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Functional studies of pathogen-specific T cell responses in patients with chronic lymphocytic leukemia

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Cover illustration: Patients with CLL facing more than cancer

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Thesis for Doctoral Degree (Ph.D.)

By

Jinghua Wu

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Popular science summary of the thesis

For patients with CLL, the challenge isn't just the cancer itself, because common infections can also become life-threatening, as the COVID-19 pandemic showed. Our immune system relies on antibodies and T cells that work together to fight infections. In CLL, long-lasting pressure from the leukemia can leave T cells "tired," so they cannot function properly. Although modern targeted drugs now control CLL more effectively, it remains unclear whether these infection-fighting T cells recover when the cancer is treated. Antibody responses are often weak in CLL, and B-cell–targeted therapies can make this worse, increasing the risk of infections. Since T cells are the main healthy lymphocytes remaining in many people with CLL, understanding whether they can still mount strong responses to pathogens is a key question this thesis explores.

In **Studies I & II**, we found that the T cells which normally protect us from infections, both the CD4 helper and CD8 killer cells, do not work properly as they should in patients with CLL. Even after modern targeted drugs successfully reduce the cancer cells, these T cells remain weakened. The CD4 helper cells lose their youthful, renewing ability, while the CD8 killer cells become short-lived and locked in their terminal stage. These findings open the door to improving future cell-based therapies.

In **Study III**, we found that even after more than two years of next-generation targeted therapy in CLL, COVID-19 continued to threaten patients' lives. Five doses of vaccine were still not enough to restore their weakened antibody (humoral) immunity. However, the strength of their T cell responses appeared to recover to near-normal levels, suggesting that cellular immunity may provide some protection even when antibody defenses remain weak.

In **Study IV**, we found that healthy people and patients with CLL who had been infected or vaccinated still had memory T cells that could recognize new hypermutated variants of the COVID-19 virus. This suggests that our body's cellular immunity remains strong and can continue to protect against new variants of the virus, even as it keeps changing, in both healthy and vulnerable populations.

In summary, people with CLL continue to have an immune system that does not fully recover, even after modern targeted treatments successfully control the cancer. Their helper and killer T cells lose some of their strength and flexibility,

making it harder to fight infections. However, despite these persistent defects, vaccination, particularly against SARS-CoV-2, can still boost T cell immunity, indicating that parts of the T cell response remain responsive and may provide some protection even when antibody defenses fail.

Abstract

CLL is the most common type of leukemia in adults and remains an incurable malignancy. Patients with CLL often suffer from non-malignant complications, particularly recurrent infections, which have been highlighted in recent years by the exceptionally high mortality rates during the COVID-19 pandemic. Increasing evidence indicates that T cells in CLL patients become functionally exhausted, potentially contributing to their impaired control of pathogenic infections. To this end, this thesis investigated the pathogen-specific T cell response, functionality, and characteristics across patients with varying tumor burden, different targeted therapies, and diverse vaccination and infection histories, against a broad spectrum of pathogens, including the most common bacteria and viruses.

In **Study I**, we assessed the magnitude and functionality of both bacteria- and virus-specific $CD4^+$ T cells and characterized their phenotypic signatures before and after BTKi therapy. We demonstrated that the impaired functionality of $CD4^+$ T cells was largely irreversible even after a reduction in tumor burden. Moreover, these cells progressively lost their stem-like memory subset during CLL progression.

In **Study II**, we investigate the phenotype and functional property of virus-specific $CD8^+$ T cells. Although the overall magnitude of these responses was largely preserved in CLL patients, their functional capacity remained persistently impaired and did not recover after tumor burden reduction. In parallel, these cells exhibited a gradual loss of self-renewing characteristics and became confined to a terminal effector state despite effective tumor reduction by next-generation BTKi therapy.

In **Study III**, we detected both humoral and cellular responses to variants of the SARS-CoV-2 spike antigen. We first showed the system and mucosal vaccine response in patents with long-term next-generation of zanubrutinib treatment.

In **Study IV**, we demonstrated that memory T cells remained capable of recognizing both the ancestral strain and the highly mutated BA.2.86 variant of COVID-19, in both healthy individuals and patients with CLL.

List of scientific papers

I. **Jinghua Wu**, Yu Gao, Curtis Cai, Maria Andersson, Rokeya Sultana Rekha, Takuya Sekine, Sarah Adamo, Kia Heimersson, Oriana Jesus Ribeiro, Marzia Palma, Peter Bergman, Rula Zain, C. I. Edvard Smith, Anders Österborg, Marcus Buggert. Systemic pathogen-specific $CD4^+$ T cell dysfunction persists despite selective BTK inhibition in CLL. Manuscript

II. **Jinghua Wu**, Curtis Cai, Maria Andersson, Mily Akhirunnesa, Thomas R. Müller, Takuya Sekine, Kia Heimersson, Akshaya Vidhya, Marzia Palma, Rula Zain, C. I. Edvard Smith, Yu Gao, Anders Österborg, Marcus Buggert. BTKi therapy uncouples tumor control from virus-specific $CD8^+$ T cell functional recovery in CLL. Manuscript

III. Andersson M*, **Wu J***, Wullimann D, Gao Y, Aberg M, Muschiol S, Healy K, Naud S, Bogdanovic G, Palma M, Mellstedt H, Chen P, Ljunggren HG, Hansson L, Sallberg Chen M, Buggert M, Ingelman-Sundberg HM, Osterborg A. Local and Systemic Immunity During Five Vaccinations Against SARS-CoV-2 in Zanubrutinib-Treated Patients With Chronic Lymphocytic Leukemia. J Hematol. 2023 Aug;12(4):170-175. doi: 10.14740/jh1140.

IV. Müller TR*, Gao Y*, **Wu J**, Ribeiro O, Chen P, Bergman P, Blennow O, Hansson L, Mielke S, Nowak P, Vesterbacka J, Akber M, Söderdahl G, Smith CIE, Loré K, Chen MS, Ljungman P, Ingelman-Sundberg HM, Ljunggren HG, Österborg A, Sette A, Grifoni A, Aleman S, Buggert M. Memory T cells effectively recognize the SARS-CoV-2 hypermutated BA.2.86 variant. Cell Host Microbe. 2024 Feb 14;32(2):156-161.e3. doi: 10.1016/j.chom.2023.12.010.

*These authors contributed equally

Other scientific papers not included in this thesis

I. Ingelman-Sundberg HM, Blixt L, Wullimann D, **Wu J,** Gao Y, Healy K, Muschiol S, Bogdanovic G, Åberg M, Kjellander C, Grifoni A, Sette A, Aleman S, Chen P, Blennow O, Hansson L, Ljunggren HG, Chen MS, Buggert M, Österborg A. Systemic and mucosal adaptive immunity to SARS-CoV-2 during the Omicron wave in patients with chronic lymphocytic leukemia. Haematologica. 2024 Feb 1;109(2):646-651. doi: 10.3324/haematol.2023.282894.

II. Adamo S, Gao Y, Sekine T, Mily A, **Wu J**, Storgärd E, Westergren V, Filén F, Treutiger CJ, Sandberg JK, Sällberg M, Bergman P, Llewellyn-Lacey S, Ljunggren HG, Price DA, Ekström AM, Sette A, Grifoni A, Buggert M. Memory profiles distinguish cross-reactive and virus-specific T cell immunity to mpox. Cell Host Microbe. 2023 Jun 14;31(6):928-936.e4. doi: 10.1016/j.chom.2023.04.015.

III. Gao Y, Cai C, Adamo S, Biteus E, Kamal H, Dager L, Miners KL, Llewellyn-Lacey S, Ladell K, Amratia PS, Bentley K, Kollnberger S, **Wu J**, Akhirunnesa M, Jones SA, Julin P, Lidman C, Stanton RJ, Goepfert PA, Peluso MJ, Deeks SG, Davies HE, Aleman S, Buggert M, Price DA. Identification of soluble biomarkers that associate with distinct manifestations of long COVID. Nat Immunol. 2025 May;26(5):692-705. doi: 10.1038/s41590-025-02135-5.

IV. **Wu J#**, Zhang L#, Shi J, He R, Yang W, Habtezion A, *et al.* Macrophage phenotypic switch orchestrates the inflammation and repair/regeneration following acute pancreatitis injury. EBioMedicine. 2020;58:102920. doi: 10.1016/j.ebiom.2020.102920

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VII. **Wu J**, Zhang R, Hu G, Zhu HH, Gao WQ, Xue J. Carbon Monoxide Impairs CD11b+Ly-6Chi Monocyte Migration from the Blood to Inflamed Pancreas via Inhibition of the CCL2/CCR2 Axis. J Immunol. 2018 Mar 15;200(6):2104-2114. doi: 10.4049/jimmunol.1701169.

co-first author

Contents

List of abbreviations

ALL	Acute lymphoblastic leukemia
Allo-HCT	Allogeneic hematopoietic cell transplantation
AML	Acute myeloid leukemia
AP-1	Activator protein 1
AIM	Activation-induced marker
APCs	Antigen-presenting cells
BCR	B-cell receptor
BTK	Bruton tyrosine kinase
BTKi	Bruton tyrosine kinase inhibitors
CAR-T	Chimeric antigen receptor T cell
CLL	Chronic lymphocytic leukemia
CLPs	Common lymphoid progenitors
CLRs	C-type lectin receptors
CML	Chronic myeloid leukemia
CMPs	Common myeloid progenitors
CMV	Cytomegalovirus
COVID-19	Coronavirus Disease 2019
CR	Complete remission
CRS	Cytokine release syndrome
DAG	Diacylglycerol
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
DMSO	Dimethyl sulfoxide
EBV	Epstein-barr virus
ER	Endoplasmic reticulum
FasL	Fas ligand
FBS	Fetal bovine serum

FDA	Food and Drug Administration
FDCs	Follicular dendritic cells
Flu	Influenza
GC	Germinal center
GVHD	Graft-vs-host-disease
H. influenzae	*Haemophilus influenzae*
HBV	Hepatitis B
HCV	Hepatitis C
HEVs	High endothelial venules
HIV	Human immunodeficiency virus
HSCs	Hematopoietic stem cells
HSCT	Hematopoietic stem cell transplantation
HSV	Herpes simplex virus
IAV	Influenza A virus
ICB	Immune checkpoint blockade
ICS	Intracellular cytokine staining
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IGHV	Immunoglobulin heavy-chain variable region
IgM	Immunoglobulin M
IP_3	Inositol 1,4,5-triphosphate
IRF4	Interferon regulatory factor 4
IRs	Inhibitory receptors
ITK	Interleukin-2-inducible T cell kinase
JCV	John cunningham virus
K. pneumoniae	*Klebsiella pneumoniae*
M-IGHV	Mutated immunoglobulin heavy-chain variable region
M. catarrhalis	*Moraxella catarrhalis*

MALTs	Mucosa-associated lymphoid tissues
MDSCs	Myeloid-derived suppressor cells
NF-κB	Nuclear factor κB
NFAT	Nuclear factor of activated T cells
NK (cells)	Natural killer (cells)
NLRs	NOD-like receptors
ORR	Overall response rate
OS	Overall survival
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PFS	Progression-free survival
PKC	Protein kinase C
PLCγ1	Phospholipase Cγ1
pMHC	peptide-MHC complexes
PRRs	Pattern-recognition receptors
RLRs	RIG-I-like receptors
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
S. pneumoniae	*Streptococcus pneumoniae*
SEB	Staphylococcal enterotoxin B
SHM	Somatic hypermutation
SLOs	Secondary lymphoid organs
TAMs	Tumor-associated macrophages
Tcm	Central memory T cells
TCR	T cell receptor
Tem	Effector memory T cells
Temra	Terminally differentiated effector memory re-expressing CD45RA T cells
Tex	Exhausted T cells

Tex-int	Intermediate exhausted T cells
Tex-term	Terminally exhausted T cells
TFS	Treatment-free survival
Tfh	T follicular helper T cells
T_H (cells)	T helper (cells)
TLRs	Toll-like receptors
Tn	Naive T cells
Tpex	Precursor/progenitor exhausted T cells
Tregs	Regulatory T cells
Trm	Tissue-resident memory T cells
Tscm	Stem-like memory T cells
TST	Tumor-specific T cells
U-IGHV	Unmutated immunoglobulin heavy-chain variable region
VZV	Varicella-zoster virus

1 Introduction

This thesis addresses a fundamental question in cancer immunology: whether cellular immunity in immunosuppressed and elderly individuals, specifically patients with chronic lymphocytic leukemia (CLL), can mount effective responses and function normally against bacterial and viral infections, and whether such immune function can truly recover after tumor remission following targeted therapy.

Currently, more than 60% cancers occur in older adults, and over 70% of cancer-related death arise in this population (1). CLL represents a prototypical cancer of older adults and serves as a valuable human disease model for studying tumor–immune interactions under immunocompromised conditions. Patients with CLL exhibit a combined state of immunosenescence and tumor-driven immunosuppression, which together exacerbate perturbations within the immune microenvironment. Given the high susceptibility to infections in these patients, both before and after receiving treatment, this thesis focused on pathogen-specific T cell responses in the context of CLL.

In **Study I**, we investigated bacteria- and virus-specific $CD4^+$ T cell before and after different generation of Bruton tyrosine kinase (BTK) inhibitor (BTKi) treatment. In **Study II**, we examined virus-specific $CD8^+$ T cell under the same therapeutic conditions. In **Study III**, we assessed immune responses to coronavirus disease of 2019 (COVID-19) vaccination in patients receiving the newer generations of BTKi therapy, and in **Study IV**, we analyzed T cell response to a highly mutated severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) variant. Together, these studies aimed to determine whether T cells can provide effective responses against diverse infections in an immunosuppressed cancer setting.

2 Literature review

2.1 Introduction to CLL

2.1.1 Leukemia definition and classification

Leukemia is a malignancy of the hematopoietic system, primarily affecting the bone marrow and lymphatic tissues. Globally, it ranks 13th in incidence and 10th in mortality (2) . The disease is categorized into four major types, including acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), CLL, and chronic myeloid leukemia (CML). Its incidence is highest in Western regions, particularly North America, Australia and Europe (**Figure 1**) (3, 4).

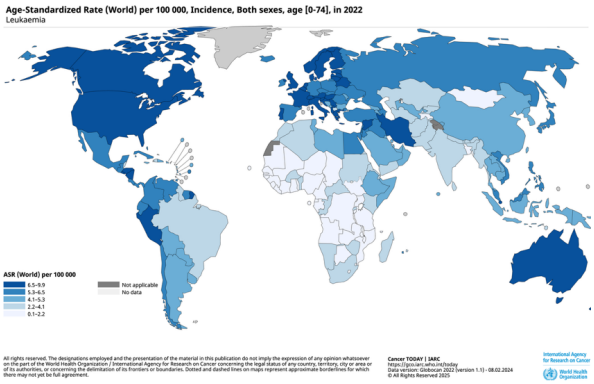

Figure1: Age-Standardized incidence rate of Leukemia in 2022 worldwide (Picture from (4))

2.1.2 Epidemiology of CLL

CLL is the most common adult leukemia in the USA and Europe, representing 95% of chronic leukemia cases and 25% of all leukemia (5). In 2019, it accounted for more than 100,000 new diagnoses and over 40,000 deaths worldwide (6). In the USA alone, an estimated 23,690 new cases were diagnosed in 2025, accounting for about 1.2 % of all cancers (7). It affects more than 200,000 people and is associated with approximately 4410 deaths in the US annually (8). In 2024, approximately 40,000 patients were estimated to receive first-line treatment for CLL across the US, UK, France, Germany, Spain, Italy, Japan, and China (9). This malignancy incidence rises steeply with age, reaching a peak in the elderly population and mainly occurs in older men, with a median age of 70 years at diagnosis and 82 years at death (7).

2.1.3 CLL features, pathogenesis, risk factors and symptoms

CLL is a B-cell hematologic malignancy defined by the clonal expansion of morphologically mature but functionally incompetent $CD5^+$ B cells (10, 11). It is

defined by a minimum number of monoclonal B cells of 5 × 10^9/L (8). Malignant cells are characterized by the elevated expression of CD5, CD19, CD20, and CD23, as well as surface or cytoplasmic kappa and lambda light chains in the peripheral blood, secondary lymphoid tissues, and bone marrow (8, 12). For differential diagnosis from other small B-cell leukemias, CLL can also be identified by markers such as CD10, CD43, CD79b, CD81, CD200, and ROR1 (13).

The pathogenesis of CLL is primarily driven by impaired apoptosis due to BCL2 overexpression and sustained proliferation mediated by antigen-independent, constitutive activation of the B-cell receptor (BCR) pathway (8). As an upstream determinant of BCR signaling, the mutation status of the immunoglobulin heavy-chain variable region (IGHV), which encodes part of the heavy-chain variable domain of the BCR, represents a robust prognostic and predictive biomarker (8). During CLL ontogeny, naïve B cells may follow two distinct routes of activation (14). Through the T cell-independent pathway, B cells are activated without germinal center (GC) entry and therefore retain unmutated IGHV genes (U-IGHV) (14, 15). These U-IGHV CLL originates from pre-GC naive B cells or from antigen-experienced B cells that developed outside GCs (**Figure 2**) (15). By contrast, in the T cell-dependent pathway, B cells migrate into GC of secondary lymphoid organs, undergo somatic hypermutation (SHM), and acquire mutated IGHV genes (M-IGHV) (**Figure 2**) (14, 15). IGHV mutational status is a critical prognostic and predictive marker: patients with M-IGHV typically show a more indolent disease course and favorable outcomes (16). Importantly, U-IGHV is included in the International Prognostic Score for Asymptomatic Early-stage CLL, together with absolute lymphocyte count ≥15 × 10^9/L and palpable lymphadenopathy (17).

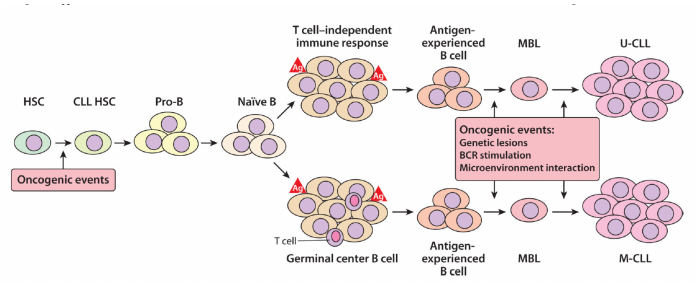

Figure2: A model for the development of CLL(Picture from (14)).

Five independent factors are associated with inferior survival in CLL: del (17p) or TP53 mutation, U-IGHV status, β2-microglobulin >3.5 mg/L, advanced clinical stage (Rai I-IV or Binet B-C), and age >65 years (11). More than 80% CLL patients

harbor genetic lesions (18) affecting cell cycle control, apoptosis, DNA damage response, RNA processing, and other signaling pathways (19). The initial genetic lesions that drive CLL development may occur in hematopoietic stem cells (HSCs), although this remains controversial. It has been reported that in some cases of allogeneic hematopoietic stem cell transplantation (HSCT), premalignant lymphoid cells were transmitted from the donor to the recipient and subsequently gave rise to CLL (15). Common abnormalities include del (13q), Trisomy 12, NOTCH1, del (11q)/ATM disruption, and del (17p) /TP53 loss (15). Del13q14 is the most frequent lesion in CLL (~50-60%) and often the unique abnormality, consistent with a role in early disease development (15, 20-22). Genetic lesions in the 3′ untranslated region of NOTCH1 confer a similarly poor prognosis as other NOTCH1 mutations in CLL (15, 20, 23). Patients with TP53 mutations or CD38 positivity experience a shorter time to first infection, reflecting increased susceptibility to infections (24).

Most CLL patients show no symptoms before diagnosis apart from increased lymphocytosis (8). Almost 70% to 80% of all CLL patients are diagnosed incidentally while being asymptomatic (8). In individuals displacying symptoms, signs like enlarged lymph nodes, liver, and spleen swelling, unintended weight loss, fever, elevated temperature, soaking night perspirations, or severe tiredness (often referred to as B symptoms) may appear. Rai stage classification defines the risk level and clinical stage for CLL (stages 0–IV). Low-risk disease is defined by lymphocytosis only, intermediate-risk by palpable lymphadenopathy or hepatosplenomegaly, and high-risk by anemia or thrombocytopenia (15).

2.1.4 Current treatment of CLL

Asymptomatic CLL patients without anemia, neutropenia, or thrombocytopenia are managed with active observation rather than therapy, as early treatment has shown no clinical benefit in overall survival in this group; therefore, most do not require therapy at diagnosis (8). Patients require treatment when CLL-related complications arise, including significant anemia or other cytopenias, massive, progressive, or symptomatic lymphadenopathy or hepatosplenomegaly, recurrent infections, or refractory autoimmune cytopenias attributable to CLL (8).

Although durable remissions can be achieved in CLL, the disease remains incurable without allogeneic hematopoietic cell transplantation (Allo-HCT). Consequently, patients often require repeated courses of therapy (8). Current CLL management relies on genetic classification to guide therapy, with key

markers such as TP53 mutations, del (17p), del13q, Trisomy 12, del11q and IGHV mutation status routinely assessed. In earlier decades, chemoimmunotherapy was the standard of care of CLL treatment. However, clinical evidence has demonstrated its inferior efficacy, with shorter progression-free survival (PFS) and overall survival (OS) compared with novel targeted therapies (25-29). Consequently, first-line management now relies on BTKi (ibrutinib, acalabrutinib, zanubrutinib, pirtobrutinib), BCL2 inhibitor (venetoclax), PI3K inhibitors (idelalisib, duvelisib), and anti-CD20 antibodies, used either alone or in combination (8). Patients will need to change the treatment strategy if there is relapsed or refractory CLL for at least 6 months (8, 10, 30).

For patients with high-risk CLL who lack other treatment options, noncovalent BTKi (pitobrutinib), Allo-HCT, or Chimeric antigen receptor T cell (CAR-T) therapy are consieded if CLL progresses after BTKi or BCL2 inhibitor therapy. However, Allo-HCT is associated with 30%-50% incidence of graft-vs-host-disease (GVHD) (31) and CAR-T is less effective in CLL than in younger B-cell malignancies such as ALL, with many clinical trails showing complete remission (CR, 28%-68%) rates significantly lower than those reported in ALL trials (62%-86%) (32). The TRANSCEND CLL 004 trial, the largest study reported to date in CLL, showed that among efficacy-evaluable patients treated at dose level 2, the CR (including incomplete marrow recovery) rate was 18%, with an overall response rate of 47% (33). These rates are considerably lower than the complete response rates achieved with CAR-T therapy in ALL, highlighting the unique resistance and functional impairment of CAR-T cells in the CLL setting.

BTKi generation

BTKi have fundamentally transformed the therapeutic landscape of CLL and other hematologic malignancies. BTK, a member of the Tec family of tyrosine kinases, is predominantly expressed in B cells, where it plays a central role in BCR signalling and survival. When activated, BTK initiates a series of downstream signalling pathways, including PI3K-AKT, PLC, PKC, IP3-mediated calcium release induced NFAT signalling and NF-κB pathways (34). BTKi block the BCR signalling cascade by binding to BTK, thereby impairing the function, proliferation, and survival of both malignant and normal B cells (35). BTKi can be broadly classified into two categories: covalent and non-covalent. Within the covalent class, notable members include ibrutinib, acalabrutinib, zanubrutinib, orelabrutinib, tirabrutinib, and evobrutinib, which irreversibly inhibit BTK enzyme activity. Over 90% of patients show a response to ibrutinib, acalabrutinib and zanubrutinib (36). The

non-covalent category reversibly inactivates BTK, such as pirtobrutinib, vecabrutinib, and ARO-531. Over the past decades, BTKi have gained prominence in the treatment of B-cell malignancies, notably revolutionizing the therapeutic approach for CLL.

Ibrutinib

Ibrutinib, as the first-generation covalent BTKi, has demonstrated robust efficacy in CLL across numerous clinical studies by targeting BTK in BCR signaling and inhibiting malignant B-cell proliferation, migration, and survival. It was initially accessed in 2013 within a phase 1b-2 multicenter study (37) and approved for relapsed or refractory CLL by Food and Drug Administration (FDA) in 2014 in a multicenter, open-label, phase 3 study (38). Early phase trials of ibrutinib demonstrated durable remissions, strong efficacy and favorable safety in relapsed or refractory CLL with an 75% PFS and 83% OS at 26 months (37). RESONATE study and RESONATE-2 study demonstrated significantly improved PFS, OS, and overall response rate (ORR) over time versus chlorambucil (chemotherapy agent) and Ofatumumab (anti-CD20 antibody) (38, 39).

However, with impressive selectivity for BTK has shown, ibrutinib can also interact with other kinases, like interleukin-2-inducible T cell kinase (ITK), to varying degrees, which is called "off-target" effects (**Figure 3**). ITK, a member of the Tec family of tyrosine kinases, acts downstream of TCR signaling and plays a dominant role in this pathway. Within TCR signaling, MAPK and NFAT has been shown digitally activated with minimal ITK dependence, whereas NF-κB activation graded and strongly regulated by ITK (40). It can lead to phosphorylation of the downstream target PLCγ1 and influence downstream DAG-MAPK/ERK and DAG-PKC-NFκB signaling as well as IP3- Ca^{2+} release (41) Thus, ibrutinib could lead to undesired effects on pathogen-specific T cell responses, which further needs to be resolved.

Acalabrutinib and zanubrutinib

Acalabrutinib and zanubrutinib, second-generation covalent BTK inhibitors, exhibit greater selectivity for BTK than ibrutinib. They were engineered to bind more selectively to cysteine 481 within the kinase domain (35). This design minimizes "off-target" effects compared with ibrutinib, with zanubrutinib showing reduced and acalabrutinib showing nearly absent "off-target" activity.

Two clinical trials (ALPINE ClinicalTrials and ELEVATE R/R study) have demonstrated such "off-target" effects are more limited for second-generation

BTKi, as compared to ibrutinib, with fewer cardiovascular adverse events, improved PFS with zanubrutinib, higher ORR with zanubrutinib, and fewer adverse events due to treatment discontinuation (42, 43). Clinical studies also indicate that ibrutinib-intolerant patients can benefit from acalabrutinib or zanubrutinib treatments, and those intolerant to either ibrutinib or acalabrutinib can also effectively use zanubrutinib (44-46). In gerneral, clinical studies have demonstrated that second-generation BTKi provide improved safety profiles and superior efficacy compared with ibrutinib. With its FDA approval for CLL on January 19, 2023, zanubrutinib has emerged as a promising therapeutic option in the CLL treatment landscape.

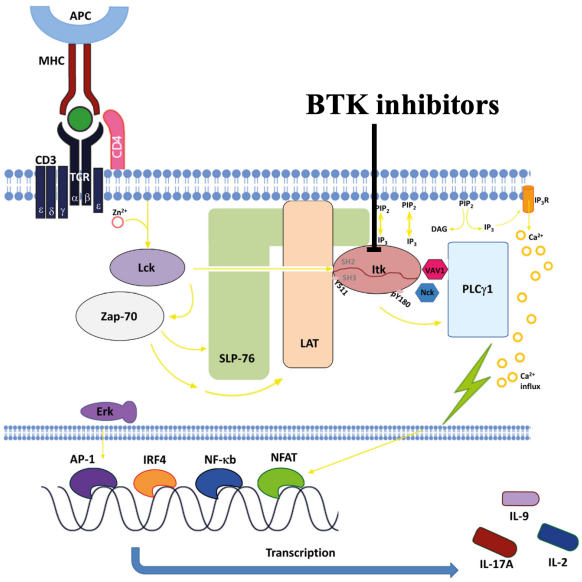

Figure3: BTK inhibitor "off-target" effect on ITK signalling (Picture adjusted from (47)).

2.2 Infections in CLL

CLL is an indolent disease in which many patients die from infections rather than from the leukemia itself (48). Nearly 80% of CLL patients suffer from infectious complications, which can lead to death (49). Infections represent the leading cause of death in CLL patients aside from the underlying haematological malignancy (50, 51). The increased vulnerability of this group was evident during the COVID-19 pandemic, as they faced heightened severity and mortality rates (52). Infections persist as a significant contributor to both morbidity and mortality among CLL patients. Infections are responsible for 60% to 80% of all deaths in these individuals (53, 54). Crassini *et al.* evaluated the impact of infections on OS

in a long-term follow-up cohort and found a significant association between infections during the first year of observation and OS (55) (**Figure4**). Comorbidity, multimorbidity and an older age are strongly associated with increased mortality in CLL, with infections contributing as a major cause (51). Together, these facts place infections as a primary clinical concern in CLL.

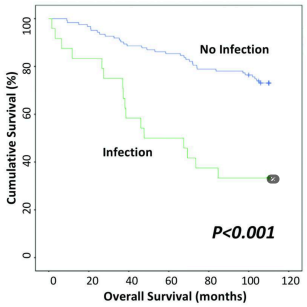

Figure4: Significant association between infection and OS in patients with CLL (Picture from(55)).

2.2.1 Immune dysfunction and infection risk in CLL

Immune dysfunction is evident from the early stages of CLL, although most patients do not require treatment at diagnosis (54). Patients with CLL have a heightened risk of infection due to impaired cellular and humoral immunity. These individuals display functional defects in B and T lymphocytes, NK cells, dendritic cells, neutrophils, and macrophages (56-58). In CLL, proliferating leukemic B cells dominate the microenvironment, suppress normal B- and T-cell function, and contribute to hypogammaglobulinemia, pseudo-exhausted immune function, and cytokine changes (48). Patients at early stages of CLL or those receiving intensive therapy or experiencing disease progression often have reduced immunoglobulin production, leading to defective clearance of bacteria and viruses and thereby further increasing infection risk (59). A Danish study reported that CLL patients with hypogammaglobulinemia have poor survival (53, 59) and deficiencies in multiple immunoglobulins and immunoglobulin G (IgG) subclasses are significantly associated with shorter treatment-free survival (TFS) (60). In addition, CLL patients show impaired neutrophil phagocytosis and defective killing of key bacterial pathogens, including *Staphylococcus aureus* and *Pseudomonas aeruginosa* (60). In most cases, the increased infection risk is associated with an older age, advanced stage, unmutated IGHV, high level of β2-microglobulin, and low level of immunoglobulin A (IgA), IgG and immunoglobulin M (IgM) (53, 55, 61, 62). Treatment-related immune suppression, especially after chemoimmunotherapy,

can further increase infection risk, although newer targeted agents may mitigate or even reverse some aspects of immune dysfunction over time (62, 63).

2.2.2 Bacterial infection in CLL patients

In patients with CLL, bacterial infections are a notable concern. A breakdown of the types of infections these patients encounter reveals that bacterial infections are the most prevalent, accounting for 67% of cases (64). It's noteworthy that only a small fraction, 3%, of these infections are categorized as severe (54). Encapsulated bacteria like *Streptococcus pneumoniae* (*S. pneumoniae*) and *Haemophilus influenzae* (*H. influenzae*) are predominant pathogens in CLL patients (54). The different treatment modalities in CLL are associated with specific infections, where some treatment even increase the risk for infection, like PI3K inhibitor treatment (65, 66). Bacterial infections are particularly common in CLL patients undergoing therapy, especially those receiving chemotherapy, anti-CD20 or BCL-2 inhibitor treatment, as well as in individuals with hypogammaglobulinemia or chemotherapy-induced neutropenia (53, 59, 61). These infections are most often caused by *Staphylococcus aureus*, *Streptococcus pneumoniae*, and a variety of Gram-negative organisms including *Pseudomonas aeruginosa*, *Escherichia coli*, *H. influenzae*, *Moraxella catarrhalis* (*M. catarrhalis*), and *Klebsiella pneumoniae* (*K. pneumoniae*) (58, 64, 67). A three-year follow-up of ibrutinib in CLL study also revealed a higher incidence of grade 3 or higher infections in relapsed or refractory patients compared to treatment-naive patients, including pneumonia, sepsis, and bacteremia (68). Among treatment-naive patients, respiratory tract bacterial infections were the most frequently reported, most caused by *Staphylococcus aureus*, *S. pneumoniae*, and *H. influenzae* (54).

2.2.3 Viral infection in CLL patients

Viral infections constitute 25% of the infections in patients with CLL (64). These patients have an increased susceptibility to viral infections, which contribute to the high mortality rate due to impaired antibody production and weakened T cell responses. These infections are caused by a wide range of viruses, including herpesviruses, respiratory viruses, hepatitis B and C viruses, and human polyomaviruses (64). Specifically, herpes simplex virus (HSV), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), cytomegalovirus (CMV), hepatitis B (HBV), hepatitis C virus (HCV), john cunningham virus (JCV), and influenza are among the most frequently detected viruses (67). Influenza has been shown to cause 25%-37% deaths in these immunocompromised individuals (49). Compared with the

general population, CLL patients have a markedly increased risk of developing severe complications from both influenza and COVID-19. Among herpesviruses, EBV, CMV, and VZV are particularly associated with severe complications in CLL patients (69). EBV is widespread, with more than 90% of adults being seropositive globally (70). In CLL patients, the concern is not primary infection but rather EBV reactivation and its link to disease progression, particularly Richter's transformation (71). Research has shown that patients with ITK mutation are more susceptible to viral infections, particularly EBV (72). CMV infection takes up to 70% of patients with CLL and is most frequently observed in those receiving alemtuzumab therapy (73). Therefore, it is crucial to closely monitor CLL patients who contract viral infections.

2.2.4 SARS-CoV-2 vaccination in CLL

Patients with CLL are immunocompromised due to both the disease itself and its treatment. During natural SARS-CoV-2 infection, they exhibit profoundly impaired immune responses, including defective humoral and cellular immunity. These defects manifest as delayed viral clearance, poor antibody generation, and dysfunctional T cell activation compared with healthy individuals (74-78). Such intrinsic immune deficits also underlie their inadequate vaccine responsiveness observed after immunization. This vulnerability became particularly evident during the COVID-19 pandemic, underscoring the critical importance of vaccination for infection prevention and mitigation. A compromised immune system, and not only B cell dysfunction alone, represents a key barrier to effective vaccination in CLL. Vaccination against influenza, pneumococcal disease, COVID-19, and varicella-zoster is recommended for all patients according to current treatment guidelines (8).

In CLL, only non-live vaccines are considered safe for individuals. SARS-CoV-2 mRNA vaccines, in which the mRNA encodes the spike protein that is translated into an antigenic protein to trigger a specific immune response, are widely used and safe. Adenoviral vector vaccines were also included for these immunocompromised patients initially, but have been replaced with mRNA vaccines. Both type of vaccines can induce a spike-specific humoral immunity or cellular immunity in patients with CLL. However, patients with CLL respond poorly to vaccines, with both humoral and cellular responses markedly lower than those observed in healthy individuals (79-83). The efficacy of the vaccination in patients with CLL is adversely affected not only by disease progression, but also by older age, current treatments, hypogammaglobulinemia,

and abnormalities in haemoglobin and platelet levels (79-84). The impaired immunity observed in CLL results from multiple deficiencies. Patients with hypogammaglobulinemia fail to produce sufficient neutralizing antibodies against viral antigens, while dysfunctional $CD4^+$ and $CD8^+$ T cells provide inadequate help to B cells and display reduced effector activity. Furthermore, diminished T cell response, cytokine production, and cytolytic capacity collectively limit the cellular immune response to vaccination in CLL (85).

Treatments such as anti-CD20 antibodies or BTKi have been shown to further compromise immune responses as compared to treatment-naïve patients, including those to vaccination, as reflected by lower seropositivity rates and weaker antibody response (80, 82, 84, 86). Similarly, the response to hepatitis B vaccination is significantly reduced in BTKi-treated CLL patients relative to untreated individuals (87). However, repeated SARS-CoV-2 vaccination has been shown to enhance immune responses, with a proportion of previous non-responders developing detectable immunity following an additional mRNA-based dose (88, 89). Patients receiving anti-CD20 therapy demonstrate particularly poor responses to various types of vaccines, which may improve gradually over time but generally remain lower than those observed in healthy individuals (86, 90). Similarly, poor responses to influenza vaccination have also been observed in lymphoma patients who received anti-CD20 antibody therapy within the past six months, indicating that such treatment markedly impairs vaccine-induced antibody production in patients with hematological malignancies (91). These findings suggest that, compared with treatment-naïve individuals, patients with CLL on different therapies are at higher risk of infection and experience reduced efficacy of SARS-CoV-2 vaccination, possibly leading to worse clinical outcomes.

2.3 Immune response

The immune system plays a vital role in preserving homeostasis and defending the host against infections and other potentially harmful antigens. It comprises two main components: the innate and adaptive immune systems. Patients with CLL exhibit intrinsic immune deficiencies that affect both arms of immunity. These include hypogammaglobulinemia, alterations in T cell subsets, and functional defects in neutrophils and monocytes/macrophages (53, 58, 92).

2.3.1 Innate immune system

The innate immune system serves as the body's first line of defense against pathogens and foreign substances. It is evolutionarily conserved and genetically encoded, enabling immediate activation without prior exposure to an antigen. Recognition of exogenous microorganisms occurs through the detection of pathogen-associated molecular patterns (PAMPs), whereas endogenous danger signals released from host's sterile tissue injury or cell death are recognized as damage-associated molecular patterns (DAMPs). Both PAMPs and DAMPs are sensed by pattern-recognition receptors (PRRs), which are encoded by the host genome and expressed on the surface or within the cytoplasm of innate immune cells. Major PRR families include Toll-like receptors (TLRs) (93), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), and C-type lectin receptors (CLRs). Engagement of these PRRs activates intracellular signalling cascades that rapidly induce antimicrobial and inflammatory responses, leading to the production of proinflammatory cytokines and type I interferons.

Innate immunity comprises diverse cell types that act in coordination to initiate protective responses. Some of them are outlined here. Macrophages, mainly derived from circulating monocytes, perform phagocytosis to engulf and eliminate pathogens within tissues. Granulocytes, including neutrophils, eosinophils, and basophils, play critical roles in inflammatory and antimicrobial responses. Neutrophils are typically the first responders at sites of infection, efficiently recognizing and destroying invading microbes. Dendritic cells (DCs) capture and process pathogens into peptide antigens for presentation to T cells, while also producing type I interferons and other cytokines to enhance antiviral defense. Natural killer (NK) cells are essential for identifying and eliminating virus-infected or malignant cells via receptor recognition. Mast cells contribute to allergic responses and parasite defense by releasing histamine and other inflammatory mediators. In addition, $\gamma\delta$ T cells, although traditionally classified within the adaptive immune system, can exhibit innate-like functions by responding rapidly to conserved microbial antigens (94).

Most innate immune cells originate from myeloid progenitors within the bone marrow. Common myeloid progenitors (CMPs) differentiate into dendritic cells, granulocytes, monocytes/macrophages, and mast cells, which subsequently mature in peripheral blood and tissues. In parallel, common lymphoid progenitors (CLPs) give rise to both adaptive immune cells, including T and B lymphocytes,

and innate lymphoid cells such as NK cells and innate lymphoid cells (ILCs) subsets.

In CLL, several components of innate immunity are functionally impaired. For instance, polymorphonuclear neutrophils exhibit defective microbicidal activity against bacteria and produce soluble factors that promote leukemic B cell survival (95). Monocytes and macrophages from CLL patients show dysregulation of phagocytosis-related proteins and genes, reflecting compromised antigen uptake and clearance capacity (96). Tumor-associated macrophages (TAMs) acquire pro-tumorigenic and immunosuppressive phenotypes that support leukemic progression (97). Circulating DCs and NKs also display defective compartments, with an impaired ability to trigger effective T cell responses (98, 99). Collectively, these innate immune defects contribute to global immune dysregulation and increased susceptibility to infections in CLL.

2.3.2 Adaptive immune system

The adaptive immune system provides antigen-specific responses and immunological memory, forming another line of defense. B and T cells are the principal adaptive immune cells. Both originate from hematopoietic stem cells in the bone marrow, where T cells migrate to the thymus for maturation, and B cells mature within the bone marrow. After maturation, these lymphocytes circulate in the bloodstream and home to secondary lymphoid organs (SLOs) such as lymph nodes, the spleen, and mucosa-associated lymphoid tissues (MALTs), including Peyer's patches (94).

As one of the main adaptive immune cell types, conventional αβ T cells differentiate into two major subsets: $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells. $CD4^+$ T cells further develop into distinct effector subsets, each characterized by specific cytokine profiles and cell-cell interactions (100). Th1 cells target intracellular bacterial and viral infections; Th2 cells mediate immune responses against extracellular helminths and are also involved in allergic inflammation; Th17 cells combat extracellular bacteria, fungi or helminth infection; T follicular helper cells (Tfh) promote B-cell antibody production and GC formation; and Treg cells suppress excessive immune activation to maintain immune tolerance. These subsets are regulated by lineage-defining transcription factors, including T-bet in Th1, GATA3 in Th2, RORγt in Th17, Bcl6 in Tfh cells and Foxp3 in Treg cells (100). Each subset plays a specialized role in coordinating the immune response and maintaining immune homeostasis.

Activation of T cells is initiated by TCR recognition of antigen-bound MHC molecules, accompanied by co-stimulatory signalling primarily mediated through the CD28-B7 interaction. Cytokine secretion not only reinforces lineage differentiation but also refines the effector functions of distinct T cell subsets. IFNγ and IL-2 produced by Th1 and $CD8^+$ T cells enhance cellular immunity, while IL-4 released from Th2 cells promotes B cell activation and class switching toward IgE production. IL-17 and IL-22 produced by Th17 cells drive neutrophil recruitment, whereas IL-21 secreted by Tfh cells supports B-cell maturation, and TGFβ from Treg cells maintains immune homeostasis (94).

$CD4^+$ T cells are activated through MHC class II molecules and primarily function as helper cells within the immune system. By releasing IFN γ and interacting with antigen-presenting macrophages, they enhance macrophage-mediated killing of intracellular pathogens. In addition, $CD4^+$ T helper (T_H) cells assist antigen-presenting B cells by promoting antibody production, class switching, and GC formation, a process largely mediated by Tfh cells that orchestrate B-cell maturation and differentiation (94).

$CD8^+$ T cells, activated through the MHCI complex, serve as cytotoxic effector cells that eliminate infected or malignant targets. They primarily recognize and kill cells harbouring intracellular pathogens, such as viruses or certain intracellular bacteria, through the release of perforin and granzymes, which induce pore formation and apoptosis in target cells. They also trigger apoptosis via Fas ligand (FasL)-Fas interactions and secrete cytokines such as TNF to amplify cytolytic responses (94).

After encountering an antigen transitted by DCs into local lymph nodes, a proportion of activated $CD4^+$ and $CD8^+$ T cells differentiate into memory T cells after clonal expansion and contraction during the effector phase (**Figure 5**) (100). These long-lived cells persist within the host and mount faster and more effective responses upon re-exposure to the same antigen (100).

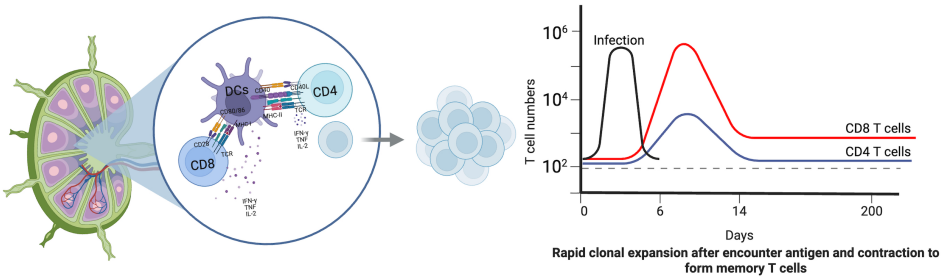

Figure5: T cells encounter antigen from DCs in lymph nodes (Picture created from biorender.com)

B cells are activated when their BCRs recognize and bind to specific antigens. Upon activation, a subset of B cells differentiates into plasma cells, which serve as specialized antibody-producing factories that secrete immunoglobulins targeting the encountered antigen. Others develop into memory B cells that provide long-term protection by rapidly responding to subsequent exposures to the same antigen (101).

In patients with CLL, malignant B cells exhibit impaired expression of co-stimulatory molecules, defective MHC-mediated antigen presentation, and multiple functional abnormalities T cells display features of exhaustion, impaired activation, reduced cytotoxic capacity, and overall functional dysfunction, with a skewing toward effector differentiation (102). In the following subsection, we will focus on the abnormalities of T cells observed in CLL.

2.3.3 Immunological memory

Immunological memory, a hallmark of the adaptive immune system, encompasses both humoral immunity, mediated by antibodies produced by B cells, and cell-mediated immunity, encompassed by T cells. Upon an initial exposure to a pathogen or antigen, the adaptive immune system generates a long-lasting protective memory response. As a result, subsequent encounters with the same antigen elicit a more rapid and robust immune response than the first exposure (100, 103). This memory response allows the immune system to "remember" the pathogen and immediately respond to it upon reinfection, which also lays the foundation for effective vaccination (103).

B cell activation and memory formation

Naïve B cells, generated from bone marrow and circulating through the SLOs are attracted into B-cell follicles by the chemokine CXCL13, which is constitutively

produced by follicular dendritic cells (FDCs) (104). This chemokine gradient guides B cells (via their CXCR5 receptor) to the follicles, where they can encounter antigens. Tfh cells, which also express CXCR5, are concurrently recruited by CXCL13 to the boundary of T cell zones and B cell follicles (T-B border) where B-T interactions occur (104)

Upon antigen recognition, naïve B cells become activated, undergo clonal expansion, and differentiate into effector and memory cells. When a naïve B cell's BCR binds its specific antigen, the B cell internalizes the antigen, processes it, and displays peptide fragments on MHC class II molecules. The B cell then migrates to the follicular border to present these peptide-MHC II complexes to $CD4^+$ T helper cells, especially Tfh cells (105). Engagement of the T cell receptor (TCR) by the peptide-MHC II, along with co-stimulatory signals, like CD40 on B cell binding CD40L on T cell, activates the B cell further. This T cell "help" is critical for B cell fate since the Tfh cell provides cytokines and additional signals that drive SHM and B cell proliferation and maturation (105). Notably, B cells primarily present antigen to $CD4^+$ T cells, but some studies have shown that B cells can also cross-present exogenous antigen on MHC class I to $CD8^+$ T cells under certain conditions (106), though this is a less common pathway.

After activation, B cells undergo clonal expansion and form a GC within the follicle. GCs are specialized microenvironments where B cells rapidly proliferate and mutate their immunoglobulin genes through SHM, leading to increased antibody affinity (107). The GC is constituted into a dark zone where proliferating B cells accumulate SHM and a light zone where B cells encounter antigen retained on FDCs and interact with Tfh cells (101). In the light zone, Tfh cells and FDCs selectively keep B cells with higher-affinity BCRs whereas others die off (101). Positively selected B cells then differentiate into one of two major paths: antibody-secreting plasma cells or memory B cells (101). Some selected B cells may also recycle back to the dark zone for additional rounds of mutation and selection, further improving antibody affinity. The end result of the germinal center reaction is the generation of long-lived plasma cells which migrate to the bone marrow or other niches and continuously secrete high-affinity antibodies and durable memory B cells that persist in lymphoid tissues or circulation (101). Memory B cells are quiescent but can rapidly reactivate upon re-exposure to the antigen, quickly proliferating and differentiating into plasma cells to produce antibodies in secondary responses.

T cell activation and memory formation

Naïve T cells constantly recirculate and enter SLOs, like lymph nodes, from the blood through specialized high endothelial venules (HEVs). This homing is guided by the CCL19/CCR7 and CCL21/CCR7 chemokine-receptor axes, through HEVs. These T cells express CD62L (L-selectin) and LFA-1, which enable them to adhere to and roll on the HEVs. Once inside the lymph node, naïve T cells scan antigen-presenting cells (APCs) for their specific antigen.

DCs carrying antigen enter lymph nodes via afferent lymphatic vessels and display antigenic peptides on MHC molecules. Naïve T cells become activated when their TCR recognizes a peptide-MHC complex on DCs or other APCs. However, TCR signaling alone is not sufficient; full T cell activation requires a second co-stimulatory signal provided by the APCs (108-110). A primary co-stimulatory interaction is CD28 on the T cell binding to B7-1/B7-2 (CD80/CD86) on the APC, which promotes T cell proliferation and survival (108-110). Once a naïve T cell receives both signals along with appropriate cytokines, like IL-2 and IL-12, it clonally expands within the lymph node. Co-inhibitory signals like PD-1, CTLA-4, LAG-3, and TIM-3 function as balanced brakes to prevent excessive activation (100). During this procedure, each T cell gains a unique TCR, which is an antigen-specific T cell, and forms a different-sized clone after expansion. Activated T cells downregulate CCR7 and L-selectin, and after several hours of proliferation, these effector T cells upregulate egress receptors and exit the lymph node via efferent lymphatics, eventually reaching the bloodstream and migrating to the site of infection. $CD8^+$ cytotoxic T cells will traffic to infected tissues to kill pathogen-infected cells, and $CD4^+$ helper T cells will migrate to assist different immune cells in peripheral sites.

Once the acute infection is resolved, the T cell population contracts as the majority of effector cells undergo apoptosis. A small fraction, approximately 5-10%, survive as memory precursor T cells that retain the capacity to de-differentiate into long-lived memory cells with stem-like properties (100, 111). These memory cells can also arise during clonal expansion following antigen stimulation, a process in which asymmetric cell division generates progeny with distinct fates (112). In this division, $TCF7^{hi}$ progenitor-like effector cells have been shown to give rise to central memory T cells (Tcm) (113). Some studies have also proposed that distinct memory precursor subsets arise independently during the contraction phase, giving rise separately to Tcm, effector memory T cells (Tem), and tissue-resident memory T cells (Trm) (114). These all process forms the basis

of the classical effector-derived, or contraction-phase, model of memory T cell differentiation. Alternatively, the linear differentiation model suggests that memory cells develop progressively along a trajectory from naive T cells (Tn) to Tcm, Tem, and finally terminally differentiated effector memory re-expressing CD45RA T cells (Temra). This model is supported by evidence of gradual changes in chromatin accessibility, transcriptomic signatures, and co-regulated gene expression clusters across these subsets (115). In contrast to both models, the predetermined fate model, proposes that the fate of memory cells is preconditioned early during the naïve T cell stage (116).

Regardless of their developmental pathway from naïve cells, memory T cells have been shown to be long-lived, quiescent, and slowly proliferative, capable of persisting in the host for years or even a lifetime (117). Although the effector genes do not express without stimulation, their epigenetical loci preserve the active ability (118). Furthermore, their chromatin loci at effector genes remain open even for a decade which allows them to respond vigorously upon re-exposure to the antigen, providing rapid cell-mediated protection (100, 117).

Circulating memory T cells and Trm are two distinct categories of memory T cells that aid the immune system in recalling and responding to past pathogen encounters (119). Depending on the homing recetor CCR7 and the differentiation marker CD45RA expression, circulating memory cells can be classified into diffferent subsets: stem-like memory T cells (Tscm, marked by $CD95^{+}$ $CD45RA^{+}CCR7^{+}$), central memory T cells (Tcm, marked by $CD45RA^{-}CCR7^{+}$), effector memory T cells (Tem, marked by $CD45RA^{-}CCR7^{-}$), and terminally differentiated effector memory T cells re-expressing CD45RA (Temra, marked by $CD45RA^{+}CCR7^{-}$) (120-122).

Tscm serves as a relatively "naïve" memory cell, as they highly express CCR7 and CD62L, which facilitate their localization within lymph nodes T cell zones (121). They possess a robust capacity for proliferation. Similarly, Tcm cells also express CCR7 and CD62L, favoring their migration to SLOs and exhibiting a state akin to Tscm, but remaining somewhat more activated. They both have low cytotoxic activity and a high proliferative capacity. However, Tcm cells, in contrast to Tscm cells, proliferate to a less extent and express the CD45RO marker, indicating a relatively more differentiated status (121).

Tem cells function as a reserve response pool, readily differentiating into effector cells upon encountering antigen stimulation (122, 123). They express inflammatory chemokine receptors, like CCR2, CCR4, and CCR5, that enable them to sense

inflammation, facilitating their rapid migration from the bloodstream to inflamed tissues (120, 124). In these tissues, Tem cells secrete various cytokines, chemokines, and cytolytic proteins to stop pathogen propagation. Temra cells represent a more terminally differentiated subtype of effector memory cells (122). They express effector molecules like perforin, granzyme B, cytokines such as IFNγ, and the cytotoxic effector marker CX3CR1 and are mostly confined to the blood during steady state (125).

In contrast to circulating memory cells, Trm cells do not traffic through the bloodstream. Instead, they establish residency within epithelial tissues, where they remain localized. This unique characteristic allows Trm cells to quickly recall and respond to previous infections at the site of initial exposure (119, 126, 127).

2.3.4 T cell response in CLL

T cell responses are thought to be dysfunctional in patients with CLL (102, 128, 129). Dysfunction in T cells results in a heightened susceptibility to infections and a reduced ability to manage the progression of CLL. T cells exhibiting exhaustion are characterized by their diminished rates of proliferation, decreased cytotoxicity, impaired activation, skewed effector phenotype, altered cytokine production, and increased expression of several inhibitory receptors, such as CTLA-4, TIGIT, PD-1, and LAG-3 (128, 130-133). Although CLL patients exhibit elevated PD-1 expression on T cells, clinical trials using anti-PD-1 blockade as a single agent have shown limited efficacy in relapsed or refractory CLL, suggesting that PD-1 overexpression does not necessarily correlate with therapeutic responsiveness (134). In contrast, anti-PD-1 therapy has demonstrated substantial efficacy in other hematologic malignancies, such as relapsed or refractory classical Hodgkin lymphoma, and benefits a small subset of CLL patients who develop Richter transformation. These observations indicate that the therapeutic efficacy of PD-1 blockade varies considerably across different tumor types (134-136). Meanwhile, in CLL, $CD4^+$ T cells have been showing defects in cytoskeleton formation and vesicle trafficking which results in an impaired T cell immunological synapse formation, and CD8 T cells show decreased cytotoxicity from gene expression analysis (137, 138). An altered T cell composition has been reported in CLL, characterized by impaired $CD8^+$ T cell expansion alongside increased frequencies of Th17, Tfh, and suppressive regulatory T cells (138-145). Both $CD4^+$ and $CD8^+$ T cells are more skewed toward effector memory phenotypes in CLL and have higher activation levels, indicated by increased HLA-DR, CD38 and CD69 expression (131, 146-149). A couple of studies have demonstrated that CLL patients have an increased

capacity to secrete cytokines like IFNγ and TNF (128, 146); however, these studies are probably a consequence of the effector memory skewage in CLL and usage of PMA/Ionomycin stimulation, which does not mimic physiological T cell activation by antigens. Thus, more precise assays are needed to evaluate the functionality of T cells in CLL more accurately.

Although T cells are generally considered exhausted in CLL, studies assessing virus-specific $CD4^+$ and $CD8^+$ T cell responses have yielded variable results. In CMV-seropositive CLL patients, both the absolute numbers and cytokine secretion of CMV-specific $CD4^+$ T cells were reported to be higher than those in age-matched seropositive controls (149). Conversely, another study observed no increase in total $CD4^+$ T cells but noted an expansion of $CD8^+$ T cells with effector memory phenotypes, including $tetramer^+$ CMV-specific subsets (148). Several reports further demonstrated that CMV-specific $CD8^+$ T cells in CLL largely preserve their functional competence, including cytokine production, cytotoxic activity, and immune synapse formation (128, 150). In contrast, EBV-specific $CD8^+$ T cells in CLL exhibit functional impairment, particularly in cytotoxic capacity, as reflected by reduced CD107a expression and diminished degranulation (151). Despite this, their cytokine production appears preserved (151). However, we and other groups found that SARS-CoV-2-specific CD4 and CD8 T cells in CLL exhibit a marked decrease in specific responses and cytokine release, highlighting their functional impairment (85, 152). Collectively, these observations highlight the heterogeneity of virus-specific T cell dysfunction in CLL and emphasize the need for further mechanistic studies to elucidate the underlying determinants of these differential responses.

2.3.5 The role of ITK in T cell development and differentiation

Since BTKi such as ibrutinib also exert off-target effects, it is important to investigate the role of ITK signalling in T-cell responses.

ITK, a member of the Tec family of tyrosine kinases, is the predominant Tec kinase expressed in T cells and plays a central role in orchestrating TCR signaling (153). Upon TCR engagement, ITK becomes phosphorylated and subsequently phosphorylates phospholipase Cγ1 (PLCγ1), leading to the production of inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (47, 153, 154). These second messengers activate protein kinase C (PKC) and induce Ca^{2+} influx, which together promote the activation of transcription factors such as activator protein 1 (AP-1), nuclear factor of activated T cells (NFAT), interferon regulatory factor 4 (IRF4), and nuclear factor κB (NF-κB) (47). This cascade ultimately drives

the expression of multiple effector genes, including IL-2, IL-9, and IL-17A (47, 155). Studies in mouse models have demonstrated that ITK is crucial for regulating the differentiation of T helper subsets, including Th1, Th2, and Th17 cells (156, 157). While ITK deficiency largely preserves Th1-associated cytokine production, such as IFNγ, it markedly diminishes Th2 cytokines, including IL-4, IL-5, and IL-13 (155, 156, 158, 159). A similar reduction in IL-17 production has also been observed in $Itk^{-/-}$ T cells (160). ITK deficiency also adversely affects $CD8^+$ T cell cytolytic activity and degranulation (161). Moreover, ITK can promote TCR/CD28-induced activation through phosphorylation of its direct substrates SLP-76 and Gads (162). Collectively, ITK regulates TCR and calcium signaling and controls T cell differentiation by modulating pathways that govern transcription factor activation and cytokine production.

2.3.6 T cell response under CLL treatment

CLL treatment often exacerbates immunodeficiency by impairing B-cell maturation and responses (163). However, accumulating evidence suggests that treatment also favors the immune response. Ibrutinib has been shown to down-regulate PD-1, CTLA-4, and IL-10, and inhibit the STAT3 pathway, thereby mitigating the immunosuppressive tumor microenvironment (164). It selectively suppresses Th2 formation but preserve Th1 immunity in CLL patients (165). Furthermore, reduced levels of inflammatory cytokines, a lower frequency of Th17 cells, and decreased circulating tumor cells have been observed in patients receiving single-agent ibrutinib therapy (166). A partial restoration of T cell functionality has been demonstrated in patients treated with BCR inhibitors, particularly those receiving rituximab-idelalisib (a PI3K inhibitor), as shown by phenotypic and immune synapse bioassays (167). Similarly, treatment with the BCL-2 inhibitor venetoclax has been associated with reduced frequencies of $PD\text{-}1^+$ T cells, and lower expression of inflammatory cytokines (168). Notably, a memory-like T cell phenotype has been correlated with improved CAR-T-mediated tumor remission in CLL patients (169). In addition, increased cytokine responses have been observed in CMV- and EBV-specific $CD8^+$ T cells following long-term ibrutinib treatment (170). Collectively, these findings indicate that BTKi and other targeted therapies can partially reshape the immune landscape in CLL, although whether the “off-target” effects of BTKi influence pathogen-specific T cell responses remains to be elucidated.

2.4 T cell dysfunction

2.4.1 T cell dysfunction and exhaustion features

T cell dysfunction may result from several distinct yet sometimes overlapping states, including exhaustion, anergy, senescence, and tolerance, among others. These states arise from different triggers, such as insufficient costimulation, chronic antigen exposure, age-related replicative stress, or immunosuppressive microenvironments. Each differs in its degree of reversibility and therapeutic responsiveness.

T cell exhaustion is the major cause of the failure to control infections and cancer. Unlike during acute infections where antigen exposure is transient, persistent exposure to chronic viral antigens or malignant cells drive T cells into a dysfunctional state, known as T cell exhaustion. T cells exposed to chronic viral infection retain the plasticity to form memory cells at early stages following removal of persistent antigenic stimulation; however, with continued stimulation, they progressively lose this potential and become fully committed to exhaustion (171). Exhausted T cells (Tex) display impaired effector functions, characterized by reduced proliferation, diminished cytokine production, compromised cytotoxicity, metabolic dysregulation, and altered transcriptional and epigenetic landscapes (172, 173). They also exhibit sustained expression of multiple inhibitory receptors (IRs), including PD-1, LAG-3, TIGIT, CTLA-4, and TIM-3, among others (172, 173). The continuous expression of these IRs enforces the dysfunctional phenotype and restricts the effector capacity of $CD8^+$ T cells (173). While PD-1 blockade can transiently reinvigorate exhausted T cells, their epigenetic landscape remains largely stable and fails to reprogram toward memory or effector states, despite some transcriptional changes favoring effector functions (174). Tex cells are regulated by several transcription factors like TOX, TCF, and NFAT and IRF4 (175–180). TOX in particular has been shown to induce a dysfunctional phenotype, inhibit T cell function and maintain an exhausted status during chronic infection, in which mice model, TOX disruption could also downregulated EOMES and upregulate TBET (180). Moreover, the epigenetics profile reflects their loss of function, as evidenced by the lack of open chromatin at effector gene loci such as *Ifng* (174).

Tex cells are a heterogenous clusters, and it can be classified into stem-like precursor/progenitor exhausted T cells (Tpex), intermediate exhausted T cells (Tex-int) and terminally exhausted T cells (Tex-term). The pre-exhausted Tpex cells express high level of TCF-1 with stemness features and augmented

proliferative and self-renewal capacity (181, 182). Their differentiation is regulated not only by TCF-1 but also by transcription factors such as BCL-6, FOXO1, ID3, MYB, STAT3, BACH2, and RUNX1 (183). With persistent antigen exposure, these cells gradually transition into Tex-term (181), characterized by high levels of IR expression, like PD-1, TIM-3 and LAG-3 and transcription factor TOX, along with reduced stemness (181). The Tpex population has been shown to respond more effectively to immune checkpoint blockade (ICB) therapy and therapeutic vaccination, contributing to improved tumor control (182, 184). It has also been demonstrated in murine models that Tex cells arise from precursor populations with stem-like characteristics, specifically the $KLRG1^{+}CD127^{+}$ subset (171). The progression from a progenitor-like to a terminally exhausted state is regulated by transcriptional factors of activated T cells (NFAT) and FOS-JUN dimers (AP-1), which coordinate downstream effectors such as IL-2, IFNG, TOX, IRF4, and others (185).

2.4.2 T cell dysfunction in cancer and chronic viral infection

T cells within the tumor microenvironment (TME) are exposed to multiple immunosuppressive pressures, including inhibitory checkpoint signaling, immunosuppressive cytokines like TGF-β, IL-10, Tregs, myeloid-derived suppressor cells (MDSCs), TAMs, chronic inflammation, dysregulated microbial activity, as well as metabolic disturbances (183). Such a stressful environment drives T cell dysfunction, leading to states such as tolerance, anergy, exhaustion, and senescence (183).

T cell dysfunction, including exhaustion and impaired effector function, is a hallmark of chronic viral infections such as human immunodeficiency virus (HIV), HCV, but can also emerge during severe acute infections, including influenza (Flu) and SARS-CoV-2. In HIV and HCV, chronic antigen exposure drives progressive T cell exhaustion, characterized by upregulation of IRs and loss of proliferative and cytotoxic capacity (186, 187). SARS-CoV-2 infection, especially in severe COVID-19, is associated with lymphopenia, T cell activation, and features of exhaustion, with evidence of direct infection and apoptosis of T cells (188, 189). Flu also induces T cell dysfunction, particularly in the context of co-infection or in individuals with underlying immune compromise (190).

During the early phase of persistent antigen exposure, T cell exhaustion remains a reversible state that can recover following antigen removal. Continued stimulation over longer periods, however, leads to stable and irreversible dysfunction. Early studies showed that the functional inactivation of antiviral T cells during chronic

infection could be reversed once antigenic stimulation was withdrawn (191). However, during prolonged antigen exposure, exhausted $CD8^+$ T cells progressively lose their memory potential and undergo lineage commitment toward a terminally differentiated (171, 191). In 2016, Andrea Schietinger *et al.* demonstrated that dysfunction in tumor-specific T cells (TST) remains plastic and reversible during the early stages but becomes fixed and irreversible under conditions of persistent antigen exposure (192). Shortly thereafter, they further revealed that TST also undergo two distinct chromatin states: a plastic dysfunctional stage that can be rescued and a fixed dysfunctional state (193). The late-stage dysfunctional T cells gain the pattern similar to terminally differentiated exhausted T cells during chronic viral infections both phenotypically and transcriptionally (185).

3 Research aims

This thesis aims to elucidate the underlying mechanisms that make patients with CLL highly susceptible to infections, to assess whether modern therapeutic interventions can restore immune competence, particularly from the T cell perspective, and to define the molecular and functional signatures of pathogen-specific T cells.

Study I, we aim to identify whether pathogen-specific $CD4^+$ T cells are functionally impaired in patients with CLL at different disease stages and under treatment with next-generation BTK inhibitors.

Study II, we aim to define the impact of next-generation BTK inhibitors on virus-specific $CD8^+$ T cell function and to establish the molecular and phenotypic signatures of these cells in CLL before and after treatment.

Study III, we aim to evaluate how zanubrutinib influences both systemic and mucosal immune responses to SARS-CoV-2 vaccination.

Study IV, we aim to determine whether human memory T cells can still recognize the highly mutated SARS-CoV-2 BA.2.86 variant, despite extensive spike-protein mutations that may enable immune escape.

4 Materials and methods

This section provides a general overview of the methods used. Detailed descriptions of each method are presented in the Materials and Methods section of the respective papers.

4.1 Ethical considerations

In this thesis, the included patient cohorts primarily consist of older adults, as CLL predominantly affects the elderly, with a median age at diagnosis of approximately 70 years. Given this demographic, the inclusion of potentially vulnerable participants was a relevant ethical consideration. Vulnerable individuals are defined as those who may lack the capacity to provide fully informed consent or make autonomous decisions. Older adults above 70 years may, in some cases, experience diminished cognitive ability, reduced comprehension of study information, or physical limitations that affect their capacity to care for themselves or engage in the consent process. Therefore, it is essential to adhere strictly to the ethical principles of justice, respect for persons, beneficence, and nonmaleficence throughout all stages of the research.

Although we are not the clinical investigators conducting these trials, we are responsible for defining the disease- and treatment-specific inclusion criteria relevant to our research. In doing so, we do not assume that consent to participate can automatically be provided by a carer or surrogate for older adults receiving care or for individuals with cognitive impairment. In cases involving vulnerable participants, informed consent must be obtained through the subject's legally authorized representative, who should receive and understand all relevant written information.

All the samples in our studies were obtained from peripheral blood, collected from participants enrolled in different cohorts. All participants were provided with detailed written and oral information and signed informed consent forms in accordance with the Declaration of Helsinki. Given the age and disease profile of the participants, particular emphasis was placed on upholding the principles of informed consent and justice. All identifying information of the cohorts was kept strictly confidential, and the use of biological samples was conducted in accordance with relevant ethical and regulatory requirements.

All studies included in this thesis were approved by the Swedish Ethical Review Authority: **Study I and II**: 2022-06064-02 and 2021-01107; **Study III**: 2021-00451; **Study IV**: 2021-00451; 2021-06046-02.

4.2 Sample collection and processing

Peripheral blood mononuclear cells (PBMCs) were diluted in PBS and isolated by density gradient centrifugation to obtain a high yield of lymphocytes from peripheral blood. Purified PBMCs were resuspended in freezing medium containing 90% fetal bovine serum (FBS) and 10% dimethyl sulfoxide (DMSO), gradually frozen, and stored in liquid nitrogen until further use.

4.3 Pathogen-specific T cell detection by activation-induced marker (AIM) assay

In **Studies I-IV**, PBMCs were thawed and stimulated *in vitro* with bacterial lysates or viral peptide pools to mimic the physiological conditions of T cell recall responses to bacterial or viral infections.

To detect surface activation markers, we included a CD40-blocking antibody during overnight stimulation to prevent CD40-CD40L interactions among PBMCs and thereby maximize AIM signal detection. DMSO was used as a negative control to subtract background responses for each stimulus, and staphylococcal enterotoxin B (SEB) served as a positive control to induce a TCR-dependent response.

In **Study I**, several clinically relevant bacterial and viral pathogens were included to model common infections observed in patients with CLL. *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, and *K. pneumoniae* were selected to mimic bacterial infections, while influenza A virus (IAV), EBV, and CMV peptide pools were used to represent classical viral infection models.

In **Study II**, the same viral peptide pools of IAV, EBV, and CMV were used to assess virus-specific $CD8^+$ T cell responses.

In **Study III**, SARS-CoV-2 Spike-Wu-Hu.1 (wild-type) and Spike-Omicron BA.1 peptide pools were employed to simulate T cell response against distinct SARS-CoV-2 strains.

In **Study IV**, Spike-Wu-Hu.1, Spike-Omicron BA.1, and Spike-Omicron BA.2.86 peptide pools were used to evaluate memory T cell responses to different SARS-CoV-2 variants.

Following stimulation with the corresponding bacterial lysates or viral peptide pools, cells were stained with a memory T cell activation panel comprising markers of memory differentiation, activation, exhaustion, and chemokine receptors, and subsequently analyzed by flow cytometry.

4.4 Pathogen-specific functional assay

In **Studies I-II**, intracellular cytokine staining (ICS) was performed on both $CD4^+$ and $CD8^+$ T cells following stimulation with bacterial lysates or viral peptide pools. Golgi Plug (Brefeldin A) and Golgi Stop (Monensin) were added to inhibit intracellular protein transport and cytokine secretion, thereby allowing cytokine accumulation within cells for detection. Specifically, brefeldin A disrupts protein transport between the endoplasmic reticulum (ER) and the Golgi apparatus, whereas monensin interferes with protein processing and vesicular transport within the Golgi, preventing the secretion of newly synthesized proteins and promoting intracellular cytokine retention. To assess degranulation, CD107a was stained immediately after stimulation to capture the cumulative degranulation signal over the entire activation period, rather than transient surface expression at a single time point. Following stimulation with the corresponding bacterial lysates or viral peptide pools, PBMCs were stained with a memory T cell ICS panel comprising markers of memory differentiation, activation, exhaustion, and cytokine production, and subsequently analyzed by flow cytometry.

4.5 Proliferation assay

In **Studies I, II** and **IV**, PBMCs were seeded at a low density and allowed to proliferate for 3-4 days under stimulation with the corresponding bacterial lysates or viral peptide pools. During this process, pathogen-specific T cells that had previously encountered their respective antigens began to proliferate. A proliferation panel was used to stain these cells for markers of memory differentiation, proliferation (Ki-67), and cytokine production, followed by flow cytometric analysis.

In **Studies I and II**, cytokine production was further assessed after 3.5 days of stimulation by restimulating the cells 12 hours before harvest. Golgi Plug and Golgi Stop were also added during this period to inhibit cytokine secretion and allow for intracellular cytokine detection.

4.6 Virus-specific $CD8^+$ T cell detection by tetramer staining

Tetramer staining enabled the detection and characterization of antigen-specific T cells, particularly $CD8^+$ T cells recognizing viral epitopes. Peptides

corresponding to immunodominant viral epitopes were loaded to form biotinylated peptide-MHC complexes (pMHC), together with fluorochrome-conjugated streptavidin to generate epitope-specific tetramers. These tetramers mimicked the natural peptide-MHC complexes displayed on infected cells, allowing specific binding to T cells whose TCRs recognize the corresponding peptide-MHC complex. Only T cells with TCRs that matched the peptide-MHC complex bound the tetramers with sufficient avidity for stable labeling and subsequent detection by flow cytometry. Therefore, by utilizing this method, virus-specific $CD8^+$ T cells were identified and characterized from PBMCs.

In **Study II**, tetramers were generated using peptides derived from pp65 and IE1 of CMV, lytic and latent proteins of EBV, and M1 ($NP_{473-481}$), nucleoprotein, and matrix protein 1 of IAV to stain patients carrying the corresponding HLA alleles. Following tetramer staining, PBMCs were further labeled with a comprehensive memory T cell exhaustion panel that included markers of memory differentiation, stemness, effector function, exhaustion, and chemokine receptor expression, and subsequently analyzed by flow cytometry.

4.7 Naïve T cell primary response *in vitro* assay

In **Studies I-II**, naïve T cells sorted from PBMCs were pretreated with different BTKi. The degree of ITK inhibition varied among these compounds, with ibrutinib exerting strong inhibition, zanubrutinib showing weak inhibition, and acalabrutinib exhibiting minimal or no inhibitory effect. After irreversible binding to ITK, primary T cell was simulated by stimulating the cells with CD2/CD3/CD28 activator in the presence of IL-2. This setup was designed to mimic a primary immune response *in vitro* and to assess how different generations of BTK inhibitors influence the initiation of T cell activation. A naïve T cell sorting panel containing memory differentiation markers was used to isolate naïve $CD4^+$ and $CD8^+$ T cells. On day 7, a detection panel including Ki-67 and CD69, along with the same markers and fluorochrome combinations as used in the sorting panel, was applied to identify proliferating clones after stimulation, followed by flow cytometric analysis.

4.8 Flow cytometry

Polychromatic flow cytometry enables high-resolution, multiparametric detection of protein expression using fluorochrome-conjugated antibodies specific to target molecules. We developed distinct flow cytometry panels tailored to specific experimental aims to comprehensively characterize

pathogen-specific T cell signature and function. In **Studies I and II**, we applied a series of panels covering memory T cell activation panel (AIM assay), memory T cell ICS panel (functional assay), proliferation panel, and naïve T cell sorting and detection panels. In **Study II**, a 29-color tetramer staining panel was additionally implemented to define virus-specific $CD8^+$ T cells at high resolution. A simplified memory T cell activation panel (AIM assay) was applied in **Study III**, while memory T cell activation panel (AIM assay), memory T cell ICS panel, and proliferation panel were used in **Study IV**. All panels were optimized for fluorochrome spillover, marker compatibility, and reproducibility across experiments.

4.9 Data analysis

All data were analyzed using FlowJo and RStudio, visualized in RStudio, formatted in Adobe Illustrator, and schematic figures were created with BioRender, with ChatGPT (OpenAI) assisting in code generation.

5 Results and discussion

Pathogen-specific CD4$^+$ T cells were analyzed in **Study I**, and virus-specific CD8$^+$ T cells were investigated in **Study II**. Owing to the outbreak of the COVID-19 pandemic, it became particularly important to assess T cell response in this vulnerable patient group. Therefore, **Studies III and IV** were designed to investigate immune responses to SARS-CoV-2 infection and vaccination.

5.1 Study I & II: Pathogen-specific T cell response and function in CLL

As infections represent a major cause of morbidity and mortality in CLL, second only to tumor burden, it is crucial to understand how pathogen-specific T cell responses and functions contribute to host defense against different pathogens in this susceptible patient population.

Previous research has provided limited insight into pathogen-specific T cell responses in CLL. Only a few studies have investigated EBV- and CMV-specific CD8$^+$ T cells, and many of these did not assess T cell responses in a TCR-dependent manner. While several studies have focused on tumor antigen-specific CD8$^+$ T cell responses, systematic analyses of pathogen-specific CD4$^+$ and CD8$^+$ T cells driven by TCR-dependent activation remain limited. Therefore, in **Study I,** we aimed to systematically characterize pathogen-specific CD4$^+$ T cell responses in patients with CLL across different pathogens, disease stages, and treatment conditions. In **Study II**, we sought to comprehensively define virus-specific CD8$^+$ T cell responses in CLL, comparing different viral antigens, disease stages, and treatment conditions.

To systematically investigate pathogen-specific CD4$^+$ T cells, we selected four commonly encountered bacterial pathogens and three common viruses, both acute virus (IAV) and chronic viruses (EBV and CMV) to mimic physiological recall responses using the AIM assay. Bacterial lysates and viral peptide pools were used to stimulate PBMCs isolated from patients with CLL and age-matched healthy donors. Early activation marker positive cells were defined as CD40L$^+$CD69$^+$ for CD4$^+$ T cells and 4-1BB$^+$CD69$^+$ for CD8$^+$ T cells. Both bulk memory T cells and pathogen-specific T cells were analyzed across different assays. To ensure that the pathogen used in AIM assay triggers a TCR-dependent recall response, we included MHC class I and II blockade controls. We excluded the *staphylococcus aureus* from our experiments because of its great superantigen activity, and most bacteria lysate did not elicit antigen-specific

$CD8^+$ T cell responses. So, we decided only include the bacteria stimulation for $CD4^+$ T cells.

Consistent with previous findings, bulk memory $CD4^+$ and $CD8^+$ T cells in CLL progressively shift toward a more Tem phenotype, accompanied by a loss of naïve T cells as the tumor progresses. They both exhibit a more differentiated, effector-like, and exhausted phenotype in active CLL, characterized by increased expression of HLA-DR, CXCR3, CX3CR1, and PD-1, along with decreased expression of CD28, CD127, and CCR7. This phenotype likely results from persistent tumor antigen stimulation combined with chronic inflammatory signaling, which together drive T cell overactivation, differentiation, and subsequent functional exhaustion within the profoundly immunosuppressive CLL microenvironment.

As a B cell malignancy, CLL profoundly affects how T cells respond to pathogens, which is particularly critical for patients' ability to combat infections. To this end, we next sought to determine the response and functional capacity of pathogen-specific T cells. We first utilized the AIM assay to assess the magnitude of pathogen-specific T cell responses. All bacteria-specific $CD4^+$ T cells exhibited a diminished response, whereas virus-specific $CD4^+$ T cells remained largely preserved. The reduced magnitude of bacteria-specific responses positively correlated with CLL tumor progression, as indicated by Rai stage. In contrast, virus-specific $CD8^+$ T cells did not show a consistent trend in response magnitude. Importantly, when focusing on the pathogen-specific populations, we found that both bacteria- and virus-specific $CD4^+$ T cells exhibited consistently impaired functionality, characterized by reduced production of Th1-, Th2-, Th17-, and Tfh-associated cytokines. Virus-specific $CD8^+$ T cells also displayed functional impairment, with decreased degranulation capacity and diminished cytokine production. The extent of this dysfunction correlated positively with tumor progression, as reflected by both Rai stage and Lymphocyte count. This consistent impairment likely underlies the increased susceptibility to infections observed in this patient population.

Interestingly, when comparing cytokine production across age-matched healthy controls, indolent CLL, and active CLL, we found that patients with indolent CLL exhibited a mild, non-significant reduction or even preserved functionality under most stimulations. Both pathogen-specific $CD4^+$ and virus-specific $CD8^+$ T cells largely maintained their cytokine production and polyfunctionality, in contrast to the pronounced impairment observed in active CLL. This finding highlights that

immune dysfunction in CLL is not uniform but strongly dependent on disease stage and activity. Accordingly, precise patient stratification is essential in cancer immunology research; without it, interpretations risk conflating preserved immune competence in indolent disease with profound dysfunction in advanced stages. This nuance may also explain why several previous studies reported intact EBV- and CMV-specific T cell responses in CLL, reflecting heterogeneity in patient cohorts rather than true biological discrepancies.

5.2 Study I & II: Pathogen-specific T cell response and function during BTKi treatment

BTKi have transformed the therapeutic landscape of CLL. Given their off-target effects on ITK, with ibrutinib exerting strong inhibition, zanubrutinib showing weak inhibition, and acalabrutinib displaying minimal or no inhibition**,** we initially hypothesized that these differential effects might modulate ITK-mediated signaling and thereby alter T cell responses and function.

Unexpectedly, we observed no major differences between ibrutinib and zanubrutinib, although ibrutinib-treated patients showed a more pronounced reduction in proliferative capacity. Across multiple assays, including AIM, functional, and proliferation assays, both inhibitors produced comparable results, showing limited evidence of reinvigoration of functional T cells, either in pathogen-specific $CD4^+$ or virus-specific $CD8^+$ populations. Both the magnitude in absolute count and functionality remained unchanged after BTKi treatment. In addition, a persistently low level of cytokine polyfunctionality before and after treatment further confirmed the sustained dysfunctionality of these cells. These findings indicate that T cell dysfunction in active CLL represents an irreversible state of functional exhaustion that persists despite tumor burden reduction.

Interestingly, we observed that CXCR3 expression was reduced in virus-specific $CD4^+$ T cells following both ibrutinib and zanubrutinib treatment, but the reduction was markedly greater with ibrutinib (data not shown). In contrast, CXCR3 expression in virus-specific $CD8^+$ T cells and bulk memory $CD4^+$ and $CD8^+$ T cells were selectively decreased only under ibrutinib treatment but remained largely preserved with zanubrutinib (data not shown). This pattern suggests that ibrutinib, but not zanubrutinib, exerts off-target inhibition on ITK, thereby dampening ITK-dependent Th1-associated signaling.

Furthermore, virus-specific $CD8^+$ T cells displayed a reduced activation profile following either ibrutinib or zanubrutinib treatment, characterized by lower

expression of activation and inhibitory markers such as HLA-DR, CD38, PD-1, TIGIT, and CD39 (data not shown). Accordingly, the frequencies of activated subsets, including HLA-DR$^+$CD38$^+$, CD38$^+$PD-1$^+$, and CD39$^+$PD-1$^+$TIGIT$^+$ T cells, were also decreased. These phenotypic ameliorations were also evident in bulk memory CD4$^+$ and CD8$^+$ T cells and likely reflect a systemic alleviation of bystander activation pressure imposed by the tumor microenvironment.

5.3 Study I & II: Signature of pathogen-specific T cells before and after BTKi treatment

We next examined pathogen-specific CD4$^+$ T cells using the AIM assay. The CCR7$^+$CD45RA$^+$CD95$^+$ Tscm population showed a negative correlation with Rai stage in CLL, indicating a progressive loss of stem-like and a concomitant enrichment of terminally differentiated effector clusters in CLL by phenograph analysis. To further assess virus-specific CD8$^+$ T cells, we performed tetramer analysis targeting epitopes from IAV, EBV (both latent and lytic), and CMV. We observed a gradual loss of stem-like features, reflected by decreased expression of CD27, CD127, LEF1, TCF1, and CXCR5, with increasing age and tumor burden. Meanwhile, virus-specific CD8$^+$ T cells exhibited a gain of terminally differentiated effector-like and exhausted phenotypes, characterized by elevated expression of GzmB, CX3CR1, EOMES, TOX, and PD-1 progressing from old healthy controls, indolent CLL, to active CLL (Acala base), especially in IAV-specific CD8$^+$ T cells. These findings reflect a general loss of stem-like features and a parallel gain of effector characteristics in both pathogen-specific CD4$^+$ and virus-specific CD8$^+$ T cells.

Interestingly, after zanubrutinib treatment but not ibrutinib, pathogen-specific CD4$^+$ T cells exhibited partial recovery of stem-like features, reflected by increased Tscm frequencies and CD127 expression, to varying degrees under different stimuli. In contrast, virus-specific CD8$^+$ T cells across all BTKi treatments, including ibrutinib, zanubrutinib, and acalabrutinib, showed no sign of stem-like feature restoration, as evidenced by AIM assay results for ibrutinib and zanubrutinib and tetramer analysis for acalabrutinib. Instead, they maintained a terminally differentiated effector-like phenotype even six months after acalabrutinib treatment, although this was accompanied by a reduction in exhaustion marker expression.

These findings reflected an irreversible loss of stem-like features and fixation in a terminally differentiated effector-like state, which may explain why the dysfunctionality of virus-specific CD8$^+$ T cells persist even after BTKi treatment.

In 2021, E. John Wherry and Debattama R. Sen 's lab has conclusively demonstrated through studies in both murine models and human cohorts, that after chronic antigen removal, phenotypic and transcriptional "recovery" may occur, however, persistent epigenetic scars remain in virus-specific $CD8^+$ T cells following the cure of chronic infection in humans and antigen clearance in mice (194, 195). In the same year, Pierre Tonnerre *et al.* confirmed that long-term cure of HCV infection is insufficient to improve or reverse the virus-specific T cell dysfunction (196). Exhausted virus-specific $CD8^+$ T cells remained persistently fixed in a dysfunctional state after the cessation of chronic antigenic stimulation, although partial recovery was observed in the expression of PD-1, CD38, HLA-DR, CD39, CD127, and EOMES (196). This provides a mechanistic rationale for the persistent T cell dysfunction in CLL even after tumor burden reduction. In-depth and comprehensive epigenetic and transcriptomic profiling will be essential to fully understand the mechanisms driving these pathogen-specific T cell dysfunction in CLL.

This persistent dysfunction suggests that patients remain immunologically vulnerable even in remission, with a reduced ability to mount effective responses to viral infections or vaccination. Moreover, exhausted and terminally differentiated T cells are poor starting material for cellular therapies, being less proliferative, less durable, and functionally impaired factors that may help explain why CLL patients historically show lower response rates to CAR-T therapy compared to other hematologic malignancies, like ALL.

It is noteworthy that pathogen-specific T cells in indolent CLL exhibit an intermediate state between those in old healthy controls and active CLL, characterized by a less terminally differentiated effector-like phenotype and partial retention of stem-like features. In 2016, Andrea Schietinger *et al.* demonstrated that dysfunction in tumor-specific T cells remains plastic and reversible during the early stages but becomes fixed and irreversible under conditions of persistent antigen exposure (192). This observation prompted us to consider whether the limited efficacy of CAR-T therapy in CLL may result from its intervention at an advanced disease stage, when T cells have already entered an irreversible dysfunctional state. Currently, CAR-T therapy is only approved for patients who have failed all prior treatments and is not used as a first- or second-line option. However, recent clinical trials, including ZUMA-7, BELINDA, and TRANSFORM, have evaluated CAR-T therapy as a second-line treatment for patients with large B-cell lymphoma (LBCL) whose initial therapy failed (197-199).

These studies demonstrated significantly improved OS, higher complete response rates, and faster recovery compared with standard care (197-199). Therefore, earlier intervention with CAR-T therapy in CLL may represent a promising strategy to enhance treatment efficacy.

5.4 Study I & II: BTKi direct effect on T cells

As the effect of BTKi observed in the cohort samples represent an integrated outcome of the overall immune response, it remains unclear whether the persistent T cell dysfunction arises from BTKi off-target activity or intrinsic T cell defects. To this end, we performed *in vitro* primary response assays and recall assays. The recall assays included AIM, functional, and proliferation assays to assess the direct effects of different generations of BTKi, namely ibrutinib, zanubrutinib, and acalabrutinib. Overall, zanubrutinib and acalabrutinib showed no inhibitory effects on TCR-dependent primary or recall responses. In contrast, ibrutinib markedly suppressed primary T cell expansion and differentiation, as evidenced by a pronounced blockade of naïve T cell activation, substantial reduction in CD3, CD4, and CD8 expression, decreased magnitude of responding T cells, diminished cytokine production, and limited proliferative capacity. This inhibitory effect was also observed in virus-specific $CD4^+$ T cells in a dose-dependent manner. In contrast to CD4, ibrutinib exerted minimal inhibitory effects on $CD8^+$ T cell recall responses, with no significant changes in cytotoxicity-associated molecule expression, such as GzmB and CD107a.

This striking inhibition observed with ibrutinib prompted us to consider that the higher incidence of infections reported in ibrutinib-treated patients may result from excessive ITK inhibition, leading to impaired T cell activation and an inadequate recall response upon re-exposure to infectious antigens. Importantly, the newer-generation BTK inhibitors, zanubrutinib and acalabrutinib, largely preserve T cell immunity with minimal interference in activation or recall responses.

5.5 Study III: T cell response to SARS-CoV-2 vaccine in BTKi treated CLL

When the COVID-19 pandemic broke out, patients with CLL were identified as a high-risk group for severe infection. This heightened vulnerability also extended to those receiving first-generation BTKi therapy, such as ibrutinib. Multiple studies have reported impaired humoral immunity in these patients, which

adversely affects their vaccine-induced responses to SARS-CoV-2 infection (80, 82, 200, 201).

In **Study III** (**Figure 6**), we aimed to evaluate the effects of the next-generation BTKi, zanubrutinib, on both circulation and mucosal immune responses following SARS-CoV-2 vaccination, with mixed regimens of Pfizer/BioNTech (BNT162b2), Moderna (mRNA-1273), and AstraZeneca (ChAdOx1) vaccines. All included patients were seronegative for SARS-CoV-2 prior to vaccination and remained uninfected throughout the study period. They had been receiving long-term treatment with zanubrutinib, with a median treatment duration of 28 months. Nine CLL patients under ongoing zanubrutinib therapy were longitudinally sampled for blood and saliva before and after vaccine doses three to five. In parallel, ibrutinib-treated CLL patients and age-matched healthy controls were analyzed 2-3 weeks after dose 5. We assessed total spike-specific antibody reactivity in serum and saliva, along with T cell responses to spike-WT (Wuhan ancestral strain) and spike-Omicron peptide stimulation.

Vaccination after dose three induced a marked increase in spike-specific total antibody levels in zanubrutinib-treated patients, consistent across both ancestral (Wuhan strain) and Omicron spike-specific IgG. However, despite this increase, spike-specific total antibody level remained markedly lower in both zanubrutinib- and ibrutinib-treated CLL patients compared with age-matched old healthy controls, in line with previous findings of impaired humoral responses in CLL. Meanwhile, mucosal IgA responses in serum and saliva were largely absent in zanubrutinib-treated patients even after multiple vaccine doses, while serum IgG levels showed modest increases in both BTKi-treated groups.

Using AIM assay, we observed that T cell reactivity to spike-WT (Wuhan ancestral strain) and spike-Omicron was largely preserved in BTKi-treated patients compared with healthy donors, suggesting that seronegative CLL patients receiving long-term zanubrutinib could mount T cell responses comparable to healthy individuals. However, as these analyses were based on relative frequencies, accurate absolute cell counts would be required to determine whether this preservation reflects true maintenance of response magnitude or a relative enrichment due to lymphopenia. In addition, we observed a robust increase in $CD8^+$ T cell reactivity after the third vaccine dose, with no further enhancement following subsequent doses, consistent with previous reports.

Collectively, these findings indicate that after five doses of SARS-CoV-2 vaccine, zanubrutinib-treated CLL patients exhibited a restoration of T cell responsiveness to normal levels comparable to healthy individuals, whereas humoral and mucosal immunity remained substantially impaired, underscoring their persistent vulnerability to SARS-CoV-2 infection despite repeated vaccination.

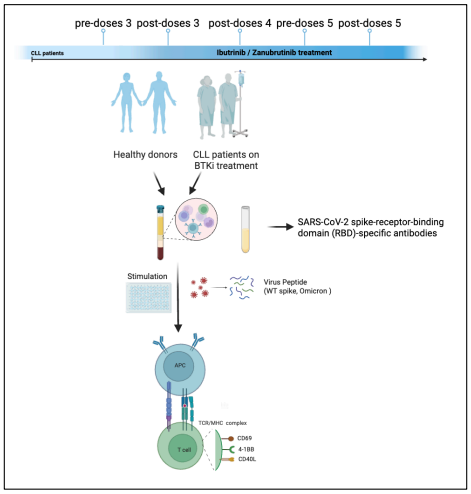

Figure6: Experimental design in Study III (Picture created from biorender.com)

5.6 Study IV: Effective cross-recognition of new SARS-CoV-2 variant BA.2.86 in memory T cells

With the continuous evolution and global circulation of SARS-CoV-2 variants (**Figure 7**), it becomes critical to determine whether pre-existing memory T cells retain sufficient capacity to recognize and respond effectively to these highly mutated strains, as emerging variants often acquire mutations to escape immune surveillance.

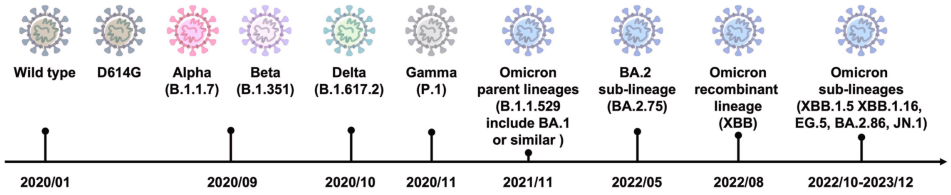

Figure7: Evolutionary path of SARS-CoV-2 (Picture from(202)).

In **Study IV** (**Figure 8**), we aimed to evaluate $CD4^+$ and $CD8^+$ T cell responses to the highly mutated SARS-CoV-2 variant BA.2.86 in immunocompetent healthy controls and patients with CLL, who had previously shown poor vaccine-induced

T cell responses and high mortality during the early pandemic. PBMCs were collected from uninfected but vaccinated healthy controls and patients with CLL, as well as from individuals with hybrid immunity, defined as those with both prior SARS-CoV-2 infection and vaccination-induced T cell responses. Samples from cohort 1 and cohort 2 were obtained at different time points following the Omicron wave. This study cohort thus reflected a real-world population with diverse vaccination histories and infection backgrounds. Moreover, the study design allowed direct comparison of T cell responses against two major SARS-CoV-2 variants that represent the most pronounced mutational leaps in viral evolution, Omicron BA.1 and BA.2.86, across cohorts including both infection-naïve and hybrid-immunity individuals.

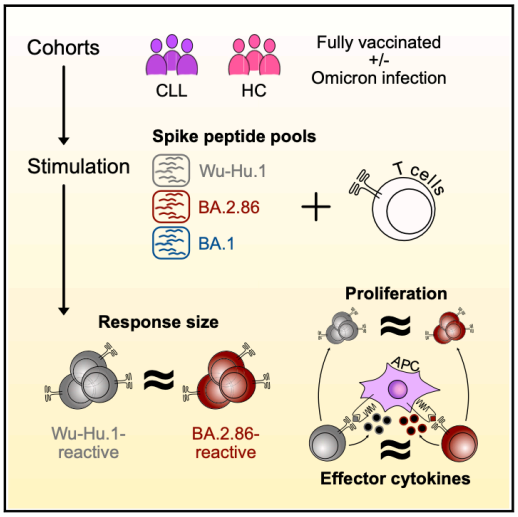

Figure8: Experimental design in Study IV (Picture from (203))

Variant spike peptide pools, including BA.1 and BA.2.86, were subdivided into regions corresponding to the homologous (ancestral) Wu-Hu.1 sequences and those covering variant-specific mutations, allowing quantification of the loss in T cell recognition for each variant. To estimate the total T cell response against the variant spike, a net response was calculated by subtracting the recognition loss in mutated regions from the overall Wu-Hu.1 full spike response. This approach normalized for peptide pool size and provided a reliable measure of the overall cross-recognition capacity of vaccine-induced T cells toward each variant.

Using AIM, functional, and proliferation assays, we evaluated and compared the magnitude and functionality of T cell responses, including proliferation, cytokine production, and polyfunctionality, across different variant peptide pools. In both

cohorts, all assays revealed no significant differences between ancestral and variant peptide pools. Calculated responses across all assays similarly showed comparable reactivity among Wu-Hu.1, BA.1, and BA.2.86. These findings demonstrate that SARS-CoV-2 spike-specific $CD4^+$ and $CD8^+$ T cells induced by prior infection or vaccination retain robust immune recognition of BA.2.86. The largely preserved response magnitude and sustained functionality suggest that cellular immunity continues to provide effective protection against this highly mutated variant.

6 Conclusions and points of perspective

The emergence of SARS-CoV-2 breakthrough infections has highlighted the heightened vulnerability of immunocompromised individuals, particularly patients with CLL. As the immune system constantly faces diverse infectious challenges, the ability of pathogen-specific T cells to mount effective responses becomes a critical determinant of host survival, especially in populations where infection-related mortality remains exceptionally high. This thesis provides a comprehensive evaluation of pathogen-specific $CD4^+$ and $CD8^+$ T cell responses in CLL across four independent studies, spanning untreated early stage, advanced active stage, different time points during BTKi therapy, and varied vaccination and infection histories. By integrating phenotypic and functional analyses, this work delineates the spectrum of T cell response impairment, the dynamics of their functional defects, the limited capacity of next-generation BTK inhibitors to restore pathogen-specific T cell function, the preserved spike-specific T cell magnitude of zanubrutinib treated patients, and the preserved cross-recognition of hypermutated SARS-CoV-2 variants in CLL. Together, our studies provide important insights into why CLL patients remain immunologically vulnerable, assessed whether BTKi therapy can restore T cell immunity and offer valuable evidence of cellular immunity protection to SARS-CoV-2 within this vulnerable patient group.

Study I demonstrated that pathogen-specific $CD4^+$ T cells in CLL are profoundly impaired in both magnitude and function, with bacterial-specific responses particularly affected. The progressive loss of cytokine production and stem-like features with increasing tumor burden highlights a direct link between malignant progression and pathogen-specific T cell deterioration. Importantly, the weaker off-target effects of BTKi treatment were insufficient to restore the functionality of these pathogen-specific $CD4^+$ T cells despite tumor reduction.

Study II revealed that virus-specific $CD8^+$ T cells in CLL display impaired functionality, characterized by reduced degranulation capacity and diminished cytokine production. This dysfunction persisted despite tumor burden reduction following BTKi treatment, although a partial recovery in response magnitude was observed. Virus-specific $CD8^+$ T cells exhibited a terminally differentiated effector-like and exhausted phenotype, accompanied by the loss of stem-like features. Second-generation BTK inhibitors exerted minimal influence on both

primary and recall $CD8^+$ T cell responses, suggesting that T cell dysfunction in CLL represents a largely irreversible state.

Both **Study I and II** revealed a persistent systematic pathogen-specific T cell dysfunction in CLL suggests that patients remain immunologically vulnerable even in remission, with a reduced ability to mount effective responses to viral infections or vaccination. This sustained functional impairment may be intrinsic, inherited from the accumulated epigenetic "scars" imprinted by repeated exposure to the chronic inflammatory tumor microenvironment.

These findings prompt a reconsideration of current cellular therapy strategies. Given that CAR-T therapy in CLL is typically administered only after multiple treatment failures, when antigen-specific T cells have already become terminally differentiated, exhausted, and dysfunctional, carrying potential "scars" that disrupt many of their intrinsic functional programs, it may already be beyond the optimal window for these cells to be functionally reprogrammed through engineering approaches. This could help explain why CAR-T therapy achieves much worse efficacy in CLL than in ALL. In ALL, the harvested T cells retain a far more stem-like, flexible, and plastic phenotype, essentially a "younger" functional state, making them more responsive to engineering and expansion. In contrast, memory T cells in CLL are highly terminally differentiated, leaving little capacity for rejuvenation or reprogramming. CAR engineering alone is insufficient to reverse this terminally exhausted phenotype. If our hypothesis holds true that the tumor microenvironment, together with age-related acceleration, reprograms and imprints antigen-specific T cells to limit their functionality, then the reinfusion of CAR-T cells may also need to be combined with agents that modulate or improve the inflammatory and suppressive microenvironment to achieve optimal therapeutic efficacy.

Study III showed that although humoral immunity remains severely compromised under BTKi therapy, cellular immunity, particularly T cell responsiveness, appears relatively preserved. This indicates that T cell-based protection may still contribute to antiviral defense in long-term treated CLL patients. However, as magnitude recovery does not necessarily correspond into restored functionality, future work should include single cell and mechanistic analyses to define the true quality of vaccine-induced T cell responses.

Study IV demonstrated that memory T cells elicited by prior infection or vaccination largely retain their ability to recognize and respond to emerging

SARS-CoV-2 variants, including both Omicron BA.1 and the highly mutated BA.2.86 strain. These results indicate that cellular immunity remains resilient and capable of providing broad cross-variant protection despite extensive viral evolution. This underscores the durability of T cell–mediated immune surveillance as a critical layer of defense in both healthy and immunocompromised individuals.

Collectively, the findings demonstrate that antigen-specific T-cell dysfunction in CLL is broad and largely irreversible, even after BTKi treatment and tumor reduction at late-stage disease. Both $CD4^+$ and $CD8^+$ T cells progressively lose stem-like potential and functional competence, contributing to the profound immune vulnerability characteristic of this disease. Future cellular therapy strategies should be reconsidered to enhance therapeutic efficacy and optimize treatment outcomes in CLL.

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8 Declaration about the use of generative AI

I declare that I have used OpenAI's GPT-4o and GPT-5 for checking the fluency, coherence, grammar, accuracy, and logic of my original text in order to improve its readability and language quality. This process involved some paraphrasing, language polishing, and suggestions on writing style based on my original work.

However, the scientific content of this thesis, including all hypotheses, result descriptions, interpretations, discussions, and conclusions, is entirely my own. All suggestions, feedback, and linguistic adjustments were reviewed and approved by me in accordance with the original source material.

I confirm that the use of these tools complies with Karolinska Institutet's research integrity guidelines, and I take full responsibility for the content of this thesis.

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