimuli [143]. Within this context, B-1 cells represent a major contributor to the B10 compartment. However, IL-10 production varies across anatomical locations and subsets, with peritoneal B-1a cells displaying the highest constitutive IL-10 levels, followed by B-1b cells, and lower levels in splenic B-1 cells [144]. Thus, even within the B-1 compartment constitutive IL-10 production varies substantially. **A deeper understanding of the developmental origins of regulatory B10 cells may help refine and stratify this functionally defined group (Paper IV).**

Natural antibodies and tissue homeostasis

B-1 cells perform essential functions that maintain tissue homeostasis under steady-state conditions [145]. A hallmark feature of this subset is the constitutive production of natural antibodies (NAbs), for which B-1 cells represent the primary source [146, 147]. These antibodies are present prior to any cognate antigen encounter and are evolutionarily conserved across species. NAbs are predominantly germline-encoded IgM antibodies characterized by low affinity but broad poly-reactivity, enabling recognition of a wide range of structurally diverse antigens [148]. Importantly, NAbs are readily detectable in germ-free animals, demonstrating that their production occurs independently of microbial exposure and is largely driven by intrinsic developmental programs [149].

NAbs provide immediate protection against invading pathogens by recognizing conserved microbial structures, thereby contributing to early immune defence before the establishment of high-affinity adaptive responses. Another defining feature of NAbs is their ability to recognize self-antigens, which is a direct consequence of the enriched self-reactive BCR repertoire of B-1 cells. This intrinsic autoreactivity is not pathological but instead serves critical homeostatic functions [145, 148].

One of the most important roles of NAbs is the recognition and clearance of apoptotic cells and cellular debris, a process that is essential for maintaining tissue homeostasis. During apoptosis, profound changes in membrane composition and lipid asymmetry lead to the exposure of intracellular membrane components that act as “eat-me” signals that are efficiently bound by natural IgM. Through this binding, NAbs opsonize apoptotic cells, facilitating their uptake by phagocytes via complement receptors and scavenger pathways [95, 150]. Efficient removal of apoptotic material is critical for preserving tissue integrity and immune tolerance. Impairments in this pathway, including defective opsonization or phagocytic clearance, have been strongly associated with immune dysregulation and the development of autoimmune diseases such as systemic lupus erythematosus [151, 152].

Beyond apoptotic cell clearance, NAbs contribute to several additional immune processes. They play a key role in complement activation, particularly through the classical pathway, enhancing opsonization and pathogen elimination. Furthermore, NAbs modulate inflammatory responses by regulating cytokine production and influencing innate immune cell activation. Emerging evidence also indicates that NAbs shape early adaptive immune responses by facilitating antigen uptake and presentation, thereby influencing subsequent B and T cell activation [150, 153-155].

The preferential selection of self-reactive BCR specificities into the B-1 repertoire underlies these diverse functional capacities. By maintaining a pool of broadly poly-reactive antibodies that recognize conserved self- and non-self-structures, B-1 cells generate a preconfigured system that supports both rapid host defence and tissue homeostasis [85, 150]. Consequently, NAbs occupy a unique position at the interface of innate and adaptive immunity, contributing to early protection against infection while promoting immune tolerance and limiting inflammation [148, 153, 154].

Human B-1 cells

B-1 cells are well defined in mice as a developmentally restricted, innate-like B cell population that bridges innate and adaptive immunity and can be reliably identified based on a combination of phenotypic markers [88]. In contrast, the identity and even existence of B-1 cell counterparts in humans has long been a subject of considerable debate [156-161].

This controversy largely stems from the absence of a clear and universally accepted phenotypic definition in humans, as well as the difficulty of directly translating mouse classification schemes to the human immune system. For example, CD5 expression, widely used as a defining marker of mouse B-1a cells, is much more broadly expressed across multiple human B cell subsets, limiting its specificity [162]. Although populations with B-1-like properties, such as CD20+, CD27+, CD43+ B cells, have been described in humans [158], their functional equivalence to mouse B-1 cells remained contested. Several studies have suggested that these cells may instead represent activated MBCs or pre-plasmablast populations, rather than a distinct B-1 lineage [156, 159, 161, 163, 164].

Recent advances have begun to provide more direct evidence supporting the existence of human B-1 cells. Transcriptomic analyses of human embryonic tissues have identified B cells with molecular signatures consistent with mouse B-1 cells, suggesting a conserved developmental origin [165]. In addition, work from the Vinuesa laboratory demonstrated that the transcription factors TCF1 and LEF1, which are critical regulators of mouse B-1a cells, are also expressed in B-1-like populations identified in human pleural fluid and CLL [106].

Together, these findings support the existence of a human B-1 cell counterpart, while also highlighting that its phenotypic definition remains less clearly delineated than in mice. Consequently, current efforts increasingly rely on functional, transcriptional and developmental criteria, rather than surface marker expression alone, to define B-1-like populations in humans. **Further characterization of the shared molecular programs between mouse B-1 cells and human B-1-like populations will be critical for defining their identity and elucidating their role in human immunity and disease (Paper II).**

IgM memory B cells

Immunological memory in the B cell compartment has traditionally been viewed through the lens of class-switched MBCs, which arise from TD GC reactions and produce high-affinity, isotype-switched antibodies that mediate durable antigen-specific immunity. However, it is now clear that immunological memory is not restricted to class-switched populations. A substantial fraction of MBCs retains IgM expression, giving rise to the concept of IgM memory. Initially, IgM memory cells were considered a distinct population associated with TI immune responses, limited SHM, and restricted CSR [166-168]. More recent work, however, has revealed considerable heterogeneity within the IgM memory compartment and challenged the notion that IgM memory represents a single developmental lineage.

A central issue lies in the definition and phenotypic identification of IgM MBCs. In mice, these cells are typically defined through lineage-tracing, adoptive-transfer,

and functional recall studies and can be further subdivided using markers such as PD-L2, CD80, and CD73, where PD-L2+, CD80+, CD73+ subsets exhibit enhanced recall capacity and represent highly experienced memory populations [169, 170]. However, naturally occurring polyclonal IgM memory populations remain relatively poorly characterized, largely because mice lack a broadly useful marker equivalent to human CD27. Consequently, much of our current understanding derives from antigen-specific experimental systems, often employing BCR-transgenic cells transferred at the time of immunization, whereas the biology of endogenous IgM memory populations remains incompletely understood.

In humans, IgM memory cells are commonly identified phenotypically as CD27+, IgM+ B cells. However, this population is increasingly recognized as heterogeneous and likely encompasses cells of multiple developmental origins [171, 172]. This ambiguity is further complicated by differences in GC origin. In mice, IgM MBCs can arise through both GC-dependent and GC-independent pathways and frequently retain the capacity to re-enter GCs during recall responses [173, 174]. Evidence from humans likewise suggests that IgM memory cells are developmentally heterogeneous. While some populations appear to be GC-derived, others may arise through alternative pathways associated with early-life immune responses or TI antigen exposure [166, 175].

A major source of debate concerns the relationship between IgM memory and the MZ compartment. Earlier models proposed that a substantial proportion of human CD27+, IgM+ B cells represented a specialized MZ population dedicated to rapid responses against blood-borne polysaccharide antigens [176, 177]. However, more recent studies suggest that human MZ B cells possess many hallmarks of adaptive immunity, including antigen experience, somatic diversification, and memory-like recall properties [178-180]. Thus, rather than representing a purely innate-like compartment, human MZ B cells may constitute an important component of the broader IgM memory landscape. In contrast, MZ B cells and IgM MBCs are generally considered more distinct populations in mice, contributing to a clearer conceptual separation between innate-like and memory compartments [181, 182]. The lack of direct human equivalents to the mouse PD-L2, CD80, and CD73 memory subsets further complicates cross-species comparisons and contributes to the ongoing controversy surrounding IgM memory [172].

These definitional and developmental differences translate into uncertainty regarding function. In mice, IgM MBCs are generally described as long-lived cells capable of re-entering GCs and supporting further affinity maturation during secondary immune responses, whereas class-switched MBCs preferentially differentiate into antibody-secreting cells [170, 174]. However, the functional properties of naturally occurring polyclonal IgM memory populations remain poorly understood. In humans, IgM+ MBCs have been implicated in both classical adaptive recall responses and rapid effector responses to microbial challenge [168, 183]. Collectively, these observations support the view that the human IgM

memory pool comprises multiple biologically distinct subsets rather than a single homogeneous lineage.

Notably, important commonalities exist between human IgM MBCs and mouse B-1 cells. Both populations exhibit rapid differentiation into PCs, preferential responses to TI antigens such as polysaccharides, and production of NABs that contribute to early host defence [168, 180, 184, 185]. Both also participate in mucosal immunity, including the generation of microbiota-reactive IgA responses [186, 187]. In addition, both populations are associated with splenic function. IgM MBCs are markedly reduced in asplenic patients [176], while B-1a cells show strong dependence on the spleen for their development and maintenance [188], although FL-derived B-1a cells can arise in its absence [189]. While these similarities have fuelled speculation that B-1-like populations contribute to the human IgM memory compartment, direct lineage relationships remain difficult to establish.

Despite these parallels, the classification of B-1 cells within the IgM memory compartment remains controversial. One point of debate concerns somatic diversification. Mouse B-1 cells were historically considered largely germline-encoded and SHM-negative, whereas human IgM memory cells typically harbour somatically mutated IG genes [87, 175]. However, this distinction has become increasingly blurred. Human IgM memory populations early in life can exhibit very limited SHM [190], whereas mouse B-1 cells accumulate mutations with age and antigen exposure [99]. These findings suggest that the degree of SHM may reflect antigenic history rather than lineage identity. A second and perhaps more fundamental distinction relates to the nature of the antigenic stimulus. B-1 cells are maintained in part through chronic stimulation by self-antigens and other endogenous ligands [85, 112], whereas classical MBCs are generally following a discrete antigen encounter and persist independently of continued antigen exposure [191, 192]. Consequently, whether B-1 cells should be regarded as a specialized form of IgM memory cell or as a distinct lineage of chronically activated innate-like B cells remains an area of active debate.

Together, these observations challenge a strict separation between innate-like and adaptive memory compartments and instead support a model in which IgM memory represents a heterogeneous continuum encompassing both GC-dependent and GC-independent populations. In line with this view, the Weill and Reynaud laboratory proposed a layered model of B-cell memory, analogous to the layered organization of the immune system itself [44], in which distinct memory compartments emerge at different developmental stages and perform specialized functions [167, 193]. Within this framework, innate-like populations such as B-1 cells and MZ-associated IgM memory may constitute an early evolutionary layer optimized for rapid responses and tissue homeostasis, whereas GC-derived IgM and class-switched MBCs represent later layers that provide increasingly refined and adaptable immunity. **Reconciling these layers across mouse and human**

systems remains a key challenge for understanding the role of IgM memory in protective immunity and disease (Papers I & II).

Chronic Lymphocytic Leukemia

General characteristics and precursor state

CLL is the most common leukemia in adults in the Western world and predominantly affects elderly individuals, with a median age at diagnosis of approximately 70 years. The disease is characterized by the clonal expansion and accumulation of small, mature, CD5+, antigen-experienced B lymphocytes in the peripheral blood, BM, and secondary lymphoid tissues [194-196]. Although the leukemic cells appear phenotypically mature, they exhibit profound abnormalities in survival, activation and interactions with the microenvironment. Clinically, CLL displays marked heterogeneity, ranging from indolent forms requiring no immediate therapy to aggressive disease characterized by rapid progression, treatment resistance and shortened survival [197, 198].

CLL is widely considered a disease that develops over decades and is preceded by monoclonal B cell lymphocytosis (MBL), a premalignant condition characterized by clonal B cell expansion detectable in otherwise healthy individuals. MBL is highly prevalent in the elderly population and shares many immunophenotypic, genetic and molecular features with CLL, suggesting that it represents an early stage in leukemogenesis rather than a distinct biological entity [199, 200]. Longitudinal studies have demonstrated that virtually all cases of CLL are preceded by an MBL phase, although only a minority of MBL clones progress to overt leukemia. These observations support a model in which transformation occurs gradually through the sequential acquisition of genetic and epigenetic alterations together with selective pressures imposed by antigen stimulation and the tissue microenvironment.

Clinical and molecular classification

Historically, CLL has been staged using the Rai and Binet classification systems, based on clinical parameters such as lymphocytosis, cytopenias, and lymphoid organ involvement [201, 202]. These systems remain valuable for assessing disease burden and predicting overall survival; however, they provide limited insight into the underlying biology of the disease and are less effective at identifying patients likely to experience rapid progression.

A major advance in CLL classification came with the recognition of IGHV mutation status as a powerful prognostic marker. Patients with mutated CLL (M-CLL), defined by <98% sequence identity of the rearranged IGHV gene to the closest

germline IGHV gene, typically exhibit an indolent disease course and prolonged survival. In contrast, unmutated CLL (U-CLL), defined by >98% IGHV germline identity, is associated with more aggressive clinical behaviour, shorter time to first treatment and inferior clinical outcome [203-207]. Beyond its prognostic value, IGHV mutation status reflects fundamental biological differences between the two disease subtypes, including their developmental history, antigen exposure, epigenetic landscape and BCR signalling properties. Thus, the distinction between M-CLL and U-CLL remains a cornerstone of modern CLL classification and provides important insights into disease pathogenesis.

Stereotyped BCRs, antigen selection and BCR signalling

A defining hallmark of CLL is the presence of stereotyped BCRs, highly similar or nearly identical IG sequences shared among unrelated patients. The existence of these stereotyped receptors provides strong evidence that CLL development is shaped by antigen selection rather than random BCR generation, implying that particular antigen-binding specificities are repeatedly recruited into the leukemic compartment [208-212].

Many stereotyped CLL BCRs display poly- or self-reactive binding properties and closely resemble receptors found within early developmental and innate-like B cell compartments, including B-1 cells in mice [213]. Importantly, CLL-associated BCRs are not unique to leukemia but can be detected within the normal B cell repertoire long before disease onset. Analysis of the human FL IG repertoire revealed innate-like B cell populations with repertoire characteristics shared with CLL [214], supporting the notion that components of the leukemic repertoire may originate from fetal B cell development. Furthermore, CLL-biased stereotyped IG rearrangements have been identified at low frequencies across multiple normal B cell subsets and are particularly enriched among CD5+ B cells [215], indicating that these receptors constitute part of the physiological B cell repertoire rather than disease-specific rearrangements. Consistent with this idea, stereotyped CLL-like sequences have also been detected in cord blood, BM and peripheral blood from healthy individuals [216], demonstrating that such receptors emerge early in ontogeny and persist throughout life. Together, these findings support a model in which CLL develops through the selective expansion and transformation of B cell clones derived from a developmentally programmed repertoire established during early life, potentially within fetal-derived or innate-like B cell lineages.

Beyond receptor structure, BCR signalling is a central driver of CLL pathogenesis. CLL cells can receive survival and proliferative signals through both antigen-dependent and antigen-independent mechanisms. A landmark study by the Jumaa laboratory demonstrated that CLL-derived BCRs are capable of cell-autonomous signalling in the absence of external antigen engagement, indicating that the BCR itself can directly promote leukemic-cell survival and proliferation [217]. This

autonomous signalling arises from receptor-receptor interactions mediated by characteristic IG motifs and represent a unique property of CLL BCRs. In parallel, signalling triggered by self-antigens and environmental antigens contributes to leukemic-cell activation, survival and interactions with the tissue microenvironment [218-220]. Thus, both intrinsic signalling capacity and antigen-driven stimulation are thought to contribute to disease initiation, maintenance and evolution.

The biological importance of BCR signalling is further illustrated by the existence of stereotyped BCR subsets. Distinct subsets are associated with characteristic clinical outcomes, signalling properties and recurrent genetic lesions. For example, specific stereotyped subsets are enriched for mutations in genes such as NOTCH1 and SF3B1, indicating that antigenic selection and genetic evolution are closely linked processes that contribute to the emergence of biologically distinct forms of CLL [208-211, 221, 222].

The central importance of BCR signalling is underscored by therapeutic advances. Pharmacological inhibition of key signalling components, most notably Bruton's tyrosine kinase (BTK), has transformed the treatment landscape of CLL. Clinical responses to BTK inhibitors such as ibrutinib and acalabrutinib provide direct evidence that continued BCR signalling is required for disease maintenance and progression [223-225]. Thus, BCR stereotypy, antigen recognition and signalling activity together form a core axis underlying CLL pathogenesis.

Genetic lesions, chromosomal alterations and clonal evolution

CLL is characterized by the gradual accumulation of genetic lesions and chromosomal alterations, rather than a single defining oncogenic event. Large-scale genomic studies have identified recurrent mutations affecting genes such as TP53, NOTCH1, SF3B1, and ATM as well as pathways including BCR signalling, NF- κ B activation, RNA processing and DNA damage response [226-230].

In addition to gene mutations, characteristic chromosomal abnormalities represent key drivers of disease biology. The most frequent alterations include:

- Deletion 13q14 – the most common lesion, generally associated with favourable prognosis
- Trisomy 12 – associated with intermediate prognosis and distinct biological features
- Deletion 11q22-23 (ATM locus) – linked to aggressive disease and lymphadenopathy
- Deletion 17p13 (TP53 locus) – associated with poor prognosis and therapy resistance [231, 232]

CLL exhibits pronounced subclonal heterogeneity, with multiple genetically distinct clones coexisting within a single patient. Longitudinal analyses have revealed dynamic clonal evolution, in which subclones expand or contract over time, particularly under selective pressures such as therapy [228, 233].

While M-CLL cases may exhibit a higher overall mutational burden, U-CLL is enriched for high-risk driver mutations, consistent with its more aggressive clinical behaviour [230, 234]. The long latency between MBL and overt disease further supports a model in which stepwise genetic evolution occurs within a long-lived, self-renewing B cell compartment.

Epigenetic landscape

Epigenetic alterations constitute a central layer of CLL biology and provide important insights into both disease pathogenesis and cellular origin. Genome-wide analyses have revealed widespread disruption of the DNA methylation landscape, characterized by extensive hypomethylation across gene bodies, intergenic regions and regulatory elements, together with single gene promoter hypermethylation affecting genes involved in tumour suppression, cell-cycle control and cellular differentiation [235-237]. These alterations contribute to aberrant transcriptional programs that promote leukemic-cell survival and shape interactions with the microenvironment.

Importantly, DNA methylation profiling has identified three major epigenetic subtypes, naïve-like, memory-like and intermediate CLL, that closely resemble distinct stages of normal B cell differentiation [235, 238]. These epigenetic classifications correlate strongly with IGHV mutation status, with U-CLL typically exhibiting a more naïve B cell-like methylation profile and M-CLL displaying features of antigen-experienced MBCs. Notably, the epigenetic subtype captures biological variation that is not fully explained by IGHV status alone, highlighting the complex relationship between cellular origin, antigen experience and disease evolution.

Longitudinal studies have further demonstrated that DNA methylation patterns remain remarkably stable throughout disease progression and treatment, indicating that much of the epigenetic landscape is established prior to malignant transformation and subsequently maintained during clonal expansion. As a result, epigenetic signatures serve as a molecular record of the developmental history of the leukemic clone. These findings reinforce the concept that disease heterogeneity is strongly influenced by cellular origin and support models in which distinct CLL subtypes arise from B cells that have traversed different developmental and antigen-exposure pathways before transformation [235, 236, 238].

Immunoregulation and the role of IL-10

CLL is associated with profound immune dysfunction, in part driven by the immunoregulatory properties of the malignant B cells themselves. A central mediator of this phenotype is IL-10, which is produced at elevated levels by CLL cells, particularly following BCR stimulation. Through its effects on both innate and adaptive immune cells, IL-10 suppresses anti-tumour immunity by inhibiting the activation, proliferation and effector differentiation of CD4⁺ and CD8⁺ T cells, while simultaneously impairing dendritic cell maturation and antigen presentation [137, 239, 240]. In addition to facilitating immune evasion, IL-10 may also act directly on leukemic cells through autocrine signalling pathways that enhance survival and reinforce the immunosuppressive phenotype of the tumour microenvironment.

Importantly, IL-10 production is a well-established feature of several innate-like B cell populations, particularly B-1 cells, which constitutively secrete IL-10 and can rapidly increase its production following stimulation through TLRs, microbial products, or BCR signalling pathways [137, 241]. In physiological settings, this function contributes to the resolution of inflammation, maintenance of tissue homeostasis, and prevention of excessive immune activation [242, 243]. These regulatory properties are particularly prominent during early life, when B cell responses are biased towards immune regulation and tolerance rather than highly inflammatory adaptive responses [241, 244]. The observation that CLL cells retain the capacity for robust IL-10 production therefore strengthens the hypothesis that the disease exploits developmental programs normally associated with innate-like and early-life B cell populations.

The immunological consequences of elevated IL-10 production are likely multifaceted. Beyond suppressing anti-tumour T cell immunity, excessive IL-10 may contribute to the broader immune defects characteristic of CLL, including impaired vaccine responses, dysfunctional T cell activation and reduced pathogen clearance. Such mechanisms likely contribute to the high incidence of recurrent bacterial and viral infections observed in CLL patients, which remain a leading cause of morbidity and mortality despite substantial advances in targeted therapy [245, 246].

Cell type(s) of origin

The cellular origin of CLL remains a central and unresolved question, with evidence supporting multiple, potentially overlapping models. A dominant model proposes that CLL arises from mature B cells at different stages of the GC reaction, with M-CLL deriving from post-GC, antigen-experienced MBCs, and U-CLL from pre-GC, naïve-like B cells [203, 204, 247, 248]. This model is supported by the

presence or absence of SHM and has long formed the basis for distinguishing the two major clinical subtypes.

However, this dual-origin hypothesis is challenged by the observation that M-CLL and U-CLL share remarkably similar global gene expression profiles, suggesting that they may arise from a common progenitor or closely related mature B cell pool, rather than from fundamentally distinct lineages [248, 249]. This has led to the alternative view that CLL may originate from a single population of antigen-experienced B cells, with divergence into M-CLL and U-CLL reflecting differences in antigen exposure, signalling history, or microenvironmental interactions rather than distinct cellular origins.

Additional evidence supporting an antigen-experienced origin comes from phenotypic analyses showing that CLL cells express activation and co-stimulatory molecules, consistent with prior antigen encounter [194]. Moreover, CLL cells uniformly express CD27, a canonical marker of human MBCs and B cell activation [194, 250], and CD27 levels have been associated with tumour burden, further linking this phenotype to disease biology [251].

Within this framework, increasing attention has been given to innate-like B cell populations, particularly as models for U-CLL. While all CLL cells are mature B cells, U-CLL shares several features with B-1-like cells, including restricted, often self-reactive BCR repertoires, poly-reactivity, and long-term persistence [248, 252]. These similarities, together with experimental evidence that B-1 cells can give rise to CLL-like disease [253, 254], suggest that innate-like differentiation states may underlie certain CLL subsets, rather than representing a distinct lineage of origin.

A complementary, but more debated, hypothesis proposes that HSCs may contribute to early CLL pathogenesis. Studies have shown that HSCs from CLL patients can harbour premalignant alterations and display a bias toward generating clonal B cell populations, suggesting the existence of a pre-leukemic stem cell compartment [255, 256]. In particular, Kikushige *et al.* demonstrated that patient-derived HSCs can generate independent B cell clones with CLL-like features, indicating that disease-initiating effects may occur prior to B cell differentiation.

However, several observations argue against HSCs as the direct cell of origin. CLL cells retain hallmark features of mature, antigen-experienced B cells, including functional BCR expression, SHM in a substantial fraction of cases, and evidence of antigen-driven selection. The presence of stereotyped BCRs further supports transformation occurring at the level of mature B cells undergoing peripheral selection [249]. Consistently, epigenetic profiling demonstrates that CLL cells closely resemble differentiated B cell subsets rather than stem or progenitor cells [235, 238]. Likewise, transcriptomic analyses demonstrate that CLL aligns more closely with mature B cell populations in healthy individuals. Notably, U-CLL maps to mature CD5+ B cells, whereas M-CLL corresponds to post-GC CD5+, CD27+ MBC subsets [257].

Together, these findings support a model in which CLL arises from mature B cells along a continuum of differentiation states, rather than from distinct cellular lineages. In this context, M-CLL and U-CLL likely represent different developmental or activation states of a related B cell compartment, shaped by antigen exposure and intrinsic signalling properties. **Further stratification and analysis of the developmental origins of CLL may therefore provide important insights into disease initiation and pathogenesis (Papers II & III).**

The TCL-1 transgenic mouse model

Animal models have been instrumental in elucidating the molecular and cellular mechanisms underlying CLL pathogenesis. Among these, the TCL-1tg mouse is the most extensively characterized and widely used model. In this model, expression of the human TCL-1 oncogene is placed under the control of the IGH intronic enhancer (E_{μ}), resulting in B cell-specific overexpression beginning at early stages of B cell development [258]. As the mice age, they progressively develop an accumulation of CD5⁺ B cells in the peripheral blood, spleen, BM and lymphoid tissues, ultimately giving rise to a leukemia that closely resembles human CLL.

TCL-1 functions as a potent oncogene primarily through its ability to enhance signalling through the PI3K-AKT pathway, a central regulator of cell survival, proliferation, metabolism and apoptosis resistance. TCL-1 directly interacts with and activates AKT, promoting increased downstream signalling and prolonged survival of B cells that would otherwise undergo normal homeostatic regulation [259-261]. In addition, TCL-1 influences multiple cellular processes, including transcriptional regulation, genomic stability, NF- κ B signalling and interactions with the tumour microenvironment, thereby creating conditions that favour leukemic transformation and clonal expansion [261, 262]. The gradual development of disease in TCL-1tg mice mirrors the prolonged natural history of CLL in humans and provides a valuable system for studying both disease initiation and progression [258, 263].

Importantly, the TCL-1tg model most closely resembles human U-CLL. The leukemic cells exhibit a CD5⁺, CD19⁺ phenotype, display stereotyped and self-reactive BCRs, depend on BCR-mediated signalling and follow an aggressive disease course similar to that observed in patients with U-CLL [252, 258]. Subsequent studies have further demonstrated that TCL-1-driven leukemia recapitulates many key features of human U-CLL, including genetic, transcriptional, structural, and immunological features [263, 264].

Owing to its reproduction of the immunophenotypic, molecular and clinical features of aggressive IGHV-unmutated disease, the TCL-1tg model is widely regarded as the gold-standard mouse model of human U-CLL and remains the most extensively used platform for studying CLL pathogenesis and therapeutic responses.

Evolutionary origins of the layered immune system

Why the immune system develops through multiple, temporally distinct waves of cell generation rather than relying on a single continuous repertoire remains a fundamental question in immunology. An evolutionary perspective provides a compelling framework to address this. All multicellular organisms have evolved defence mechanisms to ensure survival against internal and external threats. The innate immune system represents the most ancient arm of immunity, with origins tracing back approximately one billion years to early phagocytic unicellular organisms. It provides rapid, broadly reactive protection through the recognition of conserved PAMPs by PRRs, and mediates key effector functions including phagocytosis, inflammation, cytokine production and complement activation. These responses are largely executed by cells of the myeloid lineage and include mononuclear phagocytes (e.g. macrophages) and polynuclear phagocytes (e.g. neutrophils), which form the foundation of early host defence [265, 266].

In contrast, the adaptive immune system emerged later, approximately 500 million years ago in jawed vertebrates, introducing highly specific and flexible modes of antigen recognition. B and T lymphocytes express antigen receptors generated through somatic recombination, enabling the recognition of an immense diversity of antigens. These responses are further refined through processes such as affinity maturation, resulting in highly targeted and long-lived immunity [267, 268]. Importantly, innate and adaptive immune systems have co-evolved and are functionally integrated. Complex cytokine-mediated cross-talk between these systems allows for the activation, modulation and resolution of immune responses. Antigen presentation by dendritic cells of the innate immune system is essential for initiating adaptive immune responses, while conversely antibody-mediated opsonization enhances pathogen clearance by innate phagocytes [265, 269]. In addition, innate-like lymphocytes, including B-1 cells, blur the distinction between innate and adaptive immunity, highlighting the layered and interconnected nature of the immune system [270].

Within this evolutionary framework, the emergence of temporally distinct waves of immune cell development during ontogeny can be interpreted as a reflection of how evolution builds on pre-existing functional systems. Rather than replacing older immune strategies, evolutionary processes tend to preserve and adapt them, layering new functions on top of established ones. This has led to a layered organization in which evolutionary ancient innate programs are retained and supplemented by more recently evolved adaptive functions [44, 271, 272].

During fetal and neonatal life, sequential waves of hematopoiesis give rise to immune populations that are largely innate or innate-like, including tissue-resident macrophages, innate lymphoid cells and B-1 lymphocytes. These cells exhibit limited receptor diversity but are preconfigured for rapid responses, tissue

homeostasis and tolerance to self and commensal antigens, features that resemble ancestral immune strategies and are particularly suited to the developing organism. Importantly, many of these early-life-derived populations persist into adulthood and perform non-redundant roles, contributing to long-term immune function.

Subsequent waves of hematopoiesis, dominated by adult HSCs, establish the conventional adaptive immune system, characterized by highly diverse B and T cell repertoires. Crucially, these layers do not replace earlier ones but instead integrate with them, creating a composite immune system in which ancient and modern components coexist and cooperate. Thus, the layered architecture of immune development reflects both ontogenetic requirements and the evolutionary transition from primordial innate defences to highly specialized adaptive immunity.

Lineage tracing tools to study early-life-origin (ELO) B cell waves

Understanding how distinct waves of B cell development contribute to the adult immune system requires approaches that enable their study under physiological, steady-state conditions. Early life hematopoiesis generates temporally stratified B cell populations, yet their long-term persistence, turnover, and functional specialization remain poorly resolved. A central limitation has been the inability to discriminate B cells generated at different developmental stages without perturbing endogenous homeostasis. Inducible lineage tracing strategies overcome this challenge by enabling time-resolved labelling of cells and their progeny *in vivo*.

The Cre-loxP system forms the backbone of most lineage tracing approaches, allowing site-specific DNA recombination *in vivo*. Temporal control was achieved with the development of tamoxifen-inducible Cre recombinases (CreERT2), generated by fusing Cre to a modified estrogen receptor ligand-binding domain. In these systems, CreERT2 remains cytoplasmic under basal conditions and translocates to the nucleus upon tamoxifen administration, where it catalyses recombination between loxP sites [273, 274]. When combined with reporter alleles such as *R26^{fl-STOP-Tom}*, recombination irreversibly excises the STOP cassette, resulting in permanent fluorescent TdTomato (Tom) labelling of targeted cells and all their progeny [275].

To specifically interrogate B cell ontogeny, CreERT2 expression can be driven by the *Mb1* (*Cd79a*) promotor, which is active from the earliest stages of B cell lineage commitment. Mb1-based models enable highly efficient and lineage-restricted recombination across the B cell compartment, and inducible Mb1-CreERT2 systems have been used to achieve temporally controlled genetic targeting in B cells [276].

Importantly, administration of tamoxifen at defined early-life time points, such as fetal, neonatal, or early postnatal stages, enables the “time-stamping” of distinct B cell waves. B cells present during the induction window are irreversibly labelled (Tom+), whereas subsequent B cell waves remain unlabelled (Tom–). **This temporal stratification allows analysis of early-life-origin (ELO) B cell waves and facilitates direct assessment of their persistence, turnover, and functional contributions to the adult immune system under steady-state conditions (Papers I, II & IV).**

Thesis Aims

The overarching aim of this thesis has been to characterize the unique fetal/neonatal developmental window and to determine how B cells generated during early life shape the adult immune system, thereby establishing the foundation for both beneficial immune homeostasis and malignant immune transformation. This work highlights how early-life-programmed immunity forms a long-lasting layer of the adult B cell compartment with profound consequences for immune regulation and leukemia initiation. The papers included in this thesis specifically aim to:

- Dissect the developmental origin of adult B cell subsets and characterize their contribution to immune function during unperturbed hematopoiesis using lineage tracing
- Define the molecular signatures of ELO B cells and establish the link between early-life immune memory and age-associated leukemia formation using lineage tracing
- Investigate the role of LIN28B in reinitiating early-life developmental programs that renders ELO B cells susceptible to leukemic transformation later in life
- Interrogate the ontogenetic origin of immunoregulatory IL-10-producing B cells and define the underlying developmental requirements

Summaries of Included Papers

Paper I

The notion that certain immune cells are generated during restricted developmental windows has inspired the theory of a layered immune system [44]. This model proposes that the adult immune system is established through ontogenetically distinct waves of hematopoiesis, each producing immune cells with unique functions and properties. B-1 B cells are a prototypical example of such an developmentally restricted lineage, arising predominantly during early life and persisting in the organism long-term through self-renewal [85]. Historically, the study of ontogenetic B cell waves has relied heavily on transplantation-based assays [23, 56, 68, 71]. Although these approaches are powerful for assessing the differentiation potential of stem and progenitor cells, they do not fully recapitulate the behaviour of these cells under steady-state physiological conditions [277].

In Paper I, we established an *in situ* lineage tracing model based on tamoxifen-inducible Mb1-CreERT2 to label distinct early developmental B cell waves (Tom⁺), while subsequently generated B cells remain unlabelled (Tom⁻). This strategy enabled us to “time-stamp” distinct ELO B cell waves and track them over time under steady-state conditions. Using label induction from E17.5 to 5 weeks postnatally, we defined the developmental window of B-1a cells at steady-state. Most CD5⁺ and PtC-reactive B-1a cells originated from a postnatal developmental window, peaking between day 5 and day 19. Our findings challenged previous transplantation studies that suggested a predominantly prenatal origin [278]. We further identified a previously unrecognized contribution of ELO B cells to multiple adult B cell subsets. ELO B cells were enriched in BM IgM and IgA PCs. While BM IgM PCs have been implicated to derive from early generated B-1 cells, the enrichment of Tom⁺ B cells in BM IgA PCs was unexpected. Consistently, approximately 50% of the small intestine lamina propria (SI-LP) IgA PCs were Tom⁺. Given their importance in mucosal immunity, we tested whether ELO SI-LP IgA PCs persist due to longevity or active maintenance. Following PC ablation, the compartment reconstituted with similar Tom⁺ frequencies, indicating ongoing replenishment by early-life-derived progenitors. Next, we assessed Peyer's patches (PPs) as key inductive sites for IgA responses and found substantial enrichment for Tom⁺ cells among GC B cells and MBCs, but not naïve B cells. Further IgA and IgM repertoire analysis revealed high CDR3 clonal overlap within IgA⁺ subsets and

IgM⁺ subsets but not between them, indicating that ELO IgA⁺ clones are maintained across multiple compartments as a self-sustaining layer of early-life-memory. We identified commensal microbiota as the driving force behind the formation and maintenance of the early-life IgA⁺ memory compartment. Next, we wanted to assess whether early-derived or adult-derived IgA-memory differ qualitatively. We orally infected neonatal or adult mice with rotavirus (RV) and analysed virus-specific IgA PCs 2 and 9 weeks later. In adult-infected mice, IgA PCs were almost exclusively Tom⁻, indicating minimal contribution of ELO B cells to IgA memory. In contrast, neonatally infected mice showed enrichment of Tom⁺ virus-specific IgA PCs at 9 weeks, suggesting early-life imprinting of IgA PCs by neonatal exposure. Furthermore, we identified recruitment of distinct VH gene segments exclusively during neonatal infection that were not observed during adult infection. This highlights qualitative differences in neonatal BCR selection and shows how early-life-specific repertoires can shape the long-lived memory pool. Lastly, we aimed to further dissect the developmental relationships among different B cell subsets using lentiviral cellular barcoding. We observed substantial barcode overlap between peritoneal cavity (PerC) B-1a cells and SI-LP IgA PCs, indicating that these populations share a common HSPC origin. Although, they did not share CDR3 clones, we hypothesize that PerC B-1a cells and SI-LP IgA PCs arise from a shared progenitor and subsequently diverge in an antigen-driven manner.

Taken together, Paper I establishes a genetic time-stamping model that enables precise delineation of B cell developmental origin and reveals previously unrecognized heterogeneity of ELO B cells across multiple adult B cell subsets. The finding that B-1a cells arise predominantly during a postnatal developmental window, coinciding with increasing microbial antigen exposure, challenges the traditional view of B-1 cells as a naïve B cell population. Instead, B-1 cells may function as a memory reservoir of early-life (self-) antigen exposure. Furthermore, we identified a self-sustaining layer of early-life IgA memory shaped by commensal microbiota under steady state conditions and demonstrated that pathogenic challenge during the neonatal period can imprint long-term immune memory. These findings highlight how antigen encounter during a brief early-life window can exert a lasting influence on the composition and function of the adult immune system, fundamentally shaping the long-term B cell memory compartment.

Paper II

In Paper II we investigated the molecular characteristics of ELO B cells and the role of early-life-imprinted B cell memory in CLL development. The fetal and neonatal period represents a unique window that allows self-reactive BCR specificities to enter a long-lived B cell pool [279]. While these clones support early immune protection, they have an increased potential for transformation later in life. In Paper

II we built on our previous findings that ELO B cells form a layer of early-life-imprinted immunity that sets the stage for immune homeostasis and dysfunction in the adult.

To define the molecular composition of ELO B cells, we performed single-cell RNA sequencing (scRNAseq) on Tom-sorted cells from time-stamped mice under steady-state conditions. This analysis confirmed the heterogeneous distribution of ELO B cells across adult B cell subsets. Notably, we identified a distinct transcriptional profile that separated Tom⁺ from Tom⁻ B cells, enriched for genes associated with differentiation, regulatory capacity, and memory. This profile is consistent with prior antigen experience and supports the notion that the ELO B cell compartment is shaped by early microbial exposure (Paper I). To assess whether early-life-derived B cells exhibit a more memory-like chromatin state, we compared chromatin accessibility between Tom⁺ and Tom⁻ cells within the same B cell subsets. PerC B-1 cells and PPs MBCs, both enriched for Tom⁺ cells, showed increased accessibility at memory-associated chromatin regions, consistent with early-life antigen exposure. We further validated this by examining CD80 and PD-L2 expression, two well-established markers of differentiated MBCs, on postnatal day 10 time-stamped B-1 cells. Tom⁺ B-1 cells showed significantly higher expression than Tom⁻ B-1 cells, revealing a heterogeneous pool of IgM memory-like B cells. Consistently, both self-antigen-reactive (PtC) and foreign-antigen-reactive (group A *Streptococcus* (GAS)) Tom⁺ B-1 cells were significantly enriched for CD80/PD-L2 double-positive (DP) cells, indicating that early-life-derived B-1 cells archive early-life-imprinted IgM memory. We next investigated the role of antigen-experienced ELO B cells in CLL development later in life. Projection of previously published human U-CLL signatures onto our mouse single-cell data revealed enrichment within mouse B-1 subsets, particularly in Tom⁺ cells, indicating shared molecular features between human U-CLL and mouse B-1. Using the TCL-1tg CLL model crossed to our time-stamping system, we found that CLL-initiating cells arise before postnatal day 10 and originate exclusively from the Tom⁺ compartment. BCR repertoire analysis confirmed that all expanded clones were Tom⁺, demonstrating that a disease manifesting in advanced age can be driven by cancer-initiating cells established early in life. Tom⁺ clones showed higher SHM levels overall, consistent with an IgM memory identity, although dominant CLL-driving clones remained largely unmutated, as expected for the TCL-1tg model. Finally, we demonstrated that leukemia progression in the TCL-1tg model is driven specifically by aberrant expansion of early-life-imprinted, DP memory B-1 cells. Moreover, the gene-expression signature of human U-CLL patients was significantly enriched for a recently published healthy fetal human B-1 signature, supporting the possibility that human U-CLL originates from a B-1 lineage established early in life.

Collectively, Paper II demonstrates that ELO B cells carry a distinct (self-)antigen-experienced molecular signature and show elevated expression of memory-associated markers within the B-1 compartment. These findings establish early-life

B-1 cells as a reservoir of neonatal immune exposure, their antigenic history directly shaping their memory state. We therefore propose that B-1 cells be considered antigen experienced memory cells rather than naïve B cells. Antigen-driven activation during the neonatal period, mediated by both self-antigens and microbiota-derived stimuli, may be advantageous early in life by promoting the expansion and maintenance of innate-like B cell populations that provide rapid immune protection. However, the persistence of these activated, self-renewing clones throughout life may also increase the risk of pathological outcomes, including autoimmunity and leukemic transformation later in life. Using a CLL model, we showed that cancer-initiating cells arise in early life and that leukemia progression is driven by early-derived, antigen-experienced memory-like B-1 cells. Finally, the shared molecular features between mouse ELO B-1 cells and human U-CLL signatures suggest that a similar early-life-imprinted, antigen-experienced B cell layer may exist in humans and may serve as a source of leukemic expansion later in life.

Paper III

In Paper III we examined how LIN28B-mediated programming generates B cells with increased susceptibility to CLL later in life. Following our findings that ELO B cells form a self-sustaining layer of early-life-imprinted immune memory (Papers I & II) and can serve as a substrate for leukemic transformation (Paper II) we investigated how this developmental program is established. LIN28B, a key developmental master regulator [32] has previously been shown to shape fetal and neonatal lymphopoiesis by lowering BCR selection stringency [27] and enhancing protein synthesis rates during early life [38]. Previous work from the Hardy laboratory demonstrated that transfer of adult B-1a cells from TCL-1tg mice is sufficient to induce CLL in recipient animals, whereas transplantation of adult HSPCs from TCL-1tg donors is not [254], indicating that adult TCL-1tg HSPCs have lost the capacity to generate CLL-initiating cells.

To test whether LIN28B-mediated reprogramming can restore the capacity of adult TCL-1tg HSPCs to generate CLL-initiating cells, we induced transient LIN28B expression following transplantation. Longitudinal tracking showed that transient LIN28B exposure in adult TCL-1tg HSPCs led to markedly increased CLL-like B-1 expansion without affecting progenitor output or early B cell development, indicating enhanced susceptibility rather than altered hematopoiesis. This effect likely reflects LIN28B-mediated selection of early-life-like B cells that are more susceptible to TCL-1-driven oncogenic transformation. Notably, disease pathology was more heterogenous, with TCL-1tg atypical surface phenotypes and solid tumours, suggesting that transient reprogramming broadens the pool of B cells vulnerable to TCL-1-driven transformation. To further examine this, we analysed

the BCR repertoire of expanding clones and found a diverse, adult-like profile with frequent N-nucleotide additions and absence early-life-specific V gene segments V_H-11 and V_H-12, which encode specificity for the self-antigen PtC. While transient LIN28B expression promotes CLL-like expansion, it does not recapitulate the establishment of a fetal and neonatal BCR repertoire. Instead, selection from a more diverse adult-derived BCR repertoire likely underlies the broader and more heterogeneous disease pathology we observed. Finally, we combined a *Lin28b*-deficient (*Lin28b*^{-/-}) model with TCL-1tg mice to assess whether lack of endogenous *Lin28b* during early-life development reduces or delays CLL-like disease formation. There was no significant reduction or delay in CLL-like B-1 expansion in *Lin28b*^{-/-} TCL-1tg mice. This finding suggests that additional, LIN28B-independent factors contribute to the generation of early-life-derived CLL-initiating cells, highlighting a broader developmental landscape in which multiple early-life factors shape the output of ELO B cells susceptible to TCL-1-driven leukemic expansion.

In summary, Paper III demonstrates the importance of the unique early-life developmental window in generating B cells that remain susceptible to malignant transformation later in life. We showed that early-life-specific molecular programs, exemplified here by LIN28B, shape long-term B cell fate and leave a developmental imprint that increases vulnerability to oncogenic transformation. Although ectopic LIN28B expression in adult HSPCs did not recapitulate all aspects of fetal and neonatal development, it was sufficient to reinitiate the production of CLL-initiating cells prone for TCL-1-driven expansion.

Paper IV

In Paper IV we aimed to dissect the developmental origin of B cells with immunoregulatory potential. B cells play a central role in immune protection through antibody production, T cell activation, and the establishment of immune memory. Beyond these classical effector functions, B cells also exert important regulatory roles that help limit inflammation and prevent excessive immune activation. A key mechanism underlying these regulatory functions is the production of the immunoregulatory cytokine IL-10. Multiple B cell subsets have been shown to possess the capacity to produce IL-10; consequently, regulatory IL-10 producing B cells (B10 cells) are defined as a functional state rather than a unique lineage [142, 143]. Among these, B-1 cells are prominent IL-10 producers [133]. We previously demonstrated that early-derived B-1 cells retain neonatal antigen encounters and contribute to shaping the adult memory compartment (Papers I & II). However, LIN28B-mediated programming during the critical early-life window renders B-1 cells susceptible to leukemic transformation with age (Papers II & III). Here, we

investigated the regulatory potential of ELO B cells and the role of LIN28B in shaping the regulatory B10 compartment.

Revisiting a previously generated scRNAseq dataset of time-stamped B cells (Paper II), we identified significantly up-regulated *IL10* gene expression in ELO B cells. This was validated by IL-10 ELISpot and intracellular flow cytometry on sorted Tom⁺ and Tom⁻ B cells, confirming an enrichment of IL-10 producers within the ELO compartment. Next, we stratified IL-10 production across immunophenotypically defined B cell subsets of early-life and adult origin. In all subsets, early-life-derived Tom⁺ B cells showed higher IL-10 production than adult-derived Tom⁻ counterparts, with Tom⁺ B-1 cells exhibiting the highest frequencies. Thus, IL-10 production emerges as a defining hallmark of ELO B-1 cells. To further define the role of ELO B cells in adult immunity, we combined our time-stamping system with a diphtheria toxin receptor (DTR) -inducible mouse model. Time-stamped B cells constitutively expressed DTRs, enabling selective ablation of ELO B cells in adult mice upon diphtheria toxin (DT) administration. Acute depletion of the ELO B layer led to a two- to three-fold reduction in IL-10 producing cells. Given the key role of IL-10 in limiting intestinal inflammation, we next employed a dextran sulfate sodium (DSS)-induced colitis model. Targeted ablation of early-life-derived B cells significantly exacerbated acute intestinal inflammation, demonstrating a critical immunosuppressive role for ELO B cells during colitis. To assess how LIN28B regulates IL-10-producing capacity in ELO B cells, we measured early-derived Tom⁺ B cells in adult *Lin28b*^{-/-} mice. While overall adult B cell subset composition was unchanged, Tom⁺ cells were reduced across subsets, significantly in PerC B-1 cells. Additionally, *Lin28b*^{-/-} Tom⁺ B cells produced less IL-10 than controls, indicating that LIN28B is required for both the abundance and functional competence of ELO B cells. To determine whether the IL-10 reduction observed in *Lin28b*^{-/-} mice has functional consequences we returned to our DSS-induced colitis model. *Lin28b*^{-/-} mice developed significantly exacerbated intestinal inflammation, indicating that LIN28B is an early-life factor required to establish immunoregulatory ELO B cells. Finally, we tested whether restoring ELO B cells could reduce inflammation in DSS-treated *Lin28b*^{-/-} mice. Adoptive transfer of Tom⁺, but not Tom⁻, B cells ameliorated inflammatory disease, indicating that LIN28B-dependent neonatal programming is required for the regulatory function of ELO B cells in intestinal inflammation.

Taken together, Paper IV identifies ELO B-1 cells as key IL-10 producers and shows that their immunoregulatory capacity is tightly linked to the early-life developmental window governed by LIN28B. Using a DSS-induced colitis model, we further demonstrated their critical role in controlling intestinal inflammation, underscoring the importance of B cells generated during this unique early-life period in maintaining immune homeostasis. The work presented in this thesis highlights how developmental stratification of the B cell compartment serves as a powerful tool for dissecting functionally distinct immune layers with key roles in homeostasis

(Papers I & II), as well as potential for malignant transformation (Papers II & III). Paper IV further illustrates how the developmental origin of a cell fundamentally shapes its functional properties.

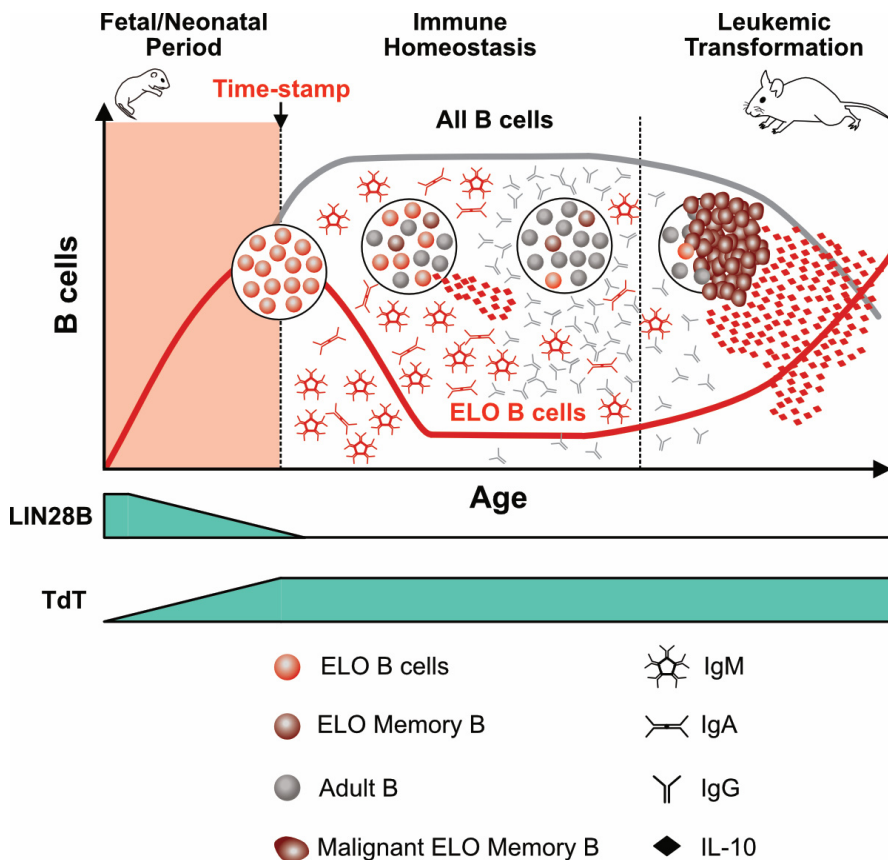


Figure 1. ELO B cells during health and disease. The fetal/neonatal period is characterized by distinct molecular programs that shape early immune development. During this time, LIN28B is highly expressed and gradually declines with age, whereas TdT expression is initially and increases later in life. ELO B cells establish a durable layer of immunity by producing natural IgM and IgA memory. Early-life-derived B-1 B cells are the primary producers of immunoregulatory IL-10, contributing to immune regulation. However, antigen-experienced, memory-like ELO B-1 cells are vulnerable to leukemic transformation later in life.

Discussion, Limitations and Future Directions

The overall aim of this thesis was to define how early-life-derived B cells shape adult immunity during both immune homeostasis and disease. The work presented here identifies distinct features of ELO B cells that equip them with key roles in immune-memory formation and immunoregulatory functions. At the same time, these same characteristics, including longevity, self-renewal capacity and long-term clonal persistence render them vulnerable for leukemic transformation later in life. Although these contrasting outcomes may appear paradoxical, providing immune protection early in life while increasing disease risk with age, they align with the evolutionary concept of antagonistic pleiotropy [280, 281]. This theory proposes that traits conferring a selective advantage early in life can be maintained through evolution despite imposing harmful consequences later in life. Because natural selection strongly favours traits that promote survival and reproductive success early in life, delayed negative effects are retained in the population even when they compromise health in old age.

Early-life-derived B-1 cells exemplify antagonistic pleiotropy at the cellular level. Their longevity, self-renewal capacity and ability to maintain clonal populations throughout life provide a durable source of rapid immune protection and NAb production. However, these same properties also permit long-term persistence of individual clones, increasing the opportunity for the accumulation of genetic and epigenetic alterations that can ultimately drive leukemogenesis. In this regard, B-1 cells share key characteristics with other long-lived, self-renewing populations, such as HSCs, where lifelong persistence enables the accumulation of genetic and epigenetic lesions that can ultimately drive age-associated malignancies [282, 283]. While B-1 cells enhance immune protection during early life, their inherently self-reactive repertoire may also contribute to autoimmune pathology when tolerance mechanisms fail [87, 254, 284, 285]. The findings presented in this thesis further support the notion that developmental origin has lasting consequences for immune cell function, influencing both protective and pathological immune responses well beyond the developmental period in which these cells were generated.

The establishment of long-lived, self-maintaining immune cell compartments during discrete early developmental windows is not unique to B-1 cells but is also well described for several other immune lineages. Innate-like T cell populations

such as $\gamma\delta$ T cells, invariant natural killer T (iNKT) cells and mucosal-associated invariant T (MAIT) cells predominantly arise during early developmental windows and are maintained throughout life [272, 286, 287]. Similarly, many tissue-resident macrophage populations originate from embryonic progenitors and colonize peripheral tissues before birth, where they are maintained through local proliferation independently of adult HSC input [288, 289]. Together, these examples suggest that the immune system utilizes a conserved developmental strategy in which cells generated during early life establish durable and functionally specialized compartments that provide rapid, context-adapted immune protection throughout adulthood.

Despite growing recognition of developmental layering across the immune system, the long-term consequences of developmental origin have been studied more extensively in tissue-resident macrophages and innate-like T cell populations than in B cells [47, 290, 291]. A major obstacle has been the lack of approaches capable of tracking discrete developmental B cell waves into adulthood. To address this gap, we employed tamoxifen-inducible lineage tracing to follow fetal- and neonatal-derived B cells and determine their contribution to the adult immune system. This strategy enabled the stratification of mature B cell populations according to developmental origin independently of conventional phenotypic classifications and revealed previously unrecognized heterogeneity within the adult B cell compartment.

Ontogenetic stratification reveals B-1 cells as an archive of early-life antigen experience

Using tamoxifen-inducible lineage tracing, we uncovered a previously unrecognized heterogeneous distribution of ELO B cells across multiple adult B cell subsets (Paper I). This approach also enabled us to dissect the developmental origin of the B-1 compartment with greater precision, demonstrating that B-1 cells are generated predominantly during neonatal life, rather than arising mainly from fetal hematopoiesis, as previously proposed [278] (Paper I). This finding has important implications for how B-1 cells are conceptualized. Traditionally, B-1 cells have been regarded as a naïve population, due to their presumed fetal origin and persistence in germ-free mice [85, 99, 181]. However, their predominant generation during the neonatal period places them within a developmental window characterised by rapidly increasing microbial exposure and the concurrent generation of other antigen-experienced B cell populations.

This revised ontogenetic model raises the possibility that at least a proportion of adult B-1 cells are not simply developmentally distinct naïve lymphocytes but instead represent long-lived cells that retain a record of antigen encounters

experienced early in life. Consistent with this hypothesis, our transcriptional analyses identified a shared signature of prior antigen experience among ELO B cells across multiple tissues (Paper II). These data suggest that long-lived ELO B cells preserve molecular evidence of previous activation even in the absence of overt infection or experimental immunization. Together, these findings support a model in which neonatal-derived B-1 cells constitute a durable archive of early-life immune experiences, linking their developmental origin to their functional state in adulthood.

Importantly, we also detected molecular overlap between a published human fetal B-1 dataset [165] and our mouse ELO PerC B-1 subset. This finding suggests that human fetal B-1 cells share key transcriptional features with ELO B-1 cells from adult mice and highlights the value of ontogenetic stratification as a strategy for cross-species comparisons. More broadly, these results demonstrate how developmental signatures derived from ontogenetically delineated mouse B cell populations can be used to infer the developmental origin of human B cell subsets.

One limitation of our scRNAseq approach is the use of a targeted gene panel focused on B cell differentiation and immune response pathways. Although this strategy improved the sensitivity of detecting genes relevant to B cell biology, it limited the discovery of novel transcriptional programs outside the predefined panel. Future studies employing whole-transcriptome single-cell sequencing will therefore be important to establish a more comprehensive molecular signature of ELO B cells. Such analyses may reveal additional developmental features unique to early-life-derived B cells and enable more precise interrogation of human B cell datasets for populations related to mouse ELO B cells.

Despite the insights provided by Mb1-CreERT2-based lineage tracing, this approach cannot determine when ELO B cells were activated and acquired their memory-like phenotype. Future studies should therefore aim to reconstruct the activation history of ELO B cells more precisely. One promising strategy would be the use of the AID-CreERT2 driver [193]. Since AID is induced upon antigen-driven B cell activation and mediates SHM and CSR, temporal labelling of AID-expressing cells could effectively "activation-stamp" B cells and provide additional resolution regarding the timing and nature of antigen encounters experienced by ELO B cells.

B-1 cells as a developmentally programmed IgM memory compartment

Our findings suggest that early-derived B-1 cells occupy a previously underappreciated position at the interface between innate-like B cell lineages and immunological memory. Although B-1 cells have traditionally been viewed as a largely naïve population, several lines of evidence from this work argue instead that they represent a developmentally programmed, antigen-experienced compartment. We observed an enrichment of the established memory-associated markers CD80 and PD-L2 within ELO B-1 cells (Paper II). Moreover, PtC⁺ ELO B-1 cells contained higher frequencies of DP memory cells than their PtC⁻ counterparts, and early-life immunization further increased the proportion of DP memory cells within the ELO B-1 compartment. Together, these findings suggest that both self-antigen recognition and encounters with foreign antigens during early life promote the acquisition of progressively more advanced memory-like features. This interpretation is consistent with previous observations that B-1 cell selection and maintenance are driven by self-antigen recognition [75, 292, 293], and that microbial exposure or early-life immunization can recruit antigen-experienced clones into the adult B-1a compartment [185].

These findings are particularly relevant in the context of IgM memory biology. In humans, IgM memory cells are readily identifiable through markers such as CD27 and constitute a well-recognized component of long-term humoral immunity [171, 172, 175]. In contrast, mouse IgM memory populations remain comparatively poorly defined, largely because mice lack a universally accepted marker equivalent to human CD27 and because much of the field has relied on antigen-specific tracking systems, adoptive transfer models, or BCR-transgenic approaches [167, 294, 295]. Consequently, the identity and developmental origin of naturally occurring IgM memory populations within the endogenous mouse repertoire remain incompletely understood. Our data suggest that B-1 cells may represent one such population.

Rather than viewing B-1 cells and IgM MBCs as distinct entities, our results support a model in which they share substantial functional and molecular overlap. ELO B-1 cells exhibit hallmarks of prior antigen experience, including enrichment of memory-associated surface markers and transcriptional signatures consistent with antigen-driven differentiation (Paper II). These characteristics position ELO B-1 cells within a natural IgM memory compartment and suggest that developmental origin provides an alternative approach for identifying memory-like B cell populations that cannot be readily resolved using conventional phenotypic markers alone.

This perspective may also help reconcile the long-standing controversy surrounding human B-1 cells [156-161]. Instead of attempting to identify a phenotypically identical counterpart to mouse B-1 cells, our data suggest that a more informative approach is to consider developmental origin and functional state. From this

viewpoint, mouse B-1 cells and human IgM MBCs may represent convergent populations that arise through distinct developmental pathways yet ultimately fulfil similar immunological roles. More broadly, these findings support an expanded view of B cell memory in which durable antigen experience is not restricted to GC-derived, class-switched populations, but can also be embedded within innate-like, developmentally programmed B cell lineages.

Taken together, our results support a unifying model in which B-1 cells are understood as part of a developmentally programmed IgM memory compartment. Ontogenetic stratification therefore not only resolves previously unrecognized heterogeneity within the B-1 lineage but also provides a conceptual bridge between mouse B-1 cells and human IgM MBCs, revealing a conserved strategy for maintaining immunological memory established early in life.

CLL: Age-associated malignancy with early-life-origin

Combining developmental time-stamping with the TCL-1tg model revealed that CLL-initiating cells arise predominantly from early life (Paper II). Notably, the expanding ELO B cell population was highly enriched for DP memory cells, suggesting that disease progression is driven by antigen-experienced, memory-like cells. These findings are in line with previous proposals that mature antigen-experienced B cells serve as the cell of origin in CLL [248, 249].

Our findings also provide developmental context for another defining feature of CLL, namely the presence of stereotyped BCRs. Although stereotyped receptors have long been interpreted as evidence of antigen-driven selection during leukemogenesis [208-211], accumulating evidence suggests the human B cell repertoire contains developmentally programmed and recurrent receptor configurations that arise early in life. Studies of cord blood have revealed conserved IG repertoires and frequent sharing of BCR clonotypes between unrelated individuals, indicating that parts of the neonatal repertoire are highly convergent rather than entirely stochastic [296]. Similarly, Soto and colleagues demonstrated the existence of extensive "public" BCR clonotypes shared between unrelated individuals, including clonotypes present in both cord blood and adult repertoires, suggesting that convergent receptor generation occurs independently of common antigen exposure and is established early during ontogeny [297]. Consistent with this concept, CLL-stereotyped-like IG sequences can be detected in cord blood as well as BM and peripheral blood from healthy individuals [216]. Furthermore, many stereotyped CLL receptors display self- and poly-reactive properties reminiscent of fetal-derived and innate-like B cell populations [211, 212]. Together, these observations suggest that at least some stereotyped CLL receptors may originate from physiological early-life B cell repertoires that are subsequently selected and

expanded during leukemogenesis. In this context, our finding that CLL-initiating cells arise from an early-life developmental window provides functional support for a model in which CLL exploits biological programs embedded within the early-life B cell compartment.

An additional feature linking CLL to early-life-derived and innate-like B cell populations is their shared capacity for immunoregulation through IL-10 production. B-1 cells constitutively produce IL-10 and rapidly upregulate its expression following innate and BCR-mediated stimulation, contributing to the control of inflammation and maintenance of immune homeostasis [137, 241-244]. Notably, when we compared IL-10 production following LPS stimulation in early-life-derived (Tom⁺) and adult-derived (Tom⁻) splenic B cells from TCL-1tg mice and control animals, the highest frequency of IL-10-secreting cells was observed within the Tom⁺ TCL-1tg compartment (Figure 2, unpublished data). These findings suggest that developmental origin influences not only leukemogenic susceptibility but also the immunoregulatory properties of CLL cells. Given the central role of IL-10 in suppressing anti-tumour immunity and shaping the immunosuppressive microenvironment in CLL [137, 239, 240], the enhanced IL-10-producing capacity of Tom⁺ leukemic cells further supports the concept that CLL co-opts developmental programs normally associated with early-life and innate-like B cell populations.

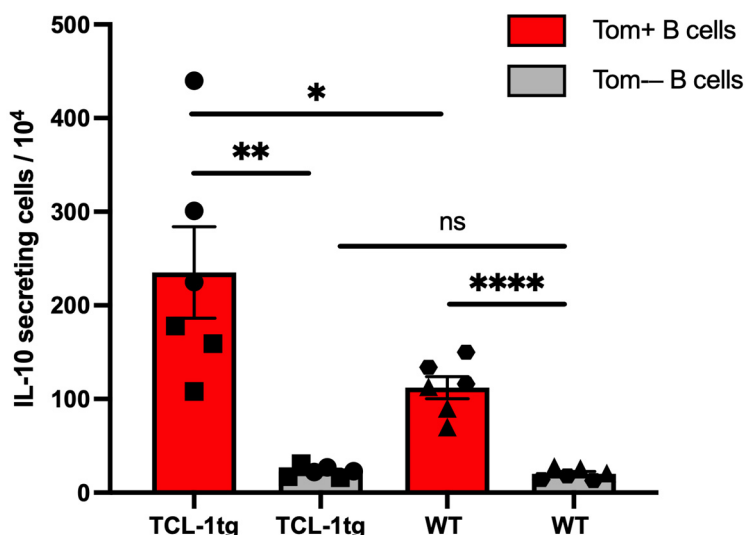


Figure 2. Increased frequency of IL-10 secreting cells among splenic TCL-1tg ELO B cells. Bar plots summarise the number of IL-10 secreting cells per 10,000 plated cells following LPS stimulation within the indicated populations. Each data point represents one technical replicate; data points sharing the same symbol correspond to replicates derived from the same individual mouse. Bars indicate mean ± SEM. Statistics were calculated with unpaired Student's *t* test. ns, not significant, **p* < 0.05, ***p* < 0.01, *****p* < 0.0001.

We further showed that the susceptibility of ELO B cells to CLL-like transformation is linked to an early-life developmental window (Paper III). Ectopic expression of the early-life-specific regulator LIN28B was sufficient to generate B cells that were vulnerable to TCL-1-driven transformation, demonstrating that developmental programming itself can establish a permissive state for leukemogenesis. Previous studies have shown that enforced LIN28B expression in adult hematopoietic cells reactivates key features of fetal B cell development, including the generation of B-1-like cells and enhanced production of autoreactive BCR specificities [27, 32, 37, 39]. Our findings extend these observations by demonstrating that reactivation of an early-life developmental program is sufficient to create a cellular state that is highly susceptible to leukemic transformation. Together, these results suggest that the developmental origin of CLL is not simply reflected by the age at which the initiating cells are generated but is encoded within a persistent molecular program that remains detectable long after birth and can actively influence malignant potential.

Previous work by Hayakawa and colleagues demonstrated that transplantation of adult TCL-1tg B-1a cells could give rise to CLL-like disease, whereas transplantation of TCL-1tg HSPCs could not [253, 254]. These studies provided important evidence linking CLL to innate-like B cell populations and suggested an early-life origin of the disease. However, the comparison involved two fundamentally different developmental stages, mature B-1a cells and hematopoietic progenitors, making the precise developmental timing difficult to resolve. Furthermore, transplantation-based approaches inherently perturb normal cellular physiology and niche interactions. In contrast, our developmental time-stamping strategy enabled direct assessment of the ontogenetic origin of CLL-initiating cells within an unperturbed in situ setting, thereby refining and extending these earlier observations.

Although developmental time-stamping enabled us to identify the ontogenetic origin of the leukemic population, it does not resolve the precise stage at which malignant transformation occurs. Future studies integrating lineage tracing with genomic and computational analyses may help reconstruct the sequential accumulation of CLL-relevant mutations across disease development. Longitudinal sampling of time-stamped TCL-1tg mice may reconstruct the mutational history leading to transformation and potentially identify the critical oncogenic events that drive disease initiation.

An additional unresolved question is whether inherited variation within IG loci influences the developmental trajectories that ultimately give rise to CLL. Recent large-scale population studies have revealed extensive germline diversity within human IG loci, including previously unrecognized allelic variants, structural variation and population-specific patterns of gene usage, while also demonstrating that such variation can substantially influence antibody repertoire formation, V gene usage and antigen recognition [298, 299]. Although these studies focused primarily

on normal immune responses rather than leukemia, they raise the intriguing possibility that inherited IG variation may influence the generation, selection, or persistence of CLL-associated stereotyped receptors. It will therefore be important to determine whether specific IG germline haplotypes predispose to particular stereotyped subsets, influence the emergence of CLL-prone clones or contribute to inter-individual differences in disease risk and progression.

Another avenue for future investigation concerns the relationship between naturally occurring early-life BCR specificities and the stereotyped receptors observed in CLL. A substantial fraction of mouse B-1 cells generated during fetal and neonatal life express BCRs recognizing PtC, a prototypical self-antigen that drives the expansion and maintenance of innate-like B cell populations. Related innate-like B cell populations have also been identified in human fetal, neonatal, and cord blood compartments, where they exhibit conserved and partially self-reactive receptor repertoires [158, 296]. Given the growing evidence linking CLL to fetal-derived and innate-like B cell repertoires, a systematic comparison between human fetal and neonatal PtC-reactive BCRs and CLL-derived BCR sequences may be particularly informative. Such analyses could reveal whether specific stereotyped CLL subsets preferentially arise from developmental programs associated with PtC recognition or other conserved self-reactive specificities. Identifying shared sequence motifs, structural features or patterns of antigen reactivity between these populations would provide further evidence that CLL originates from developmentally programmed B cell repertoires established early in life. More broadly, these studies could help define the physiological precursor populations from which different CLL subtypes emerge and clarify the role of self-antigen recognition in leukemogenesis.

Finally, an important challenge is to determine how the early developmental origin of CLL-initiating cells could be exploited therapeutically, once the leukemia has developed. Fetal and neonatal B lymphopoiesis are characterized by increased incorporation of self- and poly-reactive BCRs [27, 279], elevated metabolic activity [300], enhanced protein synthesis [38], and the establishment of populations with increased self-renewal capacity [101-104]. These characteristics persist in ELO B cells and may contribute to their susceptibility to leukemic transformation. At the same time, they may create specific therapeutic vulnerabilities. One approach would be to treat time-stamped TCL-1tg mice during a pre-leukemic phase with the BTK inhibitor ibrutinib and subsequently assess the relative persistence of Tom⁺ and Tom⁻ B cell populations. Such experiments could determine whether early-life-derived B cells exhibit differential sensitivity to BCR-signalling inhibition before overt disease develops. A deeper understanding of the biological properties that distinguish ELO B cells from their adult-derived counterparts may therefore reveal new opportunities to refine targeted therapeutic strategies.

Developmental stratification reveals immunoregulatory capacity as a hallmark of early life

In Paper IV we applied our time-stamping approach to investigate the ontogenetic basis of immunoregulatory B cell function. This strategy identified immunoregulatory capacity as a key feature of ELO B cells and highlighted their non-redundant role in constraining and fine-tuning immune responses. While previous studies have largely focused on immunophenotypic definitions of regulatory B cells, our work demonstrates that immunoregulatory function can instead be linked to developmental origin. Specifically, we identify ELO B-1 cells as developmentally programmed population with enhanced IL-10-producing capacity, thereby associating ontogeny rather than phenotype with immunoregulatory function.

Although our findings establish the developmental origin of immunoregulatory B cells, the mechanisms through which ELO B-1 cells regulate immune responses remain to be further elucidated. Future studies should address where and how these cells exert their suppressive functions and identify their primary target populations. It remains unclear whether ELO B-1 cells regulate inflammation systemically or whether they migrate to sites of inflammation and act locally. Furthermore, while the current study focused on IL-10 production, previous work has implicated B-1 cells in additional immunomodulatory pathways involving IL-27, CTLA-4, and NAb production [145, 147, 301-303]. It is therefore likely that ELO B-1 cells employ multiple complementary mechanisms to mediate immune responses. Another important question is how ELO B-1 cells interact with other immunoregulatory populations. Tregs are well-established mediators of immune tolerance [304, 305], and early-life-derived Treg populations have been shown to exert long-lasting effects on immune homeostasis [306-308]. Future studies should therefore investigate potential functional interactions between ELO B-1 cells and Tregs and determine whether ELO B-1 cells exert their effects through autocrine, paracrine or cooperative mechanisms. Such studies may help uncover whether developmental programming represents a broader principle governing the establishment of long-lived immunoregulatory networks during early life.

Taken together, the work in this thesis demonstrates that developmental lineage tracing provides a powerful tool for dissecting the functional heterogeneity of the B cell compartment. By stratifying B cells according to their developmental origin, we identified previously unrecognized functional subsets that contribute to distinct aspects of adult immunity and disease.

Limitations of the TCL-1tg model and challenges in mouse to human transferability

Despite its status as the most widely used and best-characterized model of CLL, several important limitations must be considered when extrapolating findings from TCL-1tg mice to human disease. These limitations primarily relate to differences in disease initiation, genetic complexity, immune system composition, and therapeutic responses between mice and humans.

A fundamental limitation of the TCL-1tg model is that leukemia is driven by forced overexpression of a single oncogene. In contrast, human CLL develops through a multistep evolutionary process involving the accumulation of diverse genetic and epigenetic alterations over decades. Although TCL-1 overexpression is observed in aggressive CLL and contributes to disease pathogenesis, it is not itself the initiating lesion in most patients. Consequently, TCL-1tg leukemogenesis may oversimplify the heterogeneous mechanisms responsible for CLL development in humans and may not fully capture the complex clonal evolution that characterizes the patient population [228, 258, 309].

The model also predominantly resembles U-CLL and therefore reflects only one biological subtype of the disease. Human CLL is highly heterogeneous, with major clinical and molecular differences between U-CLL and M-CLL cases. While TCL-1tg mice reproduce many features of aggressive U-CLL, they are less suitable for studying the biology of indolent M-CLL, which follows a distinct clinical course and may depend on different pathogenic mechanisms. As a result, observations obtained in TCL-1tg mice cannot necessarily be generalized to all forms of CLL [203, 204, 263].

Another important consideration is the difference between mouse and human immune systems. Although the TCL-1tg model develops within an immunocompetent host and therefore retains interactions between leukemia cells and their microenvironment, substantial species-specific differences exist in immune cell composition, cytokine signalling, innate and adaptive immune responses, and immune regulatory mechanisms. Furthermore, comparative transcriptional analyses have demonstrated that, despite broad conservation of immune cell programs across species, hundreds of immune-related genes display divergent expression patterns and regulatory networks in mice and humans. These differences may influence leukemic cell survival, immune evasion, and therapeutic responses. Consequently, mechanisms identified in mice may not always operate identically in humans, particularly when studying anti-tumour immunity or therapies designed to engage specific immune pathways [310, 311].

These biological differences are reflected in the mixed success of translating preclinical results into the clinic. Several therapeutic strategies have demonstrated

remarkable efficacy in TCL-1tg mice yet failed to achieve comparable outcomes in human trials. Conversely, some treatments that later became standards of care in CLL, such as inhibitors targeting BCR signalling pathways, showed encouraging results in the model but required extensive clinical validation before their true therapeutic value could be established. Thus, while the TCL-1tg model is extremely useful for identifying candidate pathways and generating mechanistic insights, it cannot reliably predict clinical efficacy on its own.

Finally, practical aspects of disease kinetics should be considered. Although disease development in TCL-1tg mice is relatively gradual compared with many other cancer models, it still occurs over a timescale of months rather than decades. Human CLL evolves under prolonged antigenic stimulation, ageing-related immune changes, and prolonged selective pressures that are difficult to reproduce experimentally. The accelerated disease course in mice may therefore influence patterns of clonal selection and treatment response, potentially limiting direct translation to patients.

Taken together, these limitations highlight the need for careful interpretation of findings generated in TCL-1tg mice. While the model remains indispensable for studying the biology of aggressive U-CLL and has significantly advanced understanding of CLL pathogenesis, it should be regarded as a complementary rather than fully representative system

Given these limitations of the TCL-1tg model, we sought to complement our findings using an alternative system that more closely reflects spontaneous disease development. To this end, we employed a non-transgenic model of CLL-like disease originally described by Förster and Rajewsky, in which transfer of PerC cells from aged mice into congenic neonatal recipients leads to long-term engraftment of donor-derived B cells and, in a subset of recipients, the eventual development of a CLL-like lymphoproliferative disorder [312]. Unlike the TCL-1tg model, this system does not rely on enforced expression of a defined oncogenic driver and therefore provides an opportunity to investigate disease initiation in a more physiological setting.

We combined this spontaneous CLL model with our lineage tracing approach by transferring PerC cells from aged donor mice that had been time-stamped early in life into unconditioned neonatal recipients. Recipient mice were then monitored longitudinally for donor-cell engraftment and the emergence of CLL-like expansions (Figure 3A). Notably, whenever spontaneous leukemic outgrowth occurred, the expanding cell population originated predominantly from donor-derived ELO B cells, as demonstrated by their Tom⁺ labelling (Figures 3B-C). These findings are consistent with our observations in the TCL-1tg model and support the concept that ELO B cells represent a cellular population with enhanced leukemogenic potential.

However, the spontaneous transfer model presented considerable experimental challenges. The approach requires prolonged ageing of both donor and recipient animals, resulting in exceptionally long experimental timelines. In addition, donor-cell engraftment was variable and the incidence of spontaneous CLL-like disease remained relatively low. Consequently, the number of recipients developing overt leukemic expansion was insufficient to achieve the statistical power necessary for a definitive quantitative analysis. Therefore, rather than serving as an independent validation cohort, this model should be viewed as a proof-of-principle system. Nevertheless, the findings indicate that ELO-driven CLL-like expansion is not limited to the TCL-1tg setting but can also occur in a non-transgenic model of spontaneous disease development.

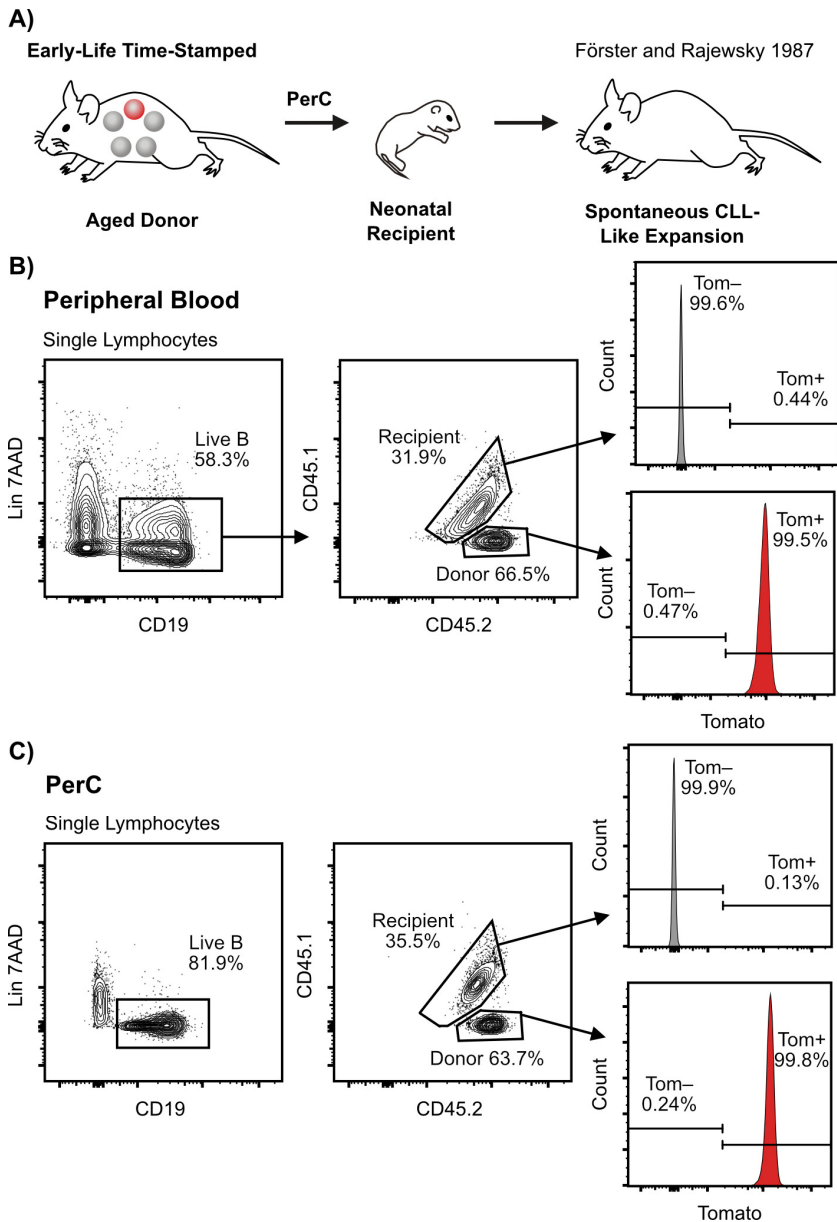


Figure 3. Spontaneous CLL model generated by adoptive transfer of aged PerC cells. (A) Schematic overview of the experimental design. Early-life time-stamped donor mice were aged prior to isolation of PerC cells, which were subsequently transferred into congenic neonatal recipients. Donor cell engraftment and the development of spontaneous CLL-like expansion were monitored longitudinally in recipient mice. (B-C) Representative flow cytometry analysis of a recipient mouse with successful donor-cell engraftment at the experimental endpoint. Shown are peripheral blood (B) and PerC (C) samples. The donor mouse was time-stamped at postnatal day 19 and aged to 14 months before PerC cell isolation and transfer. The recipient mouse was analyzed at 15 months of age at the experimental endpoint.

Limitations of SPF conditions for studying ELO B cells

Another limitation in our studies is that all experiments were performed in specific-pathogen-free (SPF) mice. SPF mice are maintained free of defined pathogens they would otherwise be exposed to in the wild. They remain the standard model for laboratory research because they improve biosafety and minimize the introduction of infectious agents as uncontrolled experimental variables. However, SPF housing also creates an immunological environment that differs substantially from natural conditions. Compared with wild or pet-store mice, SPF mice harbour a less diverse microbiota and exhibit relatively immature immune systems, including reduced frequencies of antigen-experienced lymphocytes and lower basal immune activation [313-315]. Consequently, our findings may not fully reflect how ELO B cell development, activation, and differentiation are shaped by the continuous microbial and antigenic exposures encountered in real-world settings. Indeed, the activation history and functional heterogeneity of ELO B cells are likely underestimated under SPF conditions.

The importance of environmental exposure in shaping immune function has been highlighted by studies using wildling mice, which are generated by transferring C57BL/6 embryos into wild surrogate dams and consequently acquire a natural microbiota and pathogen experience while maintaining a controlled genetic background [315]. Using this model, the Coquet laboratory demonstrated that wildling mice possess expanded populations of antigen-experienced T cells and mount robust allergic responses to house dust mite, IL-33 and *Alternaria* despite their diverse microbial exposure, challenging the notion that increased microbial experience broadly suppresses allergic immunity [316]. More broadly, environmentally enriched mouse models have repeatedly been shown to better recapitulate human immune cell composition and activation states than conventional SPF mice [313, 315]. Future studies should aim to investigate ELO B cells in wildling or similarly "naturalized" mouse models to determine how early-life microbial colonization, infections and lifelong antigen exposure influence their establishment, clonal diversity, activation history, tissue adaptation and functional specialization. Such work will be essential for establishing the physiological relevance of ELO B cells in natural immune environments and for assessing the extent to which observations made in SPF mice translate to human immunity.

Ethical considerations in animal research

All experimental work involving animals was conducted in accordance with established ethical guidelines and regulatory frameworks governing the use of laboratory animals. Central to these considerations is the principle of the 3Rs, Replacement, Reduction and Refinement, which provide a foundation for minimizing animal use while ensuring robust scientific outcomes.

Replacement was carefully considered at all stages of experimental design, with *in vitro* systems and computational approaches employed whenever feasible. However, the complex ontogeny, tissue distribution and functional specialization of the B cell subsets analysed in the present work required the use of *in vivo* models. The biological processes under investigation involve complex systemic interactions within the immune system that cannot be fully recapitulated in cell culture, thereby justifying the use of animal models in this work.

Reduction was achieved through careful experimental planning and the integration of multiple experimental readouts from individual animals when possible. Whenever feasible, tissues from the same animal were used across multiple assays, thereby maximizing data yield while minimizing the total number of animals required.

Refinement strategies were implemented to minimize animal distress and improve welfare. Animals were housed under standardized conditions with continuous monitoring, and all procedures were designed to reduce discomfort.

The studies presented in this thesis relied heavily on genetically modified mouse models, which were essential to dissect the developmental origin and function of ELO B cells. These models enabled lineage tracing, targeted gene perturbation and disease modelling in a physiologically relevant context. Such complex experimental systems cannot currently be fully substituted by alternative approaches, as they uniquely allow the study of B cell development and immune function within an intact organism. Taken together, the use of mouse models in this work was justified by the complexity of the biological systems studied. *In vivo* studies are indispensable for capturing the dynamic, multi-organ interactions that underlie immune development and disease.

Discussion of Selected Methods

Tamoxifen-inducible Mb1-CreERT2 lineage tracing

We established an inducible lineage tracing mouse model that enables temporal “time-stamping” of distinct developmental waves of B cells, allowing us to track their contribution to the adult immune system (Paper I). In contrast to transplantation-based approaches, which define developmental potential under stress by forcing donor cells to repopulate empty niches, our strategy examines ELO B cells under steady-state conditions, providing a more physiologically relevant assessment of their contribution to adult B cell subsets.

Nevertheless, several limitations of tamoxifen-inducible CreERT2 lineage tracing must be considered. A key concern relates to the biological effects of tamoxifen administration. Tamoxifen has been associated with toxicity in blood cell populations, particularly in the context of CreERT2 activation [317]. While such effects are most pronounced under regimens involving high doses and repeated administration, our experiments were conducted using comparatively low doses and limited induction schedules, which likely mitigates these risks. Tamoxifen administration also poses challenges in prenatal settings. It has been shown to interfere with pregnancy and may induce premature labour in pregnant mice [318]. Although co-administration of progesterone can partially alleviate these effects, prenatal induction remains challenging, which is why most of our experiments were performed using postnatal induction time points.

Another important consideration is the pharmacokinetic profile of tamoxifen. Although our induction protocol is designed as a short pulse-labelling event, tamoxifen and its active metabolite, 4-hydroxytamoxifen, can persist *in vivo* and thereby extend the effective labelling window beyond the intended timeframe. Indeed, even a single dose of tamoxifen can have measurable effects of up to 24 hours [319], potentially reducing temporal resolution. Finally, incomplete labelling efficiency must be taken into account. While we confirmed high labelling coverage shortly after induction (Papers I & II), a small fraction of cells is likely to remain unlabelled. Consequently, the overall contribution of ELO B cells may be underestimated due to incomplete labelling saturation.

Despite these limitations, inducible CreERT2-based lineage tracing remains one of the most powerful and accessible approaches for studying developmental dynamics

in vivo. Importantly, it uniquely enables temporal control over labelling without perturbing tissue homeostasis, thereby allowing developmental processes to be studied within their native physiological context. Alternative approaches, such as transplantation or constitutive lineage tracing, either disrupt the endogenous niche or lack temporal resolution. Therefore, although not without caveats, the CreERT2 system provided the most suitable and robust approach for dissecting distinct developmental waves of B cells in this thesis.

Flow cytometry

Flow cytometry is an integral technology in immunology and biomedical research. Its ability to resolve cellular heterogeneity at the single-cell level with high throughput has enabled numerous scientific discoveries [320, 321]. Since its beginnings approximately 60 years ago [322-324], the technology has advanced substantially and expanded to include diverse applications. One key development is fluorescence-activated cell sorting (FACS), which enables not only the analysis but also the physical isolation of live cells for downstream applications. Interestingly, the development of FACS and the concept of layered immune system formation, central to this thesis, share historical roots in the pioneering work of the Herzenberg laboratory [44, 322].

Throughout this thesis, flow cytometry constituted an essential tool for studying ELO B cells. Its ability to resolve cellular heterogeneity based on the combinatorial expression of surface and intracellular markers made it indispensable for dissecting B cell subsets across developmental stages.

Despite these strengths, several technical limitations should be acknowledged. Cell identification relies on fluorescent antibody labelling of surface markers, and the number of markers that can be simultaneously measured is constrained by the configuration of the instrument and available fluorophore combinations. Consequently, cellular populations can only be resolved to a certain degree within a single experiment. In addition, simultaneous use of multiple fluorophores leads to spectral overlap. Therefore, careful staining panel design, appropriate controls and accurate compensation are critical for reliable data acquisition. Finally, flow cytometry requires the preparation of single-cell suspensions, which inherently disrupts tissue architecture and results in the loss of spatial information regarding cellular localization and interactions [320, 321].

Despite these caveats, flow cytometry remains one of the most powerful and versatile tools for studying the immune system. Its unique combination of speed, scalability and single-cell resolution, together with the ability to isolate viable cells through FACS, makes it central to modern immunological research [320, 321]. While emerging technologies, such as spatial transcriptomics and high-dimensional

imaging provide complementary insight by adding spatial information, they currently lack the same throughput, accessibility and flexibility. Therefore, despite inherent limitations, flow cytometry remains an indispensable methodological cornerstone for immunological research and was essential for addressing the research questions in this thesis.

High-throughput sequencing approaches

The advent of high-throughput, parallel sequencing technologies has profoundly advanced molecular biology, enabling comprehensive characterization of gene expression at both bulk and single-cell levels [325]. In this thesis, both scRNAseq and bulk RNAseq were employed, each offering distinct advantages and limitations depending on the specific research objective.

scRNAseq provides high-resolution datasets that enable detailed interrogation of cellular heterogeneity within complex populations [326]. We applied a targeted scRNAseq approach (Paper II) with relevant immune genes to further dissect the ELO B cell compartment. This approach facilitated the identification of subtle transcriptional differences that would likely be obscured in bulk analyses and allowed us to identify a shared molecular signature in ELO B cells across different tissues. However, scRNAseq experiments are generally associated with higher costs and require more complex and computationally demanding downstream analyses compared to bulk RNAseq methods [327].

In contrast, bulk RNAseq offers a cost-effective and relatively straightforward approach to profiling gene expression across entire cell populations [325, 327]. In this work, bulk RNAseq was used to compare transcriptional profiles between Tom⁺ and Tom[−] B cell populations in a CLL context (Paper II). Additionally, bulk RNAseq approaches were used to analyse BCR repertoires in sorted populations (Papers I, II & III), providing insights into overall clonal composition and dominant B cell clones. Bulk BCR sequencing approaches are particularly efficient for capturing large numbers of receptor sequences at relatively low cost, enabling population-level analyses of BCR repertoires [328]. However, this approach lacks single-cell resolution and therefore cannot resolve intra-population heterogeneity or directly link gene expression to individual clonotypes [327].

Taken together, while each sequencing strategy presents inherent trade-offs between resolution, cost, and analytical complexity, the combined use of scRNAseq and bulk RNAseq in this thesis provided complementary insights. Importantly, the selected approaches were well-suited to address the specific biological questions posed, enabling both detailed cellular characterization and broader population-level analyses.

Use of AI tools

This thesis has been partly produced using the generative AI model M365 Copilot (GPT-5 chat model). Specifically, it was used to generate the cover image for the thesis. Furthermore, it was used during the writing process to assist with improving grammar, style, clarity, logical flow and paragraph structure, as well as for brainstorming ideas and identifying relevant literature. All AI-generated content was critically evaluated, edited and verified by the author, who assumes full responsibility for the final content of the thesis. In addition, M365 Copilot was used to translate the popular science summary from English into Swedish and German. These translations were subsequently reviewed and proofread by native speakers.

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