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IMMUNE CELLS IN CHRONIC LYMPHOCYTIC LEUKEMIA AND HODGKIN LYMPHOMA IN RELATION TO TUMOR BURDEN AND TREATMENT

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IMMUNE CELLS IN CHRONIC LYMPHOCYTIC LEUKEMIA AND HODGKIN LYMPHOMA IN RELATION TO TUMOR BURDEN AND TREATMENT

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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This thesis is a dedication to my late grandmother Adri who sadly succumbed to leukemia, who inspired me to be a curious and caring person and whose name I proudly carry.

POPULAR SCIENCE SUMMARY OF THE THESIS

Our immune system is the army of cells in our body that acts to protect us against infections by bacteria, viruses, and fungi. This army consists of many different disciplines. Myeloid cells are like the scouts of the immune system that are the first to recognize danger and act quickly. Lymphoid cells are the highly specialized soldiers that went through a lot of training. Sometimes, one of these lymphoid cells can choose the wrong path and become evil or malignant, causing diseases that are called lymphoid malignancies.

In this thesis, two of these lymphoid malignancies were studied. They are called chronic lymphocytic leukemia (CLL) and classical Hodgkin lymphoma (cHL). We know from previous research that these malignant cells persuade other good members of the immune system to support their evil activities and protect them from being caught by other good ones.

A drug called ibrutinib is used to treat CLL. It basically takes away the main stimulus that keeps CLL cells alive. Ibrutinib also has side effects that occur in same patients, like an irregular heartbeat (or atrial fibrillation). Ibrutinib might cause these by taking away other stimuli that are important for the normal function of other tissues, like for example the electric conduction system in the heart. In the first study, we identified several molecules that might be responsible for the irregular heartbeat that is sometimes caused by ibrutinib. We also found that most of the good immune cells that either recognized or supported the bad CLL cells go away when the CLL cells die during successful treatment.

In the second study, we tested the safety of an alternative intermittent dosing schedule for ibrutinib in CLL patients. We stopped the drug in patients that were in remission and restarted again when we saw early signs of relapse. Such cycles could in theory be repeated endlessly. We though that we might reduce side effects if we take away the drug that causes them as long as the patients are in remission. This strategy proved to be safe, meaning that none of the patient had a rapid relapse once we stopped the drug and all of them still respond to the drug when we need to give it again. This strategy reduced some of the side effects as well. However, it is too early to say if it is beneficial in the long-term.

In the last study, we investigated how the good immune cells in the blood are affected by the cHL cancer cells in the lymph nodes, by measuring their functional characteristics. Besides the known fact that cancer cells in this disease persuade local immune cells to support them, we found that some circulating immune cells were affected in a similar way. Luckily, these changes were largely resolved by standard treatment.

cells cll patients treatment ibrutinib also can disease immune cell years response clinical antigen study expression chl btk lymphoid however express stage blood plasma lymphoma common lymph risk first like tumor months might bone marrow high kinase th2 activation memory involved associated tregs antibodies factor tissue biomarkers called known one will phase protein studies reduced age paper target low year receptor circulating potential time signals progression numbers t-cell drug r/r hrs cycles th1 inhibitors tyrosine lymphocytes responses described diagnosis two genes naïve new data trial pd-1 b-cell therefore effects furthermore median interruption first-line ctls burden gene samples standard reported expressed survival well increased events immunity development mhc nodes helper treated used molecules differentiation macrophages binding found cause adverse class signaling region lineage molecule infections proteins received pathogens cases unmutated lymphocytosis present resistance leukemia malignancies even donors toxicity immunoglobulin del(17p)/tp53-mutation secondary intermittent levels subtype eosinophils lack case genetic healthy often mature still seems another surface frequency specific symptoms reduce hematopoietic number mutations subsets production early information spleen hodgkin bcr positive given cytotoxic

ABSTRACT

B-cell malignancies are a heterogeneous group of cancers that originate from lymphoid-lineage cells that are responsible for humoral immunity. When B-cell development goes wrong, chronic lymphocytic leukemia (CLL) and classical Hodgkin lymphoma (cHL), among other malignancies, may arise. Recent advances in the treatment of these diseases, e.g., ibrutinib in CLL, have greatly improved the prognosis of patients, but also introduced new challenges. Gaining knowledge about the effects that these treatments have on immune cells might help us overcome these challenges.

In Paper I, we described our efforts to further elucidate the on- and off-target mechanisms behind ibrutinib's common adverse events and immune effects by performing proximity extension assays to measure 265 plasma biomarkers and flow cytometry to assess the immune cell dynamics throughout 5 years of ibrutinib treatment in 13 CLL patients. In Paper II, we tested the safety and feasibility of a novel intermittent dosing strategy for ibrutinib. Treatment was interrupted in CLL patients who were in remission and resumed when early sign of progression occurred in a repetitive fashion. In paper III, we characterized the influences that cHL has on circulating lymphocytes in relation to clinical parameters and first-line treatment.

We found that numerous biomarkers changed during ibrutinib treatment and categorized them by cellular origin and related conditions. Interestingly, we discovered 6 potential mediators of ibrutinib-induced atrial fibrillation. We also showed that successful ibrutinib treatment leads to a reduction in all T-cell populations, including T helper 1 cells, Tregs and exhausted T cells, which paralleled the declining tumor burden. T helper 2 (Th2) cells remained relatively stable, causing Th2-skewing. Interrupting ibrutinib turned out to be safe in CLL patients that are in a sustained remission and resuming the drug upon early signs of progression induced new objective responses. Circulating T cells in cHL were found to be exhausted and terminally differentiated, B- and natural killer-cell numbers were low, and inflammation and tumor burden were related to distinct immune profiles. Standard first-line treatment reversed most changes. However, radiotherapy involving the mediastinum seemed to selectively reduce T cells.

We conclude from these studies that ibrutinib has broad immunomodulatory effects and that several investigated biomarkers might be involved in ibrutinib-induced atrial fibrillation. Moreover, ibrutinib can safely be interrupted in responding CLL patients, who remain drug-sensitive when progressive disease occurs. The risk of serious infections seems to be lower when patients are off the drug. On top of the well-studied control that malignant cells have over the immune cells in the tumor microenvironment, cHL patients have signs of systemic immunosuppression as well, which could be solved to a large extent by standard front-line treatment.

LIST OF SCIENTIFIC PAPERS

I. **Mulder TA**, Peña-Pérez L, Berglöf A, Meinke S, Estupiñán HY, Heimersson K, Zain R, Månsson R, Smith CIE and Palma M
Ibrutinib has time-dependent on- and off-target effects on plasma biomarkers and immune cells in chronic lymphocytic leukemia
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II. Lundin J, **Mulder TA**, Kättström M, Wästerlid T, Uddevik A, Mellstedt H, Heimersson K, Hansson L, Palma M and Österborg A
Temporary cessation of ibrutinib results in reduced grade 3-4 infections and durable remissions – Interim analysis of an on-off-repeat Phase 1b/2 study in patients with chronic lymphocytic leukemia
eJHaem. 2021 Aug;2(3):525-529.

III. **Mulder TA**, Andersson ML, Heimersson K, Xagoraris I, Wahlin BE, Hansson L, Rassidakis G and Palma M
Characterization of immune cells in the tumor microenvironment and peripheral blood of classical Hodgkin lymphoma patients in relation to tumor burden and treatment
Manuscript.

SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

Palma M, Hansson L, **Mulder TA**, Adamson L, Näsman-Glaser B, Eriksson I, Heimersson K, Ryblom H, Mozaffari F, Svensson A, Gentilcore G, Österborg A and Mellstedt H
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Front Immunol. 2021 Jul;12:686768.

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LIST OF ABBREVIATIONS

ABVD	Doxorubicin (adriamycin), bleomycin, vinblastine and dacarbazine
ADCC	Antibody-dependent cellular cytotoxicity
AF	Atrial fibrillation
AID	Activation-induced cytidine deaminase
ALC	Absolute lymphocyte count
Allo-HCT	Allogeneic hematopoietic cell transplantation
APC	Antigen-presenting cell
ATP	Adenosine triphosphate
Auto-HCT	Autologous hematopoietic cell transplantation
AVD	Doxorubicin (adriamycin), vinblastine and dacarbazine
BCL	B-cell lymphoma
BCR	B-cell receptor
BEACOPPesc	Escalated bleomycin, etoposide, doxorubicin (adriamycin), cyclophosphamide, vincristine (oncovin), procarbazine and prednisolone
BLIMP-1	B lymphocyte-induced maturation protein-1
BR	Bendamustine and rituximab
BTK	Bruton's tyrosine kinase
BV	Brentuximab vedotin
β2M	β2-microglobulin
CCL	C-C motif chemokine ligand
CCR	C-C motif chemokine receptor
cHL	Classical Hodgkin lymphoma
CLL	Chronic lymphocytic leukemia
CLL-IPI	Chronic lymphocytic leukemia international prognostic index
CML	Chronic myeloid leukemia
COO	Cell-of-origin
CR	Complete response
CSR	Class switch recombination
CT	Computed tomography
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte-associated antigen-4
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C motif chemokine receptor
DC	Dendritic cell

DLBCL	Diffuse large B-cell lymphoma
DN	Double-negative
DNAM-1	DNAX accessory molecule-1
EBV	Epstein-Barr virus
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EMA	European medicines agency
ETP	Early thymocyte progenitor
FasL	Fas ligand
Fc	fragment crystallizable
FcR	Fc receptor
FCR	Fludarabine, cyclophosphamide and rituximab
FDA	Food and drug administration
FDG-PET	fluorodeoxyglucose positron emission tomography
FISH	Fluorescence in situ hybridization
GC	Germinal center
GCR	Germinal center reaction
HCDR3	Heavy-chain complementarity-determining region 3
HER2	Human epidermal growth factor receptor 2
HL	Hodgkin lymphoma
HLA	Human leukocyte antigen
HMGB1	High-mobility group protein B1
HRP	Horseradish peroxidase
HRS	Hodgkin and Reed-Sternberg
HSC	Hematopoietic stem cell
IC_{50}	Half maximal inhibitory concentration
ICB	Immune checkpoint blockade
ICI	Immune checkpoint inhibitor
IFN	Interferon
Ig	Immunoglobulin
IGHV	Immunoglobulin heavy-chain variable region
IL	Interleukin
IRF4	Interferon regulatory factor 4
ITK	Interleukin-2-inducable T-cell kinase
JAK	Janus kinase
KIR	Killer immunoglobulin-like receptor

LAG-3	Lymphocyte activation gene-3
Lck	Lymphocyte-specific tyrosine kinase
LD	Lymphocyte-depleted
LLPC	Long-lived plasma cell
Lyn	Lck/Yes novel tyrosine kinase
M-CLL	Mutated *IGHV* chronic lymphocytic leukemia
M-CSF	Macrophage colony-stimulating factor
MALT	Mucosa-associated lymphoid tissue
MBL	Monoclonal B-cell lymphocytosis
MC	Mixed cellularity
MCL	Mantle cell lymphoma
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
MLP	Multi-lymphoid progenitor
MPP	Multipotent progenitor
MRD	Minimal residual disease
MSC	Mesenchymal stem cell
MZL	Marginal zone lymphoma
NF-κB	Nuclear factor-κB
NK	Natural killer
NLC	Nurse-like cell
NLPHL	Nodular lymphocyte predominant Hodgkin lymphoma
NS	Nodular sclerosis
ORR	Objective response rate
OS	Overall survival
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
PD	Progressive disease
PD-1	Programmed cell death protein-1
PD-L	Programmed cell death ligand
PEA	Proximity extension assay
PFS	Progression-free survival
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PJP	*Pneumocystis jirovecii* pneumonia

PLC-γ2	Phospholipase C γ2
PR	Partial response
PVR	Poliovirus receptor
PVRL2	Poliovirus receptor-related 2
R/R	Relapsed or refractory
RAG	Recombination-activating genes
RT	Richter transformation
SHM	Somatic hypermutation
Src	Sarcoma tyrosine kinase
Syk	Spleen tyrosine kinase
TAM	Tumor-associated macrophage
TARC	Thymus and activation-regulated chemokine
T_{CM}	Central memory T
TCR	T-cell receptor
T_{EM}	Effector memory T
T_{EMRA}	Effector memory T cells re-expressing CD45RA
Tfh	T follicular helper
Th	T helper
TIGIT	T cell immunoglobulin and ITIM domain
TIM-3	T cell immunoglobulin and mucin-domain containing-3
TKI	Tyrosine kinase inhibitor
TLR	Toll-like receptor
TME	Tumor microenvironment
T_{N}	Naïve T
TNF	Tumor necrosis factor
U-CLL	Unmutated *IGHV* chronic lymphocytic leukemia
VDJ	Variable, diversity and joining
VO	Venetoclax and obinutuzumab
WBC	White blood cell count
WM	Waldenström's macroglobulinemia
XLA	X-linked agammaglobulinemia

1 INTRODUCTION

Blood carries nutrients to our tissues and brings waste products to organs that dispose of them. On top of that, blood contains cells. These cells are called hematopoietic cells and derive from the mesoderm. They are produced in the spongy tissue in the medullary cavities of the bones, called bone marrow (1). In humans, bone marrow represents approximately 5% of the total body mass, which translates into 3 kg in a standard individual. The majority (56-82%) of bone marrow is located in the lower limbs, vertebrae and pelvis (2). All hematopoietic cells derive from the same multipotent stem cells that have self-renewing abilities, called hematopoietic stem cells (HSCs).

The hematopoietic system consists of a myeloid and a lymphoid lineage. The myeloid lineage includes red blood cells (or erythrocytes) that transport oxygen from our lungs to our tissues, platelets (or thrombocytes) that build blood clots when blood vessels get damaged to avoid excess blood loss and white blood cells (or leukocytes), like granulocytes and monocytes, that form our first line of defense against pathogens. The lymphoid lineage consists of leukocytes (or lymphocytes in this context) that form our highly adaptable and powerful second line of defense. Leukocytes from both lineages are collectively called immune cells, which protect the human body against pathogens, toxins and (potential) cancer cells (**Figure 1**).

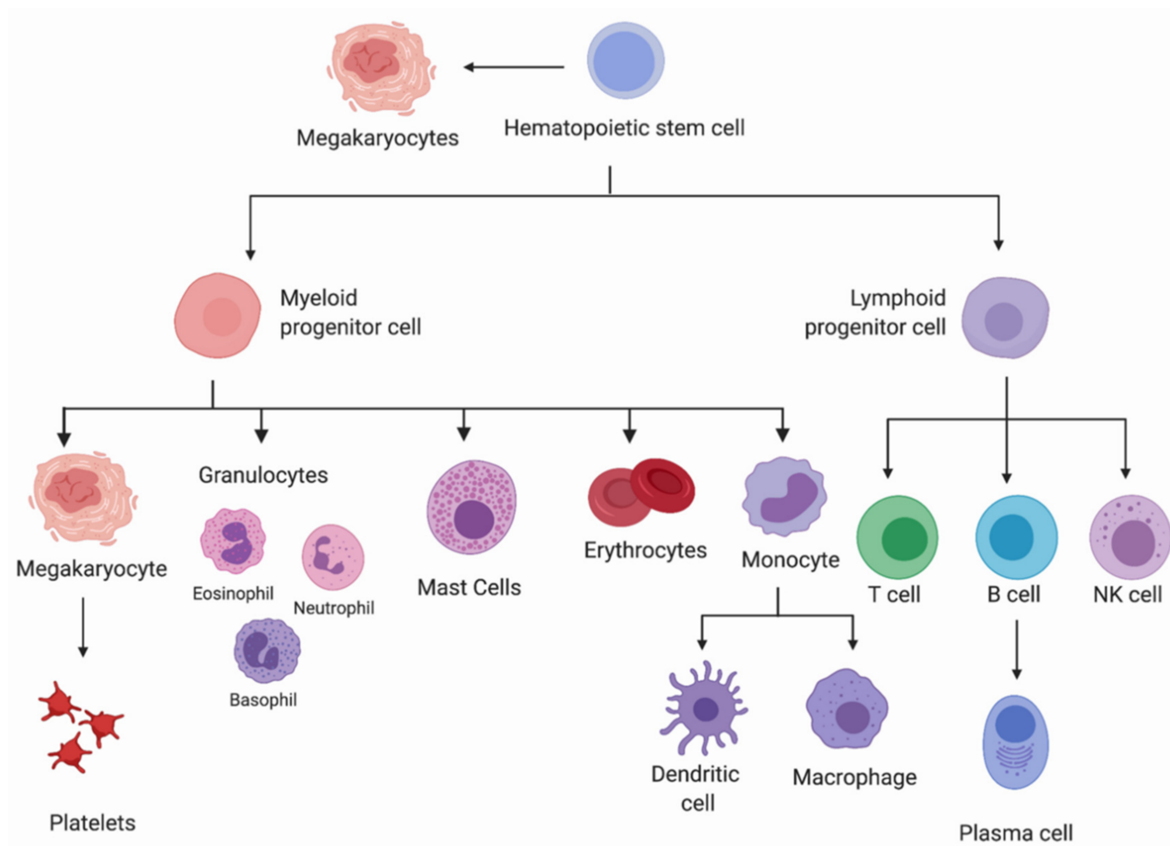

Figure 1 – The hematopoietic tree. From: Raza Y, Salman H, Luberto C. Sphingolipids in Hematopoiesis: Exploring Their Role in Lineage Commitment. *Cells*. 2021; 10(10):2507. This image was redistributed under the Creative Commons Attribution 4.0 International (CC BY 4.0) license. No changes were made.

1.1 LYMPHOPOIESIS AND LYMPHOID LINEAGE BIOLOGY

Lymphoid malignancies are a group of cancer subtypes that arise from the lymphoid lineage of hematopoietic cells. To understand the physiology of the immune system and pathophysiological mechanisms of lymphoid malignancies, the development of these cells is relevant and therefore presented here.

1.1.1 Lymphopoiesis

Hematopoietic stem cells (HSCs), which have self-renewal abilities, give rise to multipotent progenitors (MPPs), which differentiate into multi-lymphoid progenitors (MLPs) in case of early lymphoid lineage restriction (3). MLPs can become lymphoblasts, which are the cells-of-origin (COO) in acute lymphoblastic leukemia (ALL).

Stromal cell-derived interleukin (IL)-7 is the key external factor driving lymphoid commitment in MPPs and MLPs in the bone marrow. IL-7 remains a vital supporter that propels all cells of the lymphoid lineage at all their respective developmental stages and tissue niches (bone marrow, secondary lymphoid tissue and thymus) (4). The lymphoid lineage is comprised of three major cell types. B and T cells, which are part of the adaptive immune system, and NK cells, which belong to the innate immune system, although increasing evidence suggests that some NK-cell subsets have adaptive (or memory-like) capabilities (5). Unlike B cells, T-cells depend on IL-7 for their homeostasis when they are mature (4).

1.1.2 B cells

1.1.2.1 Development in the bone marrow

MLPs can differentiate into pro-B cells, which stay in the bone marrow (hence the name 'B lymphocyte' or simply B cell). Here, they develop into pre-B cells that start expressing membrane-bound immunoglobulin (Ig) isotype M (IgM) after recombination of the heavy-chain variable, diversity and joining (VDJ) gene segments (6). Through recombination of the light-chain VJ gene segments, it develops further into an immature B cell. This process of V(D)J recombination is mediated by the recombination-activating genes (RAG)-1 and RAG-2 and diversifies the antibody repertoire enormously (7) (**Box 1**). Susumu Tonegawa received the Nobel prize in physiology or medicine for his discovery of this process in 1987. At this stage, autoreactive clones are

BOX 1 — V(D)J RECOMBINATION

Immature lymphocytes have the unique capability to rearrange the variable (V), diversity (D) and joining (J) gene segments that encode the antigen-binding regions of immunoglobulins and T-cell receptors. This stochastic process enables lymphocytes to develop a highly diverse repertoire of antigen recognition molecules. The B cell's immunoglobulin consists of a heavy- and a light-chain. The heavy-chain locus contains approximately 65 V, 27 D and 6 J gene segments, resulting in roughly 10 500 different potential sequences, while the two light-chain loci κ and λ only contain 40 and 30 V, and 5 and 4 J gene segments, respectively, adding up to 320 different sequences (1). The antigen-binding domain of an immunoglobulin can therefore be made of 3,4 million unique heavy/light-chain combinations. This diversity is further increased to an estimated magnitude of 10^{11} because of the addition of nucleotides after hairpin loops are cleaved by RAG proteins, which is called 'junctional diversity' (1). The T-cell receptor is formed by an α and a β-chain. The α-chain, like the light-chains of an immunoglobin, only consists of a V and J gene segment, while the β-chain, like the heavy-chain of an immunoglobin, consists of a V, D and J gene segment. This process equips humans with an adaptive immune system than can recognize virtually any antigen.

deleted (clonal deletion). Immature B cells leave the bone marrow and enter the peripheral blood (PB) where they are called transitional B cells. Then, they occupy secondary lymphoid organs (lymph nodes, spleen, mucosa-associated lymphoid tissue (MALT), etc.) and start co-expressing membrane-bound IgM and IgD to become mature (naïve) B cells (8).

1.1.2.2 B-cell receptor signaling

Each heavy-chain of the Ig molecule of a mature naïve B cell is non-covalently bound to a heterodimer of Igα (CD79a) and Igβ (CD79b), together forming the B-cell receptor (BCR) complex. When the Ig binds its antigen, CD79a and CD79b will be phosphorylated by sarcoma tyrosine kinase (Src) family kinases lymphocyte-specific tyrosine kinase (Lck)/Yes novel tyrosine kinase (Lyn) and spleen tyrosine kinase (Syk). This phosphorylation triggers the formation of a signalosome consisting of Lyn, Syk, Bruton's tyrosine kinase (BTK) and several scaffold and adaptor proteins (**Figure 2**). Lyn also recruits costimulatory molecules, like CD19, which amplify BCR activation. BTK is phosphorylated by itself, Lyn and Syk, which stimulates BCR cross-linking together with further antigen engagement.

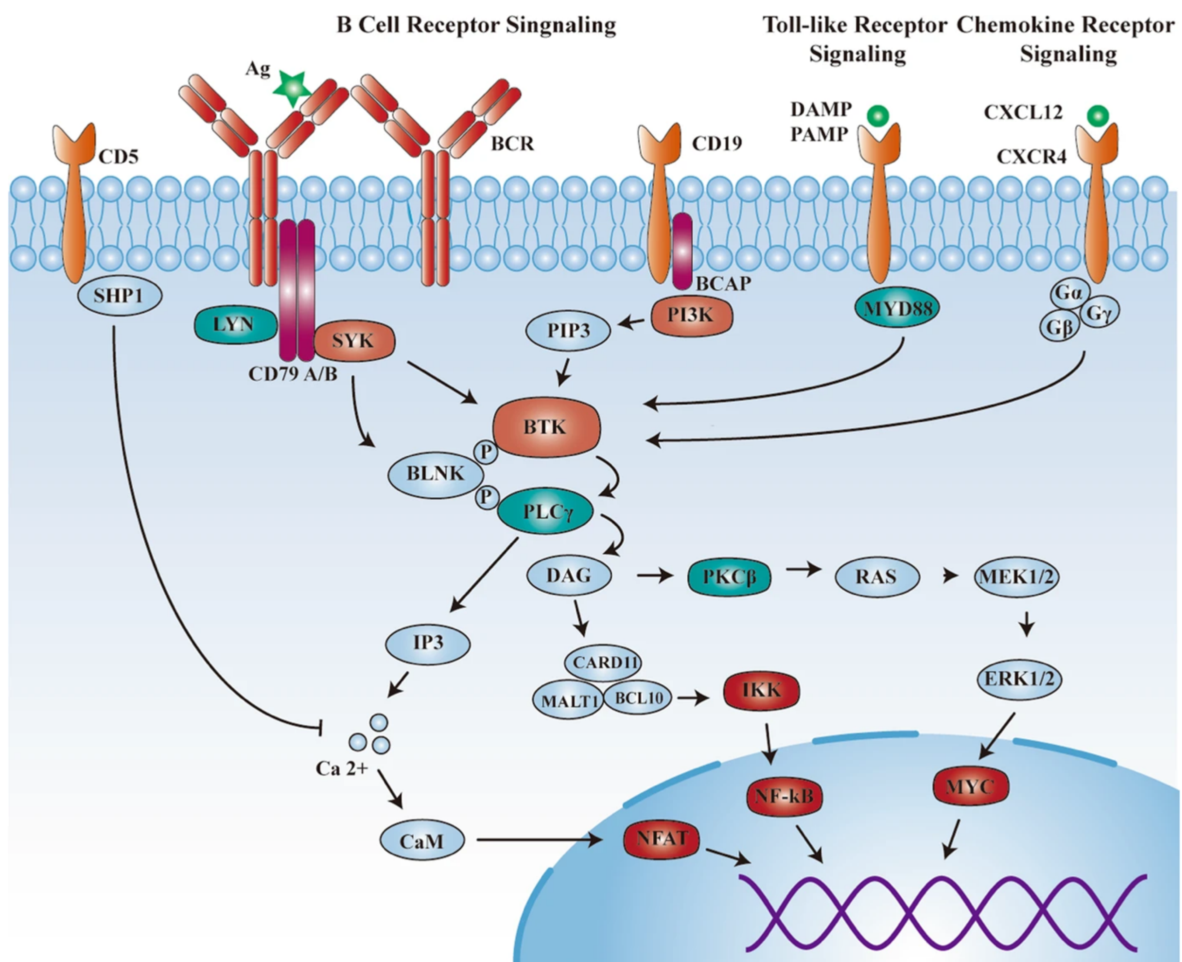

Figure 2 – Cellular signaling via BTK. From: Wen, T., Wang, J., Shi, Y. et al. Inhibitors targeting Bruton's tyrosine kinase in cancers: drug development advances. *Leukemia* 35, 312–332 (2021). This image was redistributed under the Creative Commons Attribution 4.0 International (CC BY 4.0) license. No changes were made.

Activated BTK propagates the BCR signal itself through nuclear factor-κB (NF-κB) activation and binding to phosphatidylinositol 4-phosphate 5 kinases (PIP5K). Furthermore, when phospholipase C γ2 (PLC-γ2) is recruited to the signalosome, it is phosphorylated by Syk and BTK and produces diacylglycerol (DAG). This activates protein kinase C (PKC), and inositol-1,4,5-triphosphate (IP3) from phosphatidylinositol 4,5-bisphosphate (PIP2), which causes calcium influx. Additionally, the phosphoinositide 3-kinase (PI3K) subunit p85 associates with the Src kinases Fyn, Lyn and Lyn-phosphorylated CD19, which activates PI3K subunit p110δ. Activated PI3K then phosphorylates PIP2, which turns it into phosphatidylinositol 3,4,5-triphosphate (PIP3). Then, PIP3 propagates BCR activation by recruiting more BTK and other kinases (9).

This eventually results in downstream activation of the Akt/mammalian target of rapamycin (mTOR), mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK), nuclear factor of activated T cells (NFAT) and NF-κB pathways. These initiate the transcription of a large number of genes that are involved in differentiation, proliferation, survival and migration of the B cell (**Figure 2**) (10).

1.1.2.3 The germinal center reaction

If a mature naïve B cell, which is the COO in mantle cell lymphoma (MCL) and unmutated immunoglobulin heavy-chain variable region (*IGHV)* chronic lymphocytic leukemia (U-CLL), enters the lymphoid follicle of secondary lymphoid tissue and encounters its cognate antigen while receiving sufficient help from a T helper (Th) cell (both signals are required), it will become activated and starts dividing in the dark zone of a germinal center (GC). The B cell is now called centroblast, which is the COO in Hodgkin lymphoma (HL), diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL) and Burkitt lymphoma (BL). During this GC reaction (GCR), centroblasts will undergo somatic hypermutation (SHM). During SHM, the enzyme activation-induced cytidine deaminase (AID) induces point mutations in the Ig variable region genes (11).

Centroblasts then move to the light zone, where they are called centrocytes, to interact with a T follicular helper cell (Tfh) or follicular dendritic cell (FDC) that present their cognate antigen. B cells with disadvantageous mutations that fail to bind antigens will be deleted after this interaction, and B cells with advantageous mutations that bind the antigen with more affinity than before SHM will compete. Those who fail to bind the antigen due to insufficient competitiveness will go into apoptosis as well. This process effectively selects the B cells that express Igs (also known as antibodies) with the highest affinity and specificity to the antigen, which contributes to antibody affinity maturation.

Surviving B cells will then undergo class switch recombination (CSR). Another AID-dependent process that induces genetic alterations in the heavy-chain constant region genes that switch the Ig class from IgM or IgD to IgA, IgE or IgG (12).

Unconventionally, memory B cells can also develop without the help of T cells and outside of GCs. These memory B cells express low-affinity IgM, because they have not been subjected to SHM and CSR (13).

1.1.2.4 Terminal differentiation

B cells can then differentiate further into memory B cells. These relocate either to the marginal zone of the spleen, or to the MALT, or to the subcapsular sinus of the lymph nodes and the PB. Depending on their localization, these memory B cells represent the COO of marginal zone lymphoma (MZL), MALT lymphoma and mutated *IGHV* chronic lymphocytic leukemia (M-CLL), respectively. Memory B cells that have gone through the GCR and differentiate into memory B cells have their high-affinity monoclonal antibodies bound to their surface to form the BCR. They can expand rapidly in case an antigen occurs again later in life.

After the GCR, B cells can also differentiate into plasma cells, which are the COO in multiple myeloma (MM). They reside in the medullary cord of the lymph nodes, the red pulp of the spleen and the bone marrow and secrete high amounts of their high-affinity monoclonal antibodies into the circulation (14).

1.1.2.5 Humoral immunity

Antibodies are the soluble (or humoral, *humor* is Latin for 'fluid') factors that can protect against infections by binding pathogens to either neutralize them (e.g., by preventing viruses and intracellular bacteria from entering cells) or facilitate the activation of proteins and cells that can directly eliminate extracellular pathogens and intracellular pathogens that spread through the extracellular compartment.

Complement proteins can bind to fragment crystallizable (Fc) region of the antibodies that coat the pathogen to activate the complement system. Furthermore, immune cells that express an Fc receptor (FcR), which also binds to the Fc region of the antibodies, can be activated to eliminate the coated pathogen. Macrophages, neutrophils, and eosinophils express FcαRs that recognize IgA. Mast cells, eosinophils, and basophils express FcεRs that recognize IgE. DCs, macrophages, neutrophils, eosinophils, and mast cells express FcγRs that recognize IgG. The ligation of FcRs by Igs facilitates phagocytosis and antigen presentation in dendritic cells (DC) and macrophages, while it induces the activation of respiratory burst and the secretion of granules in neutrophils, eosinophils, basophils, and mast cells. Moreover, this ligation activates antibody-dependent cellular cytotoxicity (ADCC) in NK cells, which also express FcγRs (also known as CD16) (1).

1.1.2.6 Recall response

When re-exposed to the antigen at a later stage, circulating antibodies from long-lived plasma cells (LLPCs) will act immediately and memory B cells that reside in the spleen and subcapsular sinus of lymph nodes will proliferate and differentiate to plasma cells or form

new GCs together with naïve B cells. This increases the amount of plasma cells that produce antibodies against the antigen and generates new antibodies with even higher affinity (15).

1.1.3 T cells

1.1.3.1 Development in the thymus

The MLP can also leave the bone marrow and home to the thymus (hence the name 'T lymphocyte' or simply T cell), a lobed organ in the anterior superior part of the mediastinum, if it expresses C-C motif chemokine receptor (CCR)7 and CCR9 (16). The thymus is an indispensable microenvironment facilitating the development of T cells. The early thymocyte progenitor (ETP) is the most immature T-cell progenitor in the thymus and a subset of CD4 and CD8 double-negative (DN) T cells that are also negative for CD3 and CD25 but do express CD44 (DN1). ETPs still have the potential to differentiate into natural killer (NK) cells and other lineages (17). ETPs generate DN2 cells, defined as $CD3^-CD4^-CD8^-CD25^+CD44^+$, which are still not restricted to the T-cell lineage.

1.1.3.1.1 Rearrangement of the T-cell receptor genes

Once they progress to the DN3 stage, defined as $CD3^-CD4^-CD8^-CD25^+CD44^-$, they start expressing genes that are involved in T-cell receptor (TCR) rearrangement (RAG1 and RAG2), integrity (CD3 chains) and signaling (Lck). DN3 T-cell progenitors are now committed to the T-cell lineage and undergo recombination of the TCR β-chain VDJ gene segments. The TCR β-chain is then linked to a pre-TCR α-chain to form the pre-TCR. Then, only those cells that have successfully rearranged β-chains to enable signaling through the pre-TCR survive during a process called β-selection. Surviving cells progress through the DN4 stage, also defined as $CD3^-CD4^-CD8^-CD25^+CD44^-$, and become CD4 and CD8 double-positive (DP). At this stage, VJ gene segment recombination of the TCR α-chain occurs and the TCRαβ complex is finalized (18).

1.1.3.1.2 Positive and negative selection

Only those T cells that recognize a peptide presented on self-major histocompatibility complex (MHC) molecules survive (positive selection), losing CD4 while retaining CD8 in case of MHC class I-recognition and retaining CD4 while losing CD8 in case of MHC class II-recognition. Self-antigen reactive T cells are sent into apoptosis (negative selection) (18). The two subsets of conventional mature naïve T cells are $CD8^+$ cytotoxic T lymphocytes (CTL), which are efficient killers of cells that present non-self-antigens or express non-self-MHC class I, and $CD4^+$ Th cells, which are the essential directors of the adaptive immune response that can support various immune cells through secreted cytokines (e.g., interferon (IFN)-γ) and membrane-bound ligands (e.g., CD40L).

$CD4^+$ T cells that have cytotoxic potential have also been identified (19). However, these occur mostly under inflammatory conditions and continuous antigen exposure (20).

1.1.3.2 Initial activation

In general, mature naïve T cells need two signals to become activated and undergo clonal expansion. This usually happens when an antigen-presenting cell (APC) presents their antigen to them in secondary lymphoid tissue. Signal 1 comes from the TCR complex and is triggered by the binding of the cognate antigen in the context of an MHC molecule. Signal 2 comes from the membrane-bound protein CD28 upon binding to a B7 costimulatory protein (e.g., CD80 and CD86). T cells that receive signal 1 without signal 2 are usually inactivated. Signal 2 is most often provided by a DC in addition to the presentation of the antigen on an MHC molecule (1). All APCs can process exogenous antigens to present them in the context of MHC class II to Th cells, while only a specialized subset of DCs can present exogenous antigens in the context of MHC class I to CTLs through a process called cross-presentation (21). Once adequately activated, T cells enter the circulation and can directly exert their function when they recognize the same antigen-MHC complex again anywhere in the body.

1.1.3.3 Subsets of antigen-activated T cells

A schematic representation of T-cell differentiation, including the main transcription factors that drive this process in various directions, is depicted in **Figure 3**.

1.1.3.3.1 T helper 1 and 2 cells

Upon activation, Th cells can differentiate into Th1 or Th2 cells, among other subtypes, depending on the cytokines that they are exposed to. IL-12 from APCs and IFN-γ from T and NK cells drive Th1-differentiation. Th1 cells characteristically express transcription factor ‘T-box expressed in T cells’ (T-bet) and surface molecule C-X-C motif chemokine receptor (CXCR)3. Their primary role is to support CTLs, NK cells and macrophages in fighting intracellular pathogens and cancerous cells, by secreting IFN-γ, IL-2 and tumor necrosis factor (TNF)-α, but their IFN-γ also helps B cells during CSR (22, 23).

Predominant exposure to IL-4 will cause Th2-differentiation. Th2 cells typically express transcription factor GATA-3. They play a major role in promoting CSR and antibody production by B cells through the secretion of IL-4. Furthermore, they play a vital role in the protection against extracellular pathogens (e.g., parasites like helminths) by activating eosinophils through the secretion of IL-5. Th2 cells also produce IL-13, which stimulates B cells to switch to IgE during CSR and recruits and activates eosinophils (22, 23). They have historically been regarded as tumor-promoting by dampening a cell-mediated immune response, however, they can also support antitumor immune responses (24).

1.1.3.3.2 Regulatory T cells and T helper 17 cells

Two other major Th subsets are regulatory T cells (Treg) and Th17 cells. Exposure to transforming growth factor β (TGFβ), in the absence of IL-6, drives Treg-differentiation. Tregs express master transcription factor forkhead box P3 (FOXP3) and high levels of CD25 on their surface. They secrete TGFβ and IL-10 and have an immunosuppressive and tolerogenic function (23, 25). They thereby also suppress anti-cancer immunity (26).

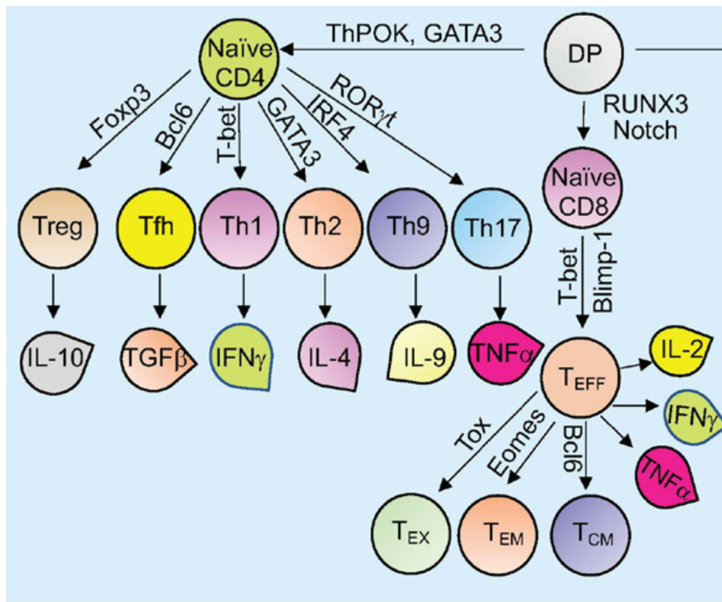

Figure 3 – T-cell differentiation. From: Dutta A, Venkataganesh H, Love PE. New Insights into Epigenetic Regulation of T Cell Differentiation. *Cells*. 2021; 10(12):3459. This image was redistributed under the Creative Commons Attribution 4.0 International (CC BY 4.0) license. The original image was cropped.

Th17-differentiation is initiated by exposure to IL-6 and TGFβ. The signature transcription factor of Th17 cells is 'retinoid-related orphan receptor γt' (RORγt) and they express CCR6 on their surface. They secrete IL-17, which attracts neutrophils, and IL-21, IL-22 and TNF-α. They are abundant in gut tissue and play an important role in the protection against extracellular bacteria and fungi at mucosal and epithelial sites (23, 27, 28). Th17 cells have a high degree plasticity and can be both tumor-promoting and tumor-suppressing depending on the signals they get from the tumor microenvironment (TME) (29).

1.1.3.3.3 T follicular helper cells

Another important Th-cell subset is the Tfh cell. As described above, it plays an important role in the affinity maturation of Igs during B-cell development in the secondary lymphoid organs. Tfh cells develop under the influence of IL-6 and IL-21, their defining transcription factor is B-cell lymphoma (BCL)6 and they typically express CXCR5 and inducible T-cell co-stimulator (ICOS) on their surface. They secrete IL-4, IL-10 and IL-21 (23, 30, 31). IL-21 induces AID expression in B cells and consequently supports SHM and CSR during the GCR (32). Tfh cells can be tumor-promoting in lymphoid malignancies and help antitumor immunity in other cancer types (33).

1.1.3.3.4 T helper 9 and 22 cells

More recently, two additional subsets have been described. Th9 cells can differentiate directly from naïve Th cells upon activation or from Th2-polarized cells when exposed to IL-4 and TGFβ. They express transcription factors GATA-3, PU.1 and interferon regulatory factor 4 (IRF4), and, as the name suggests, mainly produce IL-9. They play a pro-inflammatory role in allergic and autoimmune conditions, but also have antitumor properties (23, 34, 35).

Th22 cells are generated in environments where IL6 and TNF-α are dominant, express transcription factor aryl hydrocarbon receptor (AHR) and CCR4 on their surface and secrete

IL-22. Th22 cells play a role in mucosal immunity and support tissue repair (23, 36, 37). They might also aid tumor progression (38).

1.1.3.3.5 T helper cell plasticity and heterogeneity

Th cells are not committed to these stereotypic subsets. They can adapt their phenotype if they get the appropriate cues from the microenvironment, which cause them to switch to transcriptional programs of other subsets giving them great plasticity (39). Th cells can even co-express master transcriptional regulators from several different subsets simultaneously, which leads to functional heterogeneity (23).

1.1.3.3.6 Cytotoxic T lymphocytes

The main function of activated CTLs is to kill other cells in an antigen-dependent fashion. Upon ligation of multiple TCR molecules, an immunological synapse is formed between the CTL and its target cell, which directs the release of cytotoxic granules (40). These granules contain large amounts of perforin, which forms pores in the membrane of the target cell, and granzyme A and B, which cause DNA damage and activate caspases in the target cell. The expression of perforin and granzymes is induced by CTL master transcriptional activator T-box brain protein 2 (Tbr2), which is better known as eomesodermin and encoded by the *EOMES* gene (41). An alternative way for CTLs to trigger target cell apoptosis is through the expression of Fas ligand (FasL), which binds to Fas (also known as CD95) on target cells (42). IFN-γ, which is produced by APCs, like DCs and macrophages, Th1 cells, NK cells and CTLs themselves, is the most important supporter of the cytotoxic activity of CTLs (1).

1.1.3.4 Functional differentiation of memory T cells

Immunological memory enables a quicker and stronger response to antigens that have been encountered before. Naïve T cells (T_N), which express both CCR7 and CD45RA, that meet their cognate antigen to get activated need more time to expand. Some of the antigen-specific T cells will differentiate into memory T cells after a primary immune response. These memory T cells turn CD45RA$^-$ and start expressing CD45RO instead. Central memory T cells (T_{CM}) are still CCR7$^+$ and consequently home to lymphoid tissue. They can differentiate into effector memory T cells (T_{EM}), which lack CCR7 and are therefore able to relocate and reside in peripheral tissues (43). The CCR7$^-$ effector memory T cells re-expressing CD45RA (T_{EMRA}) are terminally differentiated and might die because of sustained antigen exposure and proliferation. Through differentiation from T_{CM} to T_{EM} and T_{EMRA}, the production of IL-2 decreases, while the production of IFN-γ and TNF-α increases (44).

1.1.3.5 Immune checkpoint molecules

1.1.3.5.1 Cytotoxic T lymphocyte-associated antigen-4

Directly upon initial activation, cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) starts being expressed by T cells. It serves to prevent excessive T-cell responses and

autoimmunity during the early phase of activation in secondary lymphoid organs (T-cell priming) by competing with CD28 for the ligands CD80 and CD86 (**Figure 4**). If a T cell binds its cognate antigen-MHC complex while CTLA-4 rather than CD28 is being ligated by CD80 and CD86, it will not be activated (45).

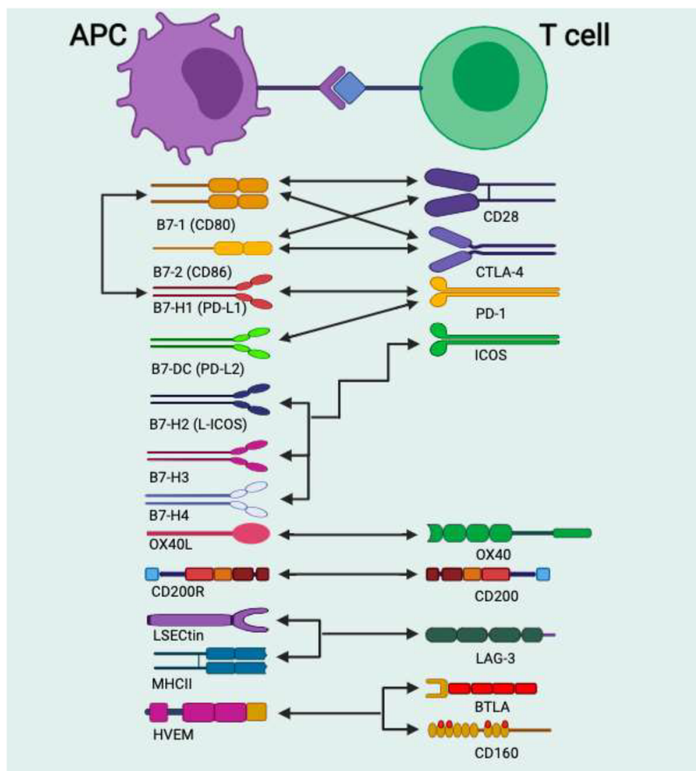

Figure 4 – T-cell co-stimulation and co-inhibition. From: Galván Morales MA, Montero-Vargas JM, Vizuet-de-Rueda JC, Teran LM. New Insights into the Role of PD-1 and Its Ligands in Allergic Disease. *International Journal of Molecular Sciences*. 2021; 22(21):11898. This image was redistributed under the Creative Commons Attribution 4.0 International (CC BY 4.0) license. No changes were made.

1.1.3.5.2 Programmed cell death protein-1

Initial T-cell activation also induces programmed cell death protein-1 (PD-1) expression. However, this checkpoint molecule plays its main role in restraining excessive T-cell responses and autoimmunity at local tissue sites (peripheral tolerance). Upon ligation by programmed cell death ligand (PD-L)1 or PD-L2 (**Figure 4**), which are upregulated in inflamed peripheral tissues, it suppresses TCR and CD28 signaling-mediated proliferation and cytokine release (46, 47).

1.1.3.5.3 T cell immunoglobulin and mucin-domain containing-3

Th1 cells, CTLs and NK cells express T cell immunoglobulin and mucin-domain containing-3 (TIM-3), which inhibits their activity upon ligation by galectin-9, carcinoembryonic antigen cell adhesion molecule-1 (Ceacam-1) (47). TIM-3 is also expressed by DCs and macrophages and influences their function upon ligation by phosphatidyl serine (PtdSer) and high-mobility group protein B1 (HMGB1) (45).

1.1.3.5.4 Lymphocyte activation gene-3

All lymphoid cells express lymphocyte activation gene-3 (LAG-3), which induces inhibitory signals in Th1 cells, as well as CTLs and Tregs (47, 48). Potential ligands are MHC class II molecules, LSECtin, galectin-3, α-synuclein and fibrinogen-like protein 1 (FGL1) (45).

1.1.3.5.5 T cell immunoglobulin and ITIM domain

Activated T cells and NK cells express T cell immunoglobulin and ITIM domain (TIGIT) (49). These cells receive inhibitory signals upon ligation of TIGIT by poliovirus receptor (PVR), also known as CD155, and poliovirus receptor-related 2 (PVRL2), also known as and CD112, which are expressed by APCs. TIGIT also competes for CD112 and CD155 with a co-stimulatory molecule called DNAX accessory molecule-1 (DNAM-1), which is also known as CD226 (48).

1.1.3.5.6 Targeting immune checkpoint molecules for the treatment of cancer

Inhibitory drugs that target all these molecules have been developed to 'release the brakes' on antitumor immunity. This type of therapy is called immune checkpoint inhibitors (ICB) and the drugs are called immune checkpoint inhibitors (ICIs). The first ICIs that gained United States food and drug administration (FDA) approval, for the treatment of melanoma initially, were ipilimumab (anti-CTLA-4) in 2011, and nivolumab and pembrolizumab (both anti-PD-1) in 2014. Many more ICIs and indications have followed since (47).

1.1.4 Natural killer cells

1.1.4.1 Development

The MLP can also give rise to a third cell type that develops without the support of IL-7, but rather relies on IL-15 (50). NK cell development seems to take place at multiple sites that provide IL-15, including the thymus, liver, secondary lymphoid tissue and even the gut and uterus (51, 52). Whether they develop in a linear or branched fashion is still unclear (53). After NK lineage restriction, the linear model assumes that NK-cell progenitors first acquire CD94 to become $CD56^{bright}$ NK cells, to subsequently turn into $CD56^{dim}$ NK cells by gaining the expression of killer immunoglobulin-like receptors (KIRs) and CD16 (53). Every individual expresses a specific set of KIRs, similar to the human leukocyte antigen (HLA) molecules they recognize (54).

1.1.4.2 Functional biology

Both $CD56^{bright}$ and $CD56^{dim}$ NK cells can be found in the PB, although the approximately 90% is $CD56^{dim}$. $CD56^{bright}$ NK cells are long-lived cytokine producers, while $CD56^{dim}$ NK cells are potent cytotoxic cells (55). Unlike B and T cells, NK cells lack a receptor that recognizes a specific antigen. Instead, they have the innate-like ability to directly respond to infected or cancerous cells. Like CTLs, $CD56^{dim}$ mainly produce IFN-γ and can serially kill target cells by forming an immunological synapse and use perforin and granzymes. Alternatively, they can induce apoptosis using FasL and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (56). $CD56^{bright}$ NK cells support the immune response by secreting IFN-γ, G-CSF, GM-CSF, IL-3 and TNF-α (57).

Unlike B and T cells, NK cells lack stochastically recombined receptors that recognizes a specific antigen and the need to be activated through recognition of this antigen. Instead,

they have the innate-like ability to directly respond to virus-infected or cancerous cells. When NK cells encounter a potential target cell, the balance between stimulatory and inhibitory signals will determine if it kills (56).

1.1.4.2.1 Activating receptors

The FcγRIII (also known as CD16), which induces ADCC upon ligation by the Fc region of an IgG molecule, is the strongest activating signal. DNAM-1 can also contribute to activating signals when triggered by PVR and PVRL2. Natural cytotoxicity receptor family members (NKp30, NKP40, NKp44, NKp46 NKp65 and NKp80), which bind to ligands that are associated with pathogens or tumors, also function as activating receptors. NKG2C, which is ligated by HLA-E and peptides from human cytomegalovirus (HCMV), and NKG2D, which is triggered by MHC class I polypeptide-related sequence (MIC)A, MICB and UL16-binding proteins (ULBPs), can serve this function as well (56, 58). The six activating KIRs are KIR2DS1-5 and KIR3DS1 (54).

1.1.4.2.2 Inhibitory receptors

HLA-E can also contribute to inhibiting NK-cell killing when it binds to its inhibitory receptor NKG2A. The recognition of self-expressed MHC class I molecules (HLA-A, -B and -C) on potential target cells is an important inhibitory signal for NK cells (56, 58). These signals are mediated by seven other KIRs, KIR2DL1-3, KIR2DL5 and KIR3DL1-3. KIR2DL4 can give both activating and inhibitory signals (54).

1.2 CHRONIC LYMPHOCYTIC LEUKEMIA

Chronic lymphocytic leukemia (CLL) is the most common leukemia in the western world, representing approximately 15% of the lymphoid malignancies. It is more common in men and the median age at diagnosis is over 70 years of age. CLL is often discovered after an incidental finding of lymphocytosis in individuals who are asymptomatic. CLL usually has a rather indolent course and many patients do not require any treatment. However, some patients have a more aggressive disease and 2 out of 3 individuals that are diagnosed with CLL will require treatment at some point during their lives. Symptoms that can occur include those consistent with anemia and thrombocytopenia (because of bone marrow failure and/or autoimmune destruction), enlarged lymph nodes, enlarged spleen and liver, infections, and constitutional symptoms.

1.2.1 Pathophysiology

The exact cause of CLL is largely unknown. It is not directly hereditary, although familial aggregation does occur, suggesting that there are some genetic risk factors. Familial and sporadic CLL are the same in terms of clinical characteristics and disease course (59). A small increased risk of developing CLL has been linked to medical exposures like blood transfusion and radiation, infectious exposures like hepatitis C virus and pneumonia, occupations like hairdressers and farmers with the exposure to chemical fertilizers and

pesticides, and exposure to substances like detergents, metals, and petroleum. The mechanisms by which these exposures cause CLL have not yet been elucidated (60, 61)

The defining feature of CLL is an increased amount of mature clonal B cells in the PB. These cells can also occupy the bone marrow, which can cause bone marrow failure and subsequent cytopenia, and secondary lymphoid tissues, which can cause lymphadenopathy and splenomegaly. CLL can arise from two COOs: 1) a mature naïve pre-GC B cell that did not undergo SHM yet and therefore has unmutated *IGHV* genes (hence called U-CLL), or 2) a mature post-GC B cell that has undergone SHM and therefore has mutated *IGHV* genes (hence called M-CLL) (62-64). Alternatively, it has been suggested that U-CLL could originate from unconventional GC-independent memory B cells (65, 66).

1.2.1.1 Monoclonal B-cell lymphocytosis

Some individuals have a clonal B-cell population in the PB with a total amount of B cells that is less than 5×10^9/L and no other symptoms. This condition is called monoclonal B-cell lymphocytosis (MBL) and because the cells often have the same immunophenotype as CLL, it is regarded as a precursor to CLL. Among individuals in the general population who are 40 years or older, 1 out of 20 has low-count ($< 0{,}5 \times 10^9$/L) MBL. Up to 2% of the individuals with a high-count ($0{,}5$-5×10^9/L) MBL, will develop CLL with a treatment-indication at some point. High-count MBL also increases the risk for secondary malignancies and infections (67).

1.2.1.2 Recurrent genetic lesions

The fact that the earliest genetic lesions and epigenetic alterations that ultimately cause CLL already appear in HSCs (68), puts the whole concept of a single COO into a different perspective (64). Compared to other cancer types, the mutational burden in CLL is relatively low (69). Nevertheless, more than 2 000 recurrent genetic alterations have been found in manifest CLL to date (70). Genomic studies often report few genes that are mutated in a large fraction of the patients, without being frequent enough to call them common drivers of the disease, and many genes that are mutated in small fractions of the patients. The most common recurrent genetic lesions are del(13q) and mutations in *NOTCH1*, *SF3B1*, *ATM*, *TP53* and *BIRC3* at varying frequencies depending on disease stage and treatment (64).

1.2.1.3 Stereotyped B-cell receptors

Interestingly, the *IGHV* genes that are used in CLL cells are not random in a large fraction of the patients. There is a restriction in their usage, which results in stereotyped BCRs that have similar *IGHV* gene usage and almost identical heavy-chain complementarity-determining region 3 (HCDR3) sequences across patients. Roughly 30% of the patients have any one of the more than 200 different stereotyped BCRs that have been identified up until now and 12% of the patients have one of the 19 major subsets (64, 71).

Several major subsets have relevant clinical profiles. Subset #1 occurs in 2,4% of all CLL patients and is associated with U-CLL and a very poor prognosis. Subset #2 has a frequency

of 2,8%, can be both unmutated and mutated and is associated with an even worse prognosis. Subset #4 occurs in 1% of all patients, is always mutated and causes a very indolent disease course with a delay of 11 years between diagnosis and first-line treatment. Subset #8 has a frequency of 0,5%, is always unmutated and causes a very aggressive disease course with a high risk for Richter transformation (RT), which we call a situation when CLL progresses to a high-grade lymphoma with a dismal prognosis (**Table 1**) (71, 72).

This phenomenon of shared stereotyped BCRs in different CLL patients suggests that these patients share an antigen that is a common trigger in CLL development.

	Subset #1	**Subset #2**	**Subset #4**	**Subset #8**
Frequency (of all CLL)	2,4%	2,8%	1%	0,5%
Gene rearrangement				
Heavy chain	V1-5-7 D6-19 J4	V3-21 CDR3 9 aa	V4-34 D5-18 J6 CDR3 20 aa	V4-39 D6-13 J5
Light chain	κ V1-39 J1-2	λ V3-21	κ V2-30 J1-2	
***IGHV* mutational status**	U	U and M	M	U
Prognosis	Very poor prognosis	Extremely poor prognosis	Very indolent disease course	Very aggressive disease with high risk for RT

Table 1 – Common stereotyped BCR subsets with prognostic implications (71, 72). aa: amino acids.

1.2.1.4 Pathological B-cell receptor activation

In CLL, BCR signaling induces tonic activation of survival and proliferation pathways, despite a low expression level of Ig on the surface (9). The main mechanisms behind this activation and their roles in CLL development are not yet fully elucidated. BCR signaling can be triggered in an antigen-independent fashion when a membrane-bound Ig molecule interacts with itself or an adjacent one. The interaction between HCDR3 and framework region 2 (FR2) has been described in this context (73, 74). On the other hand, BCR signaling can also be triggered by exogenous antigens. These can be pathogen-derived antigens or autoantigens like rheumatoid factors (RF) myosin and vimentin (74, 75).

1.2.2 Clinical management

1.2.2.1 Diagnosis

The minimal requirements to diagnose CLL are: a lymphocytosis (of $\geq 5 \times 10^9$/L) with clonal B cells, the typical expression of CD5, CD19, CD23, CD200 and lack of CD10 as measured by flow cytometry of PB and the predominance of small mature lymphocytes on a PB smear microscopy slide (76). Bone marrow aspiration and biopsy are only indicated at the time of diagnosis in case of any cytopenia.

Lymphadenopathy and/or splenomegaly, without bone marrow involvement, in combination with a lymphocytosis (of < 5 x 10^9/L) with clonal B cells that have the same immunophenotype as CLL is diagnosed as small lymphocytic lymphoma (SLL).

1.2.2.2 Treatment indication

Watchful waiting is the standard policy during the asymptomatic and indolent phase. Only if one of the following criteria of active disease is met, treatment is initiated: disease-related symptoms (weight loss, fatigue, fever and/or night sweats), occurrence or deterioration of anemia or thrombocytopenia as a result of bone marrow failure, autoimmune hemolytic anemia (AIHA) or immune thrombocytopenia (ITP) with insufficient response to corticosteroid treatment, severely enlarged spleen, severely enlarged lymph node or a fast rise in blood lymphocyte count (76).

1.2.2.3 Work-up before first-line treatment

Patients who need treatment are subjected to additional investigations. Fluorescence in situ hybridization (FISH) karyotyping, *IGHV* sequencing analysis, viral serology, and a bone marrow aspiration and biopsy are done before the initiation of first-line treatment. A computed tomography (CT) scan of the thorax and abdomen can also be done.

The most common aberrations found by FISH are (from high-risk to low-risk): del(17p), del(11q) and trisomy 12. Patients with del(13q) have an even better prognosis than those who have a normal karyotype (77), but this does not affect the choice of treatment. A del(17p) often involves the tumor-suppressor gene *TP53* and because a mutation in this gene is predictive of refractoriness to chemoimmunotherapy, the mutational status of *TP53* is subsequently assessed by DNA sequencing if FISH is negative for del(17p). The presence of del(17p)/*TP53*-mutation is the strongest negative prognostic indicator in CLL. Moreover, U-CLL has an inferior prognosis compared to M-CLL, making the *IGHV* mutational status another important prognostic indicator that can be assessed before the initiation of first-line treatment (62, 63).

1.2.2.4 Clinical staging and prognostication

Two clinical staging systems are used in CLL. In the original 5-stage system according to Rai et al., isolated lymphocytosis indicates stage 0, lymphadenopathy indicates stage I, splenomegaly and/or hepatomegaly indicate stage II, anemia (hemoglobin < 110 g/L) indicates stage III and thrombocytopenia (thrombocytes < 100 x 10^9/L) indicates stage IV (78). This was later modified to a 3-stage system where the previous stage 0 indicates a low risk, the previous stages I and II indicate an intermediate risk and the previous stages III and IV indicate a high risk (76).

In the 3 stage-system according to Binet et al., CLL patients with stage A have less than 3 involved nodal areas, those with stage B have 3 or more involved nodal areas and those with stage C have anemia (hemoglobin < 100 g/L) and/or thrombocytopenia (thrombocytes < 100 x 10^9/L) (79).

From a meta-analysis including 3 472 treatment-naïve patients, a CLL international prognostic index (CLL-IPI) was developed and validated. Five independent variables were found that predict overall survival (OS): age (≤ 65 vs. > 65 years), β2-microglobulin (β2M) concentration (≤ 3,5 vs. > 3,5 mg/L), clinical stage (Rai 0/Binet A vs. Rai I-IV/Binet B-C), *IGHV* mutational status (mutated vs. unmutated) and *TP53* status (no abnormalities vs del(17p) and/or *TP53* mutation).

Weighted risk scores were assigned based on the Cox regression coefficients. Age > 65 years and clinical stage Rai I-IV/Binet B-C received a score of 1, β2M concentration > 3,5 mg/L and unmutated *IGHV* received a score of 2 and deleted or mutated *TP53* received a score of 4. The aggregates were categorized as low (0-1), intermediate (2-3), high (4-6) and very high (7-10) risk. These risk categories were confirmed in internal and external validation sets (80).

Independent validations in an Italian cohort of 858 patients and a Danish unselected nationwide cohort of 1 514 patients have also confirmed these risk categories (81, 82). Information about the number of signaling pathways that is disrupted by frequently recurring mutations rather than the presence of individual recurrent mutations might further improve the prognostication of CLL patients using CLL-IPI (70).

1.2.2.5 First-line treatment

1.2.2.5.1 In the absence of del(17p)/*TP53*-mutation

In patients who lack del(17p)/*TP53*-mutation, chemoimmunotherapy is the primary treatment. Fludarabine, cyclophosphamide and rituximab (FCR) are the first choice for patients up to 70 years of age who lack comorbidities (83-87). FCR pushed the OS of CLL ever closer to that of the general population (88). Patients with comorbidity or an age over 65-70 years are treated with bendamustine and rituximab (BR) instead (89). Chlorambucil in combination with an anti-CD20-antibody is an alternative for this group of patients (87, 90).

The 1 year fixed-term treatment with venetoclax (a BCL2-inhibitor) and obinutizumab (anti-CD20-antibody) (VO) gives patients with comorbidity a better progression-free survival (PFS) than treatment with chlorambucil and obinutuzumab, in particular those with unmutated *IGHV* genes (91).

Ibrutinib in combination with rituximab gives a better OS than FCR in fit patients up to 70 years of age and a better PFS than FCR to patients with unmutated *IGHV* genes (92). Likewise, ibrutinib gives a better PFS than BR in patients who are older than 65, especially in those with unmutated *IGHV* genes (93). However, ibrutinib treatment is not cost-effective in the first-line treatment of patients who lack del(17p)/*TP53*-mutation (94), and therefore currently not subsidized in this setting in Sweden.

1.2.2.5.2 In the presence of del(17p)/*TP53*-mutation

For all the patients that do have del(17p)/*TP53*-mutation, a BTK-inhibitor like ibrutinib (95, 96) or acalabrutinib (97, 98) is the standard first-line treatment. The choice between these

two seems to rely on which toxicity profile fits best to the patient. If toxicity forces discontinuation, one could switch to the other. If both of them are inappropriate because of toxicity, treatment with venetoclax (99, 100) or the combination of idelalisib (a PI3K-inhibitor) and rituximab (101) will be considered. Even time-limited treatment with VO is an option in this group of patients (91, 102).

The introduction of multiple new drugs with high efficacy has reduced the need to consider allogeneic hematopoietic cell transplantation (allo-HCT) as a part of the primary treatment. However, it should still be considered for younger patients without comorbidity who have del(17p)/*TP53*-mutation. If at all, it is usually applied in this subgroup of patients after at least one line of treatment has failed.

1.2.2.6 Second- and further-line treatment

In case of relapsed or refractory (R/R) CLL after initial treatment with FCR or BR and a long response duration, the same treatment can be repeated if patients still lack del(17p)/*TP53*-mutation and if comorbidity and age still allow (103-105). Second-line treatment with a BTK-inhibitor should be initiated in patients with a short duration of response or appearance of del(17p)/*TP53*-mutation. Both ibrutinib (106-108) and acalabrutinib (109) are options, and their efficacy should be considered equal in R/R CLL (110). Patients who are refractory to, or relapse during, first-line treatment with a BTK-inhibitor, should switch seamlessly to venetoclax (100, 111-113). R/R CLL patients should receive a BTK-inhibitor in case they progress on venetoclax and have not received a BTK-inhibitor before (100, 114, 115). Allo-HCT should be considered in all young and fit patients with R/R CLL. The availability of a suitable donor and the risk for treatment-related toxicity obviously play a role in the choice for this treatment.

The transformation of CLL into, most frequently, a DLBCL, or, less frequently, a HL, is called RT and can occur at any disease stage. It is mostly a clonal expansion from the original CLL. RT has a dismal prognosis and should be treated in the way that is suitable for the subtype it transformed into (116).

1.2.3 Ibrutinib

As the first-in-class inhibitor of BTK, ibrutinib received FDA approval for the treatment of CLL in 2014 and has revolutionized the treatment of this disease.

1.2.3.1 X-linked agammaglobulinemia

In 1952, Colonel Ogden Carr Bruton described the absence of gamma globulin in the serum of an 8-year-old boy, who had suffered from frequent infections since his birth and repeatedly failed to build an antibody response to vaccinations (117). His discovery led to the identification of a congenital immunodeficiency syndrome that was eventually given the name X-linked agammaglobulinemia (XLA) (118). The gene that causes this disorder was first cloned in 1993 (119). It encodes a tyrosine kinase that is expressed in B cells, which was given Col. Bruton’s name. Patients with XLA have no BTK expression and lack or have very

few circulating B cells and Igs. Additionally, they lack plasma cells (120). B cells from XLA patients are halted in differentiation at an immature stage in the bone marrow, although few immature B cells that express high levels of IgM might enter the circulation in some cases (121, 122).

Normally, BTK is expressed in all lineages of the hematopoietic system, though plasma cells and T cells are the exception (123). This TEC-family member is an essential non-receptor tyrosine kinase in the BCR signaling pathway in B cells (124), as described in detail in chapter 1.1.2.2 and **Figure 2**.

1.2.3.2 Target binding

Ibrutinib (previously referred to as PCI-32765) was originally developed as a small molecule BCR-signaling inhibitor to treat rheumatoid arthritis in 2007 (125). It is a potent, and in fact irreversibly binding, inhibitor of BTK, with a half maximal inhibitory concentration (IC_{50}) for this kinase of 0.5 nM (125, 126). It also inhibits other kinases with similar cysteine residues, like epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2), interleukin-2-inducable T-cell kinase (ITK), Janus kinase (JAK)3 and TEC, with an IC_{50} of 5.6, 9.4, 10.7, 16.1 and 78.0 nM, respectively (126). Ibrutinib was shown to induce apoptosis in DLBCL and CLL cell lines (127, 128). In dogs with spontaneously developing lymphoma, ibrutinib could potently block BTK-phosphorylation and induce objective responses (126).

1.2.3.3 Early clinical trials and approval

A phase 1 trial with ibrutinib in patients with R/R B-cell malignancies was initiated based on these encouraging preclinical data and showed acceptable toxicity profiles and an impressive objective response rate (ORR) of 60%. Clinical responses were observed in CLL, DLBCL and MCL, among other diagnoses. Excitingly, this drug was the first treatment that had equally positive response rates in patients with and without del(17p)/*TP53*-mutation (129). Ibrutinib, which is an orally available drug that has a half-life of 2-3 hours, achieved complete BTK occupancy that persisted for 24 hours (highlighting its covalent binding) when dosed daily in this trial (130). Subsequent phase 2 and 3 clinical trials followed and led to ibrutinib's FDA approval for the treatment of R/R MCL in 2013, CLL in 2014, Waldenström's macroglobulinemia (WM) in 2015, R/R MZL in 2017 and R/R chronic graft-versus-host-disease (cGVHD) later that year.

1.2.3.4 Redistribution lymphocytosis

Interestingly, within hours after the start of treatment, a transient increase in lymphocytosis is observed in CLL patients. This phenomenon is caused by an efflux of CLL cells from the lymphoid organs into the PB and therefore known as 'redistribution lymphocytosis' (131). Before ibrutinib was first tested in patients, it was already known that BCR signaling through BTK increases integrin activity and homing in response to chemokines (132-134). Indeed, ibrutinib treatment inhibits the integrin-dependent adhesion of CLL cells (131). Furthermore,

the surface expression of CXCR4 is reduced by ibrutinib treatment, thereby inhibiting CLL cells from homing back to their protective and supportive microenvironments, which initially causes redistribution lymphocytosis and eventually deprives them from survival signals and leads to apoptosis (135). Ibrutinib reduces proliferation and triggers apoptosis of CLL cells in all compartments (136).

1.2.3.5 Adverse events

Ibrutinib has a low toxicity profile in comparison to chemoimmunotherapy, but it is given continuously until disease progression (137). Atrial fibrillation (AF) is observed with a cumulative incidence of 10-20% and a median time to onset of approximately 8 months. Risk factors are a prior history of AF and other cardiac conditions (138-141). The onset of AF does not necessarily force the discontinuation of ibrutinib, but it might form an indication for antiarrhythmic or anticoagulant drugs to prevent cardiovascular complications (137). The etiology of AF in ibrutinib-treated patients is still elusive. HER2 is expressed in cardiomyocytes and mutated forms have been associated with dilated cardiomyopathy and atrial conduction blockades in mice (142, 143).

Bleeding and bruising complications are also common and might be caused by thrombocyte dysfunction via the inhibition of BTK and TEC (144). The cumulative incidence seems to plateau after 6 months and the median time to onset is 49 days (145). The risk of bleedings (grade ≤ 2) is 2.7 times higher in ibrutinib-treated patients than in patients receiving other treatments for various B-cell malignancies (146).

Serious bacterial and fungal infections are relatively common in ibrutinib-treated patients (147). When disease progression is disregarded, infections are the most frequent reason to cease treatment (148). Invasive fungal infections, most commonly aspergillosis, that mainly occur within the first months of treatment, are particularly concerning (149). The susceptibility to invasive aspergillus infection could be caused by compromised cytokine secretion through BTK-inhibition in macrophages (150).

Other common adverse events are diarrhea, fatigue, hypertension, rash, and arthralgia (151).

1.2.3.6 Drug resistance

Ibrutinib resistance, which leads to progression during continuous treatment, has become a significant clinical problem. If disease progression occurs early, RT is the most likely cause. CLL progression with pre-existing or acquired mutations in the BCR-signaling pathway is the main cause of later progression under ibrutinib (148, 152). The most common resistance-inducing variant is the substitution of cysteine 481 for a serine (C481S) in the BTK protein, which is where ibrutinib covalently binds to its target (**Figure 5**) (152). Another recurring target for mutations that cause resistance is the gene that encodes PLC-γ2, the downstream substrate of BTK (152). About 20% of the patients progress while on ibrutinib within 4 years of treatment, 85% of which have a mutation in *BTK* or *PLCG2*, which arise at a median of 9

months before clinical progression (153). Approximately 60% of the patients will most likely develop resistance to ibrutinib eventually (154, 155).

1.2.4 Other inhibitors of Bruton's tyrosine kinase

Since the approval of ibrutinib in 2013, several next-generation BTK inhibitors have been marketed and many more are being developed for the treatment of lymphoproliferative disorders, including more selective and reversibly (non-covalently) binding compounds (154). All covalently binding BTK inhibitors will fail in patients harboring a high frequency of the resistance-inducing mutation BTK^{C481S}. However, the non-covalently binding compounds are most likely still efficacious as they do not bind BTK at this specific cysteine residue in the adenosine triphosphate (ATP)-binding pocket of the molecule (156, 157).

1.2.4.1 Covalently binding inhibitors of Bruton's tyrosine kinase

Acalabrutinib received approval for the treatment of CLL from the FDA in November 2019 and from the European medicines agency (EMA) in November 2020. This was primarily based on two phase 3 trials in treatment-naïve and R/R patients (97, 98). Compared to ibrutinib, acalabrutinib has a non-inferior efficacy and a lower rate of AF (15,6 vs. 9,0 %, respectively). However, it has a higher frequency of headache and cough (110). The lower toxicity might be a consequence of a higher selectivity for BTK. Acalabrutinib's IC_{50}s for other kinases like, for example, ITK and EGFR are a 1000-fold higher than ibrutinib's (154).

Zanubrutinib has been approved for the treatment of WM by the FDA and EMA, and for the treatment of MCL and MZL by the FDA only. However, this drug is still under consideration by these agencies for the treatment of CLL. Zanubrutinib showed promising clinical efficacy in phase 1 trials (158, 159). Two phase 3 trials with zanubrutinib treatment in CLL are currently ongoing. One compares it to BR in treatment-naïve CLL (NCT03336333) and the other compares it to ibrutinib in R/R CLL (NCT03734016). Zanubrutinib has a similar selectivity profile compared to ibrutinib, with a higher IC_{50} for only HER2 and JAK3 (154). Since HER2 has been implicated in the development of AF (143), it might not be surprising that zanubrutinib had much lower rates of AF than ibrutinib (2 vs. 15%, respectively) in a phase 3 study in WM (160).

Other covalently-binding BTK-inhibitors, like orelabrutinib (ICP-022), spebrutinib (AVL-292, CC-292), tirabrutinib (ONO/GS-4059) (161), and TG-1701, are still in early clinical development (154, 162). All of them have favorable selectivity profiles, but it is too early to comment on their toxicity and efficacy (154).

1.2.4.2 Non-covalently binding inhibitors of Bruton's tyrosine kinase

Pirtobrutinib (LOXO-305), which is a non-covalently binding and very selective BTK-inhibitor (154), has a positive safety profile. In a clinical study in 323 patients with various B-cell malignancies, the most common adverse events were: fatigue (20%), diarrhea (17%), bruising (13%) and neutropenia (13%). AF occurred in only 2 (1%) patients, who both had a previous history of this condition. Furthermore, after a median follow-up of only

6 months, this phase 1/2 trial showed an ORR of 63%, with 0% complete response (CR), in all 139 evaluable R/R CLL patients and an ORR of 71% in the 24 of them that had BTK^{C481S} (163). Pirtobrutinib even had an impressive 67% ORR (13% CR) in the 17 patients with previously treated RT (162). Phase 3 studies have been initiated, comparing pirtobrutinib to the investigator's choice of either BR or idelalisib and rituximab, in patients who have previously been treated with a BTK-inhibitor (NCT0466038), comparing the combination of pirtobrutinib, venetoclax and rituximab to the combination of venetoclax and rituximab alone in R/R CLL (NCT04965493), and comparing pirtobrutinib to BR in treatment-naïve CLL (NCT05023980).

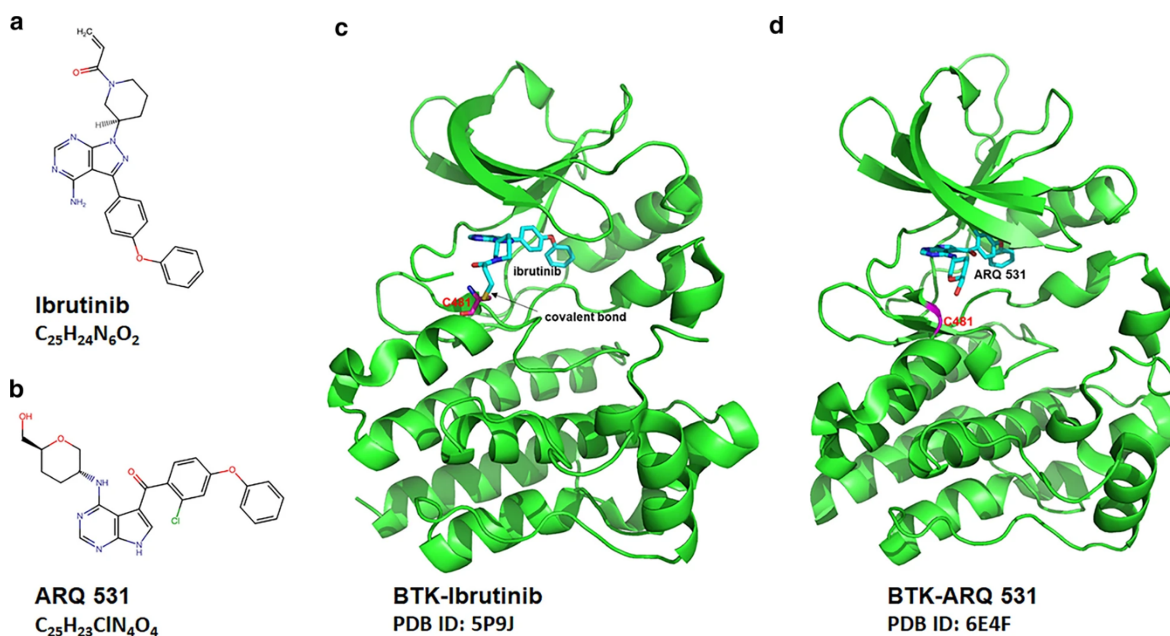

Figure 5 – Chemical structures of ibrutinib and ARQ 531 in relation to BTK. From: Gu, D., Tang, H., Wu, J. et al. Targeting Bruton tyrosine kinase using non-covalent inhibitors in B cell malignancies. *J Hematol Oncol* 14, 40 (2021). This image was redistributed under the Creative Commons Attribution 4.0 International (CC BY 4.0) license. No changes were made.

Nemtabrutinib (ARQ 531, MK-1026) binds to the ATP-binding region on the BTK molecule (**Figure 5**). It showed favorable toxicity (no AF, 10% nausea, 10% diarrhea) and an ORR of 27% in 26 R/R CLL patients in a phase 1 trial (164). A phase 1/2 (NCT03162536) and a phase 2 (NCT04728893) trial are ongoing.

The development of fenebrutinib (GDC-0853), despite showing clinical efficacy in 7 CLL patients (165), and vecabrutinib (SNS-062), despite persistent BTK inhibition and low toxicity in vivo (162) , has been terminated.

1.3 HODGKIN LYMPHOMA

Approximately 5-10% of all newly diagnosed lymphoid malignancies is HL. It usually manifests as a palpable lymphadenopathy, sometimes in combination with constitutional symptoms. The age distribution of newly diagnosed HL cases is remarkably bimodal with a first incidence peak between the age of 20 and 30 years and a second incidence peak around

the age of 50 years or older (166). This disease has two extraordinary histological features: 1) the malignant Hodgkin and Reed-Sternberg (HRS) cells are large, with multiple or bilobed nuclei and prominent nucleoli, which gives them an owl's eye appearance, and 2) malignant cells represent merely 1% of the cells in the tumor tissue and are embedded in an inflammatory infiltrate of immune cells (**Figure 6**). The tumor cells actively shape their microenvironment to receive survival signals and protection from immune surveillance (167).

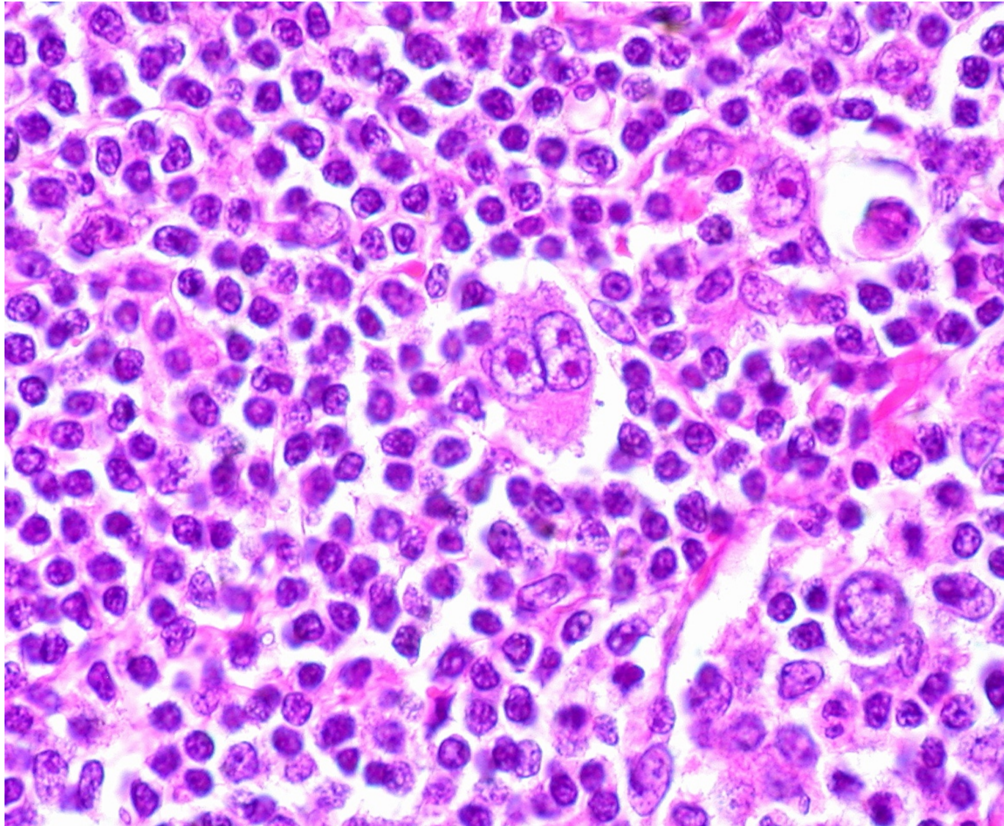

Figure 6 – Histological image of an HRS cell embedded in a lymphocyte-dominated infiltrate. From: Ed Uthman. Hodgkin Disease, Reed-Sternberg Cell. August 2009. Source: flickr.com. This image was redistributed under the Creative Commons Attribution 2.0 Generic (CC BY 2.0) license. No changes were made.

1.3.1 Pathophysiology

The main cause of classical HL (cHL) is unknown. However, up to 30% of the patients have Epstein-Barr virus (EBV) positive HRS cells. In a longitudinal study among 38 555 individuals with EBV serology-confirmed infectious mononucleosis, there was an increased risk of developing EBV-positive HL compared to what would be expected in the normal population (relative risk of 4,0). The median interval between the onset of infectious mononucleosis and the diagnosis of EBV-positive HL was 4,1 years (167). However, the absolute risk of developing HL after infectious mononucleosis is still only 0,1% (168).

This disease develops from 'crippled' pre-apoptotic GC B cells that have gone through the GCR but acquired unfavorable mutations in the *IGHV* genes that made them bind antigen with lower affinity and were therefore given apoptotic signals. This theory is evidenced by

the presence of mutations that insert stop codons in in-frame *IGHV* rearrangements, which leave the Ig dysfunctional (169).

1.3.1.1 Histological subtypes

The common immunohistochemistry findings in all cHL subtypes are HRS cells that are highly positive for CD30, moderately positive for paired box-5 (PAX-5), CD15 and IRF4, and negative for CD45, BOB1 and Oct (170). They generally lack the expression of common B-cell markers and all other lineage markers (171). B lymphocyte-induced maturation protein-1 (BLIMP-1), CD20 and T-cell antigens are, however, expressed in a minority of cases.

1.3.1.1.1 Nodular sclerosis classical Hodgkin lymphoma

Nodular sclerosis (NS) cHL is the most common subtype with a frequency of 75-80%. The microscopic morphology is characterized by nodules of lymphocytes that are separated by bands of dense collagen fibers. Classic 'owl's eyed' cells HRS cells are uncommon in this subtype and the lacunar variant of HRS cells is seen instead. Other cells in the TME are lymphocytes, plasma cells, eosinophils, neutrophils, and macrophages. Interestingly, this subtype is mostly seen in patients under the age of 45 years and marginally more common in women. Favored anatomical sites are the mediastinum and upper thoracic regions, while extranodal sites are only rarely involved (170).

1.3.1.1.2 Mixed cellularity classical Hodgkin lymphoma

About 10-15% of cHL cases is from the mixed cellularity (MC) subtype. It has a rather overwhelming and diffuse infiltrate of immune cells that obscure the nodular morphology. Fine interstitial fibrosis, rather than sclerotic bands, can occur, and classic HRS cells are unmistakably present. Unlike NS cHL, MC cHL is less common in young adults and more common in men. This subtype is often diagnosed at more advanced stages with involvement of lymph nodes, spleen and/or bone marrow (170).

1.3.1.1.3 Lymphocyte-rich classical Hodgkin lymphoma

With a large infiltrate of reactive immune cells in either a diffuse or focal morphological pattern and sometimes at interfollicular sites, lymphocyte-rich (LR) cHL occurs in about 6% of all cHL patients. The TME is obviously predominated by T and B cells, but few eosinophils, neutrophils and plasma cells can also occur. HRS cells are rare, lacunar cells are few and fibrosis can occur in a focal pattern. In this subtype, germinal centers can appear in the nodules. This subtype is less aggressive than NS cHL and often diagnosed at early stages in patients older than 45 years, although extranodal involvement, B symptoms and bulky disease might also be present at diagnosis in some cases. It is rarely located in the mediastinum and rather favors subdiaphragmatic regions.

1.3.1.1.4 Lymphocyte-depleted classical Hodgkin lymphoma

The least common (< 5%) subtype of cHL is lymphocyte-depleted (LD) cHL. Lymphocytes seem to have been replaced by diffuse fibrosis with occasional necrosis. HRS are suddenly the predominant cells in the TME, while non-malignant cells are sparse. It often occurs in elderly patients and seems to be more common in the non-industrialized regions of the world. The typical localization is the abdomen, while involvement of the spleen, liver and bone marrow is not uncommon either. Subsequently, patients with LD cHL usually already have advanced disease at diagnosis. In relation to NS cHL, this subtype has a bad response to treatment (170).

1.3.1.1.5 Nodular lymphocyte predominant Hodgkin lymphoma

In about 5-10% of HL cases, a distinct pattern occurs with a nodular aspect of the lymph node tissue, a network of DCs and many remaining B cells. This subtype is called non-classical or nodular lymphocyte predominant Hodgkin lymphoma (NLPHL) and should be considered a separate disease entity. Unlike HRS cell, the tumor cells in NLPHL, called LP-cells, do express B-cell markers like CD20 and lack expression of CD30 and CD15 (172).

1.3.1.2 Genomic landscape

Genes that encode elements of the NF-κB signaling pathway are recurrently mutated in cHL These include *MAP3K14* and *REL*, and NF-κB inhibitors *NFKBIA*, *NFKBIA* and *TNFAIP3*. JAK-signal transducer and activator of transcription (STAT) pathway is another common target of genetic alterations, including *JAK2*, *SOCS1* and *PTPN1* (170, 173). Genetic aberrations in *CIITA, B2M* led to a reduced expression of MHC class I and II molecules (174, 175). Copy number gains of chromosome 9p24.1 involve *JAK2*, *PDL1* and *PDL2* (176).

1.3.2 Clinical management

1.3.2.1 Diagnosis

An excisional lymph node biopsy and a fluorodeoxyglucose positron emission tomography (FDG-PET)-CT-scan are required to diagnose HL. The common immunohistochemical staining of certain markers in combination with the typical morphology, as described above, will deliver the diagnosis. FDG-PET-CT is necessary for correct staging (177).

1.3.2.2 Staging

In the Cotswolds-modified Ann Arbor classification of HL disease stage, a patient has stage I when a single lymph node region or secondary lymphoid structure is involved, stage II when two or more lymph node regions or secondary lymphoid structures on the same side of the diaphragm are involved, stage III when any number of lymph node regions or secondary lymphoid structures on both sides of the diaphragm are involved, and stage IV when extranodal sites are involved. Annotations include an “X” in case of bulky disease, an “E” in

case of a limited extranodal extension from a contiguous nodal site, and an “A” in case B-symptoms or absent or “B” when B-symptoms are present (178).

Limited- and advanced stage disease are distinguished for the purpose of treatment. Patients with stage I and IIA have limited-stage disease, and patients with IIB, III and IV have advanced-stage disease.

Risk factors for limited-stage cHL are: bulky disease, the involvement of more than 2 lymph node regions and an erythrocyte sedimentation rate of ≥ 50 mm. Risk factors for patients with advanced-stage cHL are: male gender, > 45 years of age, stadium IV, hemoglobin < 105 g/L, serum albumin < 40 g/L, white blood cell count (WBC) > 15 x 10^9/L, and absolute lymphocyte count (ALC) < 0,6 x 10^9/L and/or ALC/WBC < 0,08, according to the international prognostic score (IPS) for HL (179).

1.3.2.3 Primary treatment

Classical HL has a relatively good prognosis. After first-line treatment with chemoradiotherapy, 85-90% of the patients can nowadays be cured (180, 181).

1.3.2.3.1 Limited-stage disease

Patients with stage IA and IIA who lack all risk factors will be treated with 2 cycles of doxorubicin (adriamycin), bleomycin, vinblastine and dacarbazine (ABVD), and 20 Gy per 10 fractions of radiotherapy (181, 182). For patients in this group who are older than 70 years, bleomycin should be omitted from both cycles and 30 Gy per 15 fractions of radiotherapy should be given (183).

Patients with stage IA and IIA who have at least one risk factor and patients with stage IB will be treated with 4 cycles of ABVD and 29,75 Gy per 17 fractions of radiotherapy (182). For patients in this group who are older than 60 years, bleomycin should be omitted from the last 2 cycles (183-185). For those who are older than 70 years, bleomycin should be omitted from all four cycles and 30 Gy per 15 fractions of radiotherapy should be given (183).

1.3.2.3.2 Advanced-stage disease

Young patients (≤ 60 years of age) with advanced-stage cHL, are treated with 2 cycles of escalated bleomycin, etoposide, doxorubicin (adriamycin), cyclophosphamide, vincristine (oncovin), procarbazine and prednisolone (BEACOPPesc). Then a response assessment is done with FDG-PET-CT (interim PET or PET2). PET2-negative patients receive an additional 2 cycles of BEACOPPesc, while PET2-positive patients receive an additional 4 cycles of BEACOPPesc (186). Patients with a positive PET4 have refractory disease.

Young patients who are deemed not to tolerate BEACOPPesc are treated with 2 initial cycles of ABVD instead. PET2-negative patients receive 4 additional cycles of AVD, while PET2-positive patients receive 6 14-day cycles of BEACOPP or 4 cycles of BEACOPPesc

(187, 188). A new interim PET is done when half of the additional cycles has been completed. Patients whose interim PET is still positive at this stage have refractory disease.

Advanced-stage patients between 61 and 70 years of age are treated with 2 cycles of ABVD, followed by 4 cycles of AVD (184). Patient who are older than 70 are treated with 6 cycles of AVD (184, 188).

1.3.2.4 Refractory or relapsed disease

In the rare case of R/R disease, salvage chemotherapy followed by high-dose chemotherapy and autologous hematopoietic cell transplantation (auto-HCT) is the standard treatment (189). In case of R/R disease after auto-HCT, the CD30-targeting antibody-drug conjugate brentuximab vedotin (BV) is added to the salvage regimen. After failure to this line of treatment, BV monotherapy, PD-1 blockade, allo-HCT and experimental therapies are the final treatment options with curative intent (190).

2 LITERATURE REVIEW

A review of the literature that forms the background to the constituent papers that are part of this thesis is presented in this chapter.

2.1 IMMUNE DEFECTS IN CHRONIC LYMPHOCYTIC LEUKEMIA

CLL has a multidimensional TME. The main sites of proliferation for CLL cells are the affected lymph nodes and the spleen (191-193). The bone marrow can serve as a tissue niche where CLL cells receive survival signals and protection from chemotherapy-induced apoptosis (194, 195). Even though enormous numbers of CLL cells can be found in the PB, this compartment appears to play a relatively small role in CLL pathogenesis. CLL cells seem to circulate through the PB and re-enter lymphoid organs in a cyclic fashion (196). CCR7, CXCR4 and CXCR5 are highly expressed on CLL cells and critical for homing to lymphoid organs up a gradient of C-X-C motif chemokine ligand (CXCL)12, CXCL13, and C-C motif chemokine ligand (CCL)19 and CCL21, respectively (196, 197).

2.1.1 Nurse-like cells and mesenchymal stem cells

CLL cells recruit monocytes through the production of CCL3 and CCL4 (198) and re-educate them by secreting HMGB1 and macrophage colony-stimulating factor (M-CSF) (199, 200). These monocytes acquire a tumor-associated macrophage (TAM)-resembling phenotype by upregulating CD68 and CD163 (201), attract CLL cells to lymphoid organs by secreting CXCL12 (also known as SDF-1) and CXCL13 (also known as BCL and BCA-1), and support their survival. Hence, they are called nurse-like cells (NLCs) (202-204). Mesenchymal stem cells (MSCs) in the bone marrow protect CLL cells similar to NLCs (194, 205). They also use CXCL12 as a chemoattractant and give similar survival signals (204, 206). However, upon interaction with CLL cells, MSCs trigger the transcription of *FOS*/*JUN* and *TCL1*, whereas NLCs induce the expression of genes involved in the BCR and NFκB signaling pathways (207, 208).

2.1.2 T cells in the tumor microenvironment

Interestingly, CLL cells upregulate CXCR4 and CXCR5 (receptors for CXCL12 and CXCL13, respectively) and secrete chemokines that attract T cells (CCL3 and CCL4) when the BCR gets triggered (192, 209). This is consistent with the immunological function of the COO of CLL: when a naïve B cell recognizes its antigen, it homes to the lymph node (by expressing CXCR4 and CXCR5) and attracts T cells (by expressing CCL3 and CCL4). Their interaction with T cells will then elicit a comprehensive adaptive immune response in two ways. Activated B cells are stimulated to go through the GCR upon the receival of cytokine support from Th2 cells and recruited T cells can meet their antigen on an APC and get activated themselves.

Indeed, the expression of CCL3 (a ligand of CCR4) has been associated with high infiltration of chronically activated $CD57^+$ T cells in CLL lymph nodes (210). Proliferating CLL cells

express CD38 and colocalize with Th cells (191). CLL cells also attract CCR4-expressing T cells by secreting CCL22 and get anti-apoptotic signals from them through CD40 ligation (211, 212). Furthermore, T cells secrete IL-4 and IFN-γ, which support survival of CLL cells by upregulation of BCL-2 (213, 214). Unsurprisingly, increased levels of CCL3 and CCL4 can be detected in the plasma of CLL patients (198, 207). High CCL3 levels in plasma are associated with shorter time to initial treatment, highlighting the relevance of T cells in the TME for the progression of this disease (215).

2.1.3 Systemic immune defects

Besides manipulating immune cells to create a protective and supportive environment in lymphoid organs, CLL cells also affect systemic immunity (216). Infections with bacterial, viral, and fungal pathogens are more frequent in CLL patients (217-220) and are caused by acquired immunodeficiencies (221). CLL is associated with T-cell lymphocytosis with a relative enlargement of the CTL compartment and a subsequently low CD4/CD8-ratio in the PB (222-224). This CTL-specific expansion seems to follow the CLL clone and consists of dominant clones, which could implicate that they are tumor-reactive (225-227). The fact that different CLL patients with the same stereotyped BCR also share gene restrictions of the TCR in T cells, further supports this hypothesis (228, 229).

2.1.3.1 T cells

Circulating Th cells have impaired cytokine secretion and are functionally suppressive (230). Absolute numbers of all circulating Th subsets (Th1, Th2, Th17 and Treg) are increased (231). CLL patients have a shift toward Th2 polarization, which parallels disease progression (232). The fraction of Tregs is increased in the PB (231, 233). This increment in Tregs correlates with disease progression (234, 235). Th cells are also more shifted toward differentiated $CD45RA^{-}$ central and effector memory phenotypes, which could be explained by chronic antigen stimulation in an inflammatory TME (231, 236, 237).

Cytotoxic T lymphocytes in the blood have a reduced expression of activating TCR components, like the ζ-chain of CD3 and CD28 (238). T cells also have a reduced expression of genes that are involved in cell differentiation and effector processes, like formation of the cytoskeleton, relocation of vesicles and cytotoxicity (239). Especially CTLs cells are hampered in executing their cytotoxic effector function by defects in the immune synapse machinery (240, 241).

T cells are generally suppressed and express pseudo-exhaustion markers like PD-1, 2B4, BLIMP-1 and CD160 (231, 237, 241, 242). CLL cells induce this T-cell disfunction by expressing PD-L1, CD200, B7-H3 and herpesvirus entry mediator (HVEM) on the cell surface (243, 244). Functional T-cell exhaustion is further intensified in affected lymphoid organs through these inhibitory molecules (239, 245). Circulating $PD\text{-}1^{+}$ T cells often co-express other immune checkpoint molecules, like LAG-3, TIM-3 and TIGIT (246, 247). This might explain why PD-1 blockade is not efficacious in CLL (248, 249).

2.1.3.2 Natural killer cells

The NK cell population is expanded in the PB of CLL patients, but the target recognition receptor expression is downregulated (250, 251). How CLL cells suppress NK cells is not yet well understood. Possibly through the expression of HLA-G, a more tolerogenic subtype of MHC class 1, and through the expression of 4-1BB ligand (252, 253). As with T cells, CLL cells can disturb the formation of an immune synapse by NK cells (254). Since patients with a low NK/CLL-cell ratio have a shorter time to first-line treatment, NK cells might contribute to disease progression (255).

2.1.3.3 B cells

Humoral immunity is also compromised in CLL patients. Tumor cells are believed to inhibit antibody production by non-malignant B cells, resulting in clinically relevant hypogammaglobulinemia (230, 256, 257), which affects up to 85% of the patients (258). Ig-substitution reduces the incidence of bacterial infections in CLL (258).

2.1.3.4 Myeloid cells

Myeloid-derived suppressor cells (MDSCs) are generated from immature myeloid cells in various inflammatory conditions and suppress other immune cells through the production of soluble metabolic factors, such as arginase-1, indolamine 2, 3-dioxygenase (IDO), nitric oxide (NO) and reactive oxygen species (ROS), suppressive cytokines TGF-β and IL-10 and the induction of Tregs (259). They have also been found to inhibit T-cell responses and induce Tregs in CLL (260).

2.2 IMMUNE MODULATION BY IBRUTINIB

The expression of BTK and other TEC-family kinases in hematopoietic cells is a likely explanation of ibrutinib's broad effects on innate and adaptive immune cells (261). BTK itself mediates downstream signals from receptors for chemokines, the Fc-region of antibodies, pathogens and antigens (262). Considering its increasing use for the treatment of CLL patients, who have immune dysfunctions that are caused by the disease and previous lines of treatment, it is clinically relevant to gain understanding of the effects that ibrutinib has on the immune system (95). Indeed, immunological alterations are already observable within hours to days after the initiation of ibrutinib treatment (263, 264).

2.2.1 On-target inhibition of humoral immunity

As exemplified by patients with XLA, the inactivation of BTK has a profound effect on B cells and humoral immunity. In CLL patients, absolute numbers of non-malignant B cells remain low after 2 years of ibrutinib treatment. The serum levels of IgG and IgM decrease, while IgA levels surprisingly increase. Furthermore, the patients, who had an increase in serum IgA levels had less infections (265, 266). Another study also reported an increase in IgA after 1,5 years of treatment, while IgG and IgM remained stable (267). Although a seasonal influenza vaccine could partly raise the seroprotection rate in CLL patients treated

with ibrutinib, this drug seems to hamper B-cell responses against new antigens (268, 269). Accordingly, the percentage of non-malignant B cells that have gone through SHM and the *IGH* diversity did not increase during 2 years of ibrutinib treatment in another study (270).

As BTK is not expressed in terminally differentiated plasma cells (271, 272), it is unlikely that ibrutinib has a major effect on the production of antibodies that are specific for antigens that have been encountered earlier in life by LLPCs. However, this has not been studied in depth yet.

2.2.2 Off-target effects on T cells

T cells lack BTK (271), but they do express ITK (273), which is one of ibrutinib's main off-target kinases. ITK facilitates Th2 development (274) and Treg differentiation at the expenses of Th17 differentiation in mice (275). Accordingly, *ex vivo* treatment with ibrutinib impedes Th17 differentiation from naïve $CD4^+$ T cells (276). In primary human T cells, ibrutinib was shown to reduce the production of IL-6, IL-10 and TNF-α (128).

Absolute T-cell numbers, which are elevated in CLL patients, normalize after 6 months to 1 year of ibrutinib treatment (263, 266, 267, 277-281). However, one study described an increase of both Th cells and CTLs during the first 6 months of ibrutinib treatment (282). The *ex vivo* proliferation and function of T cells seems to improve during ibrutinib treatment as well (280).

The TCR repertoire diversity might increase after 1 year of ibrutinib treatment, potentially reflecting the disappearance of dominant tumor-reactive clones (277). However, ibrutinib could also increase clonality at the time of response in treatment-naïve patients (283). Ibrutinib seems to further enhance the memory differentiation of T-cell populations that is already associated with CLL (282).

Indeed, as suggested by data from knock-out mice, ibrutinib-treated CLL patients have a helper T-cell population that is Th1 skewed, because Th2-differentiation is inhibited by the inactivation of ITK (284). The consistently reported reduction of Th2-type cytokines in plasma of ibrutinib-treated patients confirms these data (263, 277, 282, 284, 285). This shift could potentially favor anti-viral and antitumor T-cell immunity. A recent study in a cohort of 71 CLL patients, who were treated with ibrutinib and rituximab for 6 months and continued on ibrutinib maintenance, reported an increase in the frequency of Th1 cells and a decrease in the frequency of Th2 cells after 1 year (286).

A reduction in Tregs has also been reported during the first year of ibrutinib treatment (267, 280-282, 285). Parallel to preclinical data, Th17 cell numbers and associated cytokines (IL-17, IL-21, and IL-23) are reduced in PB from ibrutinib-treated CLL patients (263, 277, 285, 287). Ibrutinib has also been shown to reduce the levels of many other circulating chemoattractants and inflammatory cytokines already within hours after treatment initiation in CLL patients (263, 264).

Ibrutinib might even reverse T-cell dysfunction in CLL, as it has been shown to reduce the expression of molecules involved in chronic activation, like CD38, CD39 and HLA-DR, and immune suppression, like CTLA-4 and PD-1 in T cells, and B- and T-lymphocyte attenuator (BTLA), CD200, IL-10 and PD-L1 in CLL cells (263, 280-282, 287-289). Because of these immunomodulatory effects, the potential synergy of combing ibrutinib with immunotherapies that boost T-cell responses is being investigated broadly for the treatment of CLL and beyond (290).

2.2.3 Influences on natural killer cells

There is only little data available on ibrutinib's effects on NK cells. BTK is required for NK-cell activation in mice (291, 292), and ITK enhances FcR-initiated cytotoxicity in human NK cells (293). Accordingly, ibrutinib impairs ADCC by NK cells (294). Stable absolute numbers of NK cells throughout the first year of ibrutinib treatment have been reported (267, 280). The $CD56^{bright}$ NK cells might, however, decrease (280). The potential effects of ibrutinib on NK cells deserve more thorough investigation.

2.2.4 On- and off-targets in myeloid cells

TEC family kinases are also expressed in myeloid cells, where they are involved in toll-like receptor (TLR)- and FcR-signaling (261). Inhibition of BTK in primary monocytes using ibrutinib results in reduced production of IL-1β, IL-6 and TNF-α upon FcR stimulation, though it does not affect phagocytosis (150, 295, 296). Conversely, ibrutinib might also increase the numbers and function of monocytes (280, 287, 297). Interestingly, ibrutinib treatment dose-dependently reduced the expression of CXCL12, CXCL13, CCL19 and vascular endothelial growth factor (VEGF) in macrophages. Thus, ibrutinib might even reduce the attraction and protection of CLL cells by NLC (298).

Furthermore, MDSCs, which are potent suppressors of antitumor immunity in a wide array of cancer subtypes including CLL (260), express BTK and ibrutinib treatment impairs their expansion and function (299). In ibrutinib-treated CLL patients, the absolute number of MDSC decreased during the first year and remained low after 4 years of treatment (280, 281).

Dendritic cells from patients with XLA and ibrutinib-treated primary DCs insufficiently produce inflammatory cytokines after TLR-stimulation (300, 301). Likewise, ibrutinib inhibits TLR-mediated DC maturation and activity (302). It also seems to inhibit two FcR-mediated effector functions: antibody-dependent cellular phagocytosis by macrophages (303-306), and IgE-mediated mast cell activation (307, 308)

2.2.5 Consequences for vaccine efficacy

Because of the inhibition of B cells and modulation of APCs and T cells by ibrutinib, the efficacy of vaccines, which relies on an interplay between these cells, is impacted as well. This puts CLL patients, who already suffer from many immune defects, particularly at risk.

Influenza and pneumococcal vaccines are less efficacious in CLL, but the best possible responses are achieved when tumor burden is still low and before the initiation of CLL treatment (309-311). Ibrutinib hampers seroconversion after vaccination against influenza, pneumococcal bacteria, hepatitis B virus and varicella zoster virus (268, 269, 312, 313). Accordingly, recent messenger RNA vaccines against coronavirus disease 2019 (COVID-19) have reduced antibody-mediated responses in patients with CLL, especially during treatment in general and during treatment with ibrutinib in particular (314-324). T-cell responses to these vaccines are also reduced in patients with CLL (316, 319, 324).

2.3 INTERRUPTING TREATMENT WITH TYROSINE KINASE INHIBITORS

We gain ever more long-term experience with the continuous administration of ibrutinib since its approval in 2013. An increasing number of patients have now been taking ibrutinib for 5 years or more within standard care. The continuous treatment with a drug that can cause AF, hemorrhagic complications and infections in a predominantly elderly population can be challenging. Moreover, the poor outcome of patients who relapse because of resistance is still a big concern. After 4 years of ibrutinib treatment, 40% of the patients will have stopped taking it because of resistance or intolerance (325). Besides, the costs of this drug are enormous, as it is prescribed daily until further notice (94).

2.3.1 Interrupting ibrutinib treatment

Temporary dose interruptions or complete discontinuation of ibrutinib are not uncommon and are sometimes necessary to mitigate serious adverse events or to allow for invasive elective procedures implying risk for bleeding complications.

One study that disregarded the reason for discontinuation reported a worse OS in all patients that had an interruption of more than 14 consecutive days (including permanent cessation) (326). A retrospective analysis from a clinical trial indicated that a dose intensity of 95% or less is associated with worse PFS but not OS and that patients with an interruption of ≥ 8 consecutive days had an inferior PFS (327). However, these data were based on a median of 9 months of ibrutinib treatment, when many patients are yet to acquire a clinical response and many more are still deepening their remission.

Another study, which had a median follow-up of 5 years, showed no inferior outcomes for patients who interrupted ibrutinib for ≥ 8 consecutive days (328). In a study with a median follow up of 2 years, 44% of patients were reported to have had temporary interruptions, with a median time to the first interruption of 19 months, and interruptions were linked to inferior event-free survival (EFS) but not OS (329).

Interruptions that last a week or a month can cause B symptoms that are sometimes accompanied by lymphadenopathy and/or lymphocytosis (disease flare) in 25% of the cases, though resuming ibrutinib dismisses these symptoms (330). Although rare, rapid disease

progression (rebound) can occur following ibrutinib discontinuation because of non-tolerability (331).

Data on the impact of interrupting ibrutinib in selected CLL patients that have achieved and are maintaining an objective response are currently lacking. Ibrutinib discontinuation, other than temporary interruptions to treat or prevent complications, during clinical response have not yet been reported in MCL or WM patients either.

2.3.2 Stopping tyrosine kinase inhibitors in chronic myeloid leukemia

On the other hand, strategic discontinuation of tyrosine kinase inhibitors (TKIs) has been investigated in chronic myeloid leukemia (CML). However, the data from these studies must be interpreted with great caution in the context of ibrutinib treatment in CLL.

TKIs that target the Philadelphia chromosome t(9;22)(q34;q11) gene-fusion product breakpoint cluster region-tyrosine-protein kinase ABL1 (BCR-ABL) are the backbone of CML therapy. Imatinib was the first of this TKI-family. Like ibrutinib, imatinib induces clinical responses in most patients, but very deep responses are rare and acquired resistance is common (332).

The intermittent dosing of imatinib (1-month on and 1-month off) in patients aged ≥ 65 years, who achieved a complete cytogenetic response after 2 years of continuous treatment, was associated with loss of response, but all patients could regain complete cytogenetic responses and no progression to blastic phase or leukemia-related deaths were reported (333). Similarly, a study of intermittent imatinib dosing (3-weeks on and 1-week off) in pediatric CML reported loss of response in 53% of the patients after a median of 5.3 years, but all patients regained response after reinitiating treatment and were still alive at the end of follow-up (at a median of over 12.6 years post diagnosis) (334).

Permanent discontinuation, or the so-called treatment-free remission, after reaching a deep molecular response with first- and second generation TKI is emerging as a safe policy in the treatment of CML (335).

2.4 IMMUNE MODULATION IN HODGKIN LYMPHOMA

In HL, only approximately 1% of the cells in the affected lymph nodes are tumor cells, the rest of the TME being a mix of T cells, NK cells, B cells, plasma cells, macrophages, neutrophils, eosinophils and mast cells and other stromal cells like fibroblasts and endothelial cells. These TME cells may be remnants of the original tissue architecture and are probably recruited by the malignant cells to which they provide growth and survival signals (336).

2.4.1 Immune cells in the tumor microenvironment

T cells are the most common TME cells in HL. HRS cells secrete IL-4, IL-5, IL-10, IL-13, thymus, and activation-regulated chemokine (TARC) and other CCR4 ligands, to attract Th2 and Tregs (337-343). CTL are less frequent in the TME and functionally suppressed by IL-10

and TGF-β, which are secreted by HRS, Th2 and Tregs (344, 345). Paradoxically, a high infiltration of CTL and a low infiltration of Tregs have consistently been associated with poor prognosis (174, 346).

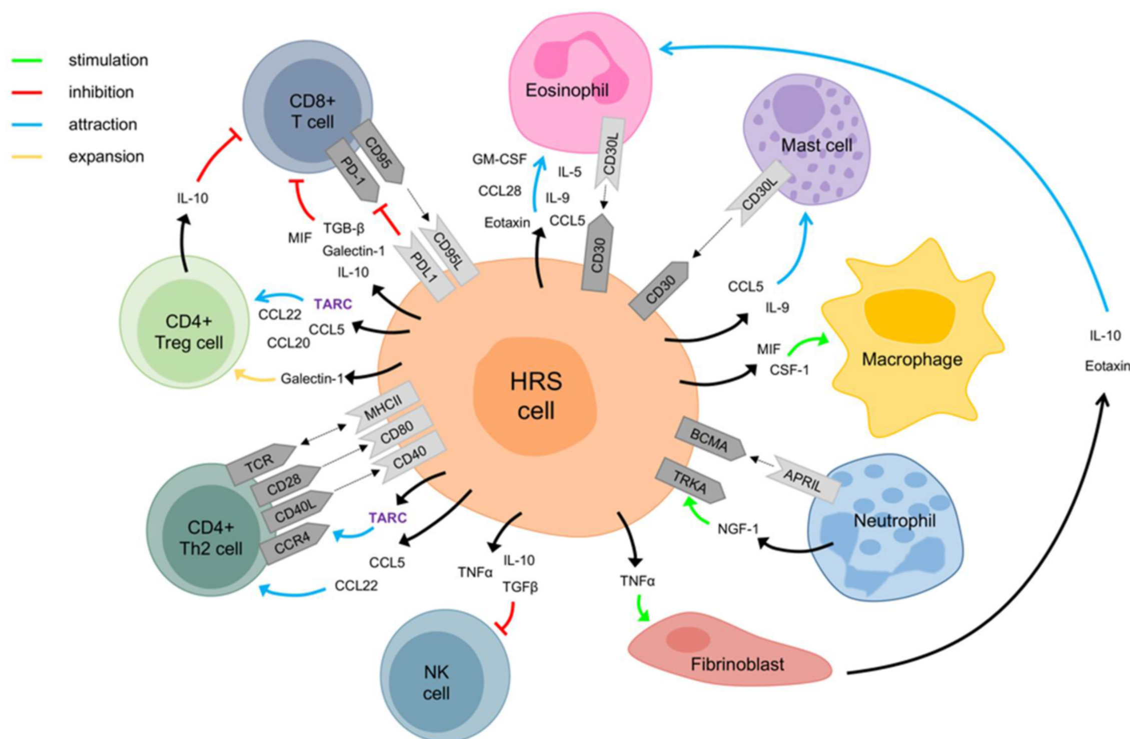

Figure 7 – Cellular interactions in the tumor microenvironment of cHL. From: Zijtregtop EAM, van der Strate I, Beishuizen A, Zwaan CM, Scheijde-Vermeulen MA, Brandsma AM, Meyer-Wentrup F. Biology and Clinical Applicability of Plasma Thymus and Activation-Regulated Chemokine (TARC) in Classical Hodgkin Lymphoma. *Cancers*. 2021; 13(4):884. This image was redistributed under the Creative Commons Attribution 4.0 International (CC BY 4.0) license. No changes were made.

HRS cells also produce M-CSF and Fractalkine to recruit macrophages (347-349). Tumor-associated macrophages are associated with treatment failure and inferior OS (350, 351). HRS cells also produce IL-13 and fibroblast growth factor (FGF) to attract fibroblasts, which is most pronounced in the NS cHL subtype (352, 353). In turn, fibroblasts help to recruit Th2 cells and eosinophils by secreting CCL11 (also known as eotaxin) (354). Eosinophils are also attracted by HRS cell-secreted IL-5, CCL5 and CCL28 (355-357). Mast cells are also recruited by CCL5 (343). Neutrophils are drawn into the TME by HRS cell-secreted and macrophage-secreted IL-8 (**Figure 7**) (356). B cells are present in the TME of HL as well and might be remainders of the original lymph node tissue (358).

2.4.2 Local expression of programmed death ligands

HRS cells express PD-L1 (359). Unlike many other cancer types, HRS cells do so because of a genetic predisposition. Most untreated HL patients have a genetic aberration in the loci for *JAK2*, *PD-L1* and *PD-L2* on 9p24.1, which leads to constitutive expression of PD-L1. This genetic alteration is associated with advanced-stage disease and a shorter PFS (360). PD-L1 is also being expressed by TAMs, which might even be the major source of PD-L1 in the

TME of HL (361). This could be the reason why patients with R/R HL respond exceptionally well to treatment with PD-1 blockade (362).

2.4.3 Systemic effects

The cellular content of the HL TME and the interaction between these cells has been investigated extensively. However, the immune cell profiles in the PB of HL patients are understudied.

Elevated serum levels of TARC and CD163 can be used as predictive biomarkers for response to treatment (363-365). A high (≥ 1.1) lymphocyte/monocyte-ratio in the total blood count at diagnosis is associated with positive clinical outcomes (366). HL patients have increased frequencies of circulating Tregs (367). PB T cells from HL patients have a reduced expression of the traditional Th1-cyokines IL-2, TNF-α and IFN-γ after *ex-vivo* stimulation (368). Furthermore, the genotype of circulating Th1 cells in HL is characterized by impaired proliferation and induced immunosuppression (369). Compared to healthy donors, HL patients have a bigger fraction of circulating PD-1^{+} T cells (359).

Recently, it has been reported that the frequencies of T cells, Th cells and specifically Tregs are decreased in cHL (370). Furthermore, T_{EM} cells are increased in treatment-naïve cHL patients and PD-1 blockade redressed T-cell exhaustion (370, 371).

3 RESEARCH AIMS

3.1 PAPER I

Ibrutinib's On- and Off-target Effects on Plasma Biomarkers and Immune Cells in CLL

- To gain further insights into the on- and off-target effects of the long-term treatment with ibrutinib that mediate its antitumor activity and cause adverse events that can force treatment discontinuation.
- To define the immunological effects of long-term ibrutinib treatment in CLL.

3.2 PAPER II

Intermittent Ibrutinib Treatment in Responding CLL Patients

Primary research aims:

- To evaluate the safety and feasibility of intermittent ibrutinib treatment in CLL patients who are in a sustained remission on ibrutinib.
- To assess the time to treatment failure (non-tolerability, non-responsiveness, or rapid rebound following an interuption) of intermittent ibrutinib treatment in these patients.

Secondary research aims:

- To determine the ORR at each cycle, time to an objective response at each cycle, duration of each interruption, total number of interruptions until treatment failure, cumulative dose of ibrutinib received until treatment failure, and OS, within this study.
- To delineate the immunological effects of discontinuing ibrutinib in CLL patients who are in a sustained remission on ibrutinib.

3.3 PAPER III

Circulating Lymphocytes in Classical Hodgkin Lymphoma

- To establish the disease-related alterations in the quantity and functional phenotype of circulating lymphocytes, in relation to their frequency in affected lymph nodes, the inflammatory state, and tumor burden, in previously untreated cHL patients.
- To elucidate the effects of standard first-line treatment on the quantity and functional phenotype of circulating lymphocytes in cHL patients.

4 MATERIALS AND METHODS

4.1 PATIENTS AND HEALTHY DONORS

To answer questions and test hypotheses that arise in the clinic, material from the applicable patient populations is indispensable. We further rely on the altruism of patients, in being willing to participate in clinical trials, to investigate the safety and efficacy of new drugs and alternative treatment strategies, while the benefit for the individual patients is often expected but essentially unconfirmed.

Thirteen patients with CLL who began ibrutinib treatment at the Hematology Department at Karolinska University Hospital were included and followed longitudinally in the study that was published in **Paper I**. Eleven of these patients were followed for up to 5 years as long as they were receiving ibrutinib. PB samples were taken from these patients at baseline and at the following intervals from the start of treatment: 4 weeks, 10 weeks, 16 weeks, 22 weeks, 8 months, 1 year, 2 years, 3 years, 4 years, and 5 years. The other 2 patients started a combination treatment in the context of another study after 1 month of ibrutinib monotherapy. Cryopreserved plasma samples from before the initiation of treatment and 9 hours, 2 days, and 4 weeks thereafter, which have been used in a previous study (264), were used from these 2 patients. Relevant clinical information was acquired from the patients' medical records. As controls in this study, we used cryopreserved plasma from 8 patients with BTK mutation-confirmed XLA and PB samples from 9 age- and sex-matched healthy donors.

Twenty-two patients with CLL who were maintaining a stable partial response (PR) or CR on ≥ 6 months of ibrutinib treatment were enrolled in the clinical trial reported in **Paper II**. Patients were treated at 3 different centers, including the Karolinska University Hospital. Ibrutinib was initially started in R/R cases and treatment-naïve cases with del(17p)/*TP53*-mutation if treatment was indicated according to the international workshop on CLL (iwCLL) guidelines (372). Relevant clinical information was acquired from the patients' case report forms. PB samples were obtained before the first interruption and 2 weeks, 4 weeks, 3 months, 6 months and 1 year thereafter, as long as the interruption lasted.

Forty-eight consecutive patients with treatment-naïve cHL who were diagnosed at the Hematology Department at Karolinska University Hospital were included in the study that was described in **Paper III**. Relevant clinical information was acquired from the patients' medical records. PB samples were obtained before the start of standard first-line treatment, right after the end of treatment and 6 months after the end of treatment. Flow-cytometry data from disintegrated excisional lymph node biopsies, which is routinely generated for diagnostic purposes, were obtained from the Department of Clinical Pathology and Cancer Diagnostics at Karolinska University Hospital. To identify disease-related alterations in these cHL patients, we used PB samples from 20 age- and sex-matched healthy donors as controls.

4.1.1 Handling of peripheral blood samples

Blood was drawn from the individuals in our studies by venipuncture and collected in sodium-heparin coated tubes. Blood plasma was separated by centrifugation and cryopreserved undiluted at -80°C. Some measurements were directly done in heparinized whole blood. Otherwise, peripheral blood mononuclear cells (PBMCs) were isolated from the blood by density gradient centrifugation using a Ficoll-Hypaque gradient and washed in phosphate-buffered saline (PBS). Isolated PBMCs were either analyzed freshly or cryopreserved in fetal bovine serum (FBS) containing 10% dimethyl sulfoxide (DMSO) at -196°C (liquid nitrogen).

4.2 PROXIMITY EXTENSION ASSAY

In **Paper I**, relative protein concentrations were measured in plasma samples using the proximity extension assay (PEA). This technique allows for the parallel measurement of up to 96 proteins in as little as 1 μL of sample. It is based on monoclonal antibodies, which recognize the target proteins, that are conjugated to unique complementary oligonucleotides. Only if pairs of monoclonal antibodies bind the same target protein, the bound complementary oligonucleotides will be in close enough proximity to hybridize. By adding DNA polymerase, the hybridized oligonucleotides will be extended to an amplicon. The 96 proteins are read out simultaneously by quantitative polymerase chain reaction (qPCR), in which the number of cycles is associated with the concentration in the sample. Software is used to translate this into relative concentrations (373).

4.3 FLOW CYTOMETRY

To measure the protein expression on the surface membrane of living cells and in the cytoplasm of fixed cells, we used flow cytometry in **Paper I, II** and **III**. To use this technique, one must stain the proteins with specific monoclonal antibodies that are conjugated to a known fluorochrome.

After staining, a suspension of single cells can be run through the flow cytometer. Fluidics will take the cells through a 'flow cell' one by one, where they will pass through several laser beams that have a specific wavelength, which will excite the fluorochromes. The wavelength by which the fluorochromes get excited dictates the wavelength of the fluorescent light they emit. Excitation emission spectra are known characteristics for every fluorochrome. The emitted fluorescence passes through filters and their intensity is measured in detectors.

If panels are designed to minimize the overlap of emitted signals, one can determine the expression of multiple proteins on a single-cell basis by the intensity of the emitted fluorescence that corresponds to the given fluorochrome that the proteins of interest were labelled with. The combination of proteins that are expressed on the same cell indicates the cell type. Across all the constituent papers of this thesis, the main cell types were consistently defined as described by Maecker et al. (374) and listed in **Table 2**.

Cell type	Immunophenotype
B cells	$CD3^{-}CD19^{+}$
NK cells	$CD3^{-}CD56^{+}$
CD56^{bright} NK cells	$CD3^{-}CD56^{bright}$
CD56^{dim} NK cells	$CD3^{-}CD56^{bright}$
T cells	$CD3^{+}$
Naïve T cells *	$CD3^{+}CCR7^{+}CD45RA^{+}$
Central memory T cells *	$CD3^{+}CCR7^{+}CD45RA^{-}$
Effector memory T cells *	$CD3^{+}CCR7^{-}CD45RA^{-}$
Effector memory T cells re-expressing CD45RA *	$CD3^{+}CCR7^{-}CD45RA^{+}$
T helper cells	$CD3^{+}CD4^{+}$
T helper 1 cells	$CD3^{+}CD4^{+}CXCR3^{+}CCR6^{-}$
T helper 2 cells	$CD3^{+}CD4^{+}CXCR3^{-}CCR6^{-}$
Regulatory T cells	$CD3^{+}CD4^{+}CCR4^{+}CD25^{+}CD127^{low}$
T helper 17 cells	$CD3^{+}CD4^{+}CXCR3^{-}CCR6^{+}$
Cytotoxic T lymphocytes	$CD3^{+}CD8^{+}$

Table 2 – Definition of common lymphocyte subset by their immunophenotype. * can occur within both the $CD4^{+}$ and $CD8^{+}$ T cell populations.

4.4 AN ATYPICAL CLINICAL TRIAL

To evaluate the safety and feasibility of intermittent ibrutinib treatment in CLL patients who are in a sustained remission on ibrutinib, we conducted a phase 1b/2 clinical trial of which **Paper II** is an early report. This trial had a single-arm, open-label, and multi-center design. Patients who had achieved a stable clinical PR according to iwCLL criteria (372) or better after at least 6 months of ibrutinib were eligible.

Ibrutinib was interrupted at inclusion. As soon as early signs of progressive disease (PD) occurred, defined as any of the following: new lymphadenopathy ≥ 2 cm, > 50% growth of existing lymphadenopathy, > 50% increase in ALC, ALC > 30 x 10^9/L, ibrutinib treatment was resumed. It was interrupted again when a new stable PR was reached. Such cycles could be repeated indefinitely until permanent discontinuation would be forced by non-tolerability or non-responsiveness. Patients who would suffer from rapid rebound after stopping ibrutinib, would receive continuous ibrutinib or alternative therapy outside this study.

Phase 1 trials are usually conducted to test new drugs in a limited number of patients to test their safety and identify common adverse events. Phase 2 trials then follow with a larger number of patients to assess efficacy. In the case of this study, however, we tested an alternative dosing strategy of an existing drug in patients that are already taking it, meaning that our experimental intervention was to stop the drug at a strategic point. This creates an apparent paradox, as one could argue that disease progression is an adverse event of our experimental intervention and that the rates of adverse events and resistance indicate its efficacy.

4.5 ENZYME-LINKED IMMUNOSORBENT ASSAY

Another way to measure protein concentration is the classic enzyme-linked immunosorbent assay (ELISA), which was used to measure TARC in **Paper III**. Like PEA, ELISA utilizes the antigen recognition of monoclonal antibodies to bind the protein of interest. But, unlike PEA, one can measure truly absolute concentrations of the protein with this technique.

ELISA is usually done in a 96-well plate and can be executed in various ways (e.g., direct, indirect, sandwich, competitive). In our study, we used a sandwich ELISA, which works as follows. Each well is first coated and incubated with capture antibodies that recognize the protein of interest. Then, the plate is blocked to reduce unspecific binding and thereby reduce the signal-to-noise ratio. Then, the sample is added. The protein that is present in the sample is then sandwiched by the capture antibody on the surface of the wells and the soluble biotinylated detection antibody that is added. In the next step, streptavidin-bound horseradish peroxidase (HRP) is added to bind the biotin on the detection antibody. Then a substrate to HRP is added to start the enzymatic reaction that creates chemiluminescence. This reaction can be terminated before read-out. The intensity of chemiluminescence is detected per well using a chemiluminescence plate reader set to a specific wavelength. The sample concentrations are extrapolated from a curve of standards of known concentration. Correct incubation and washing between the steps are vital for assay performance.

4.6 ETHICAL CONSIDERATIONS

The patients in these studies got standard treatment for their diseases or experimental treatment in a clinical trial. The use of tissue or blood from patients and even the use of clinical information about these patients raises ethical questions. Obviously, applying an experimental treatment strategy on patients does this too. Here follows a reflection on the ethical questions that arose during the studies that have been described in this thesis.

4.6.1 Drawing extra blood samples for research purposes

All the studies in this thesis involved using PB samples from patients and/or healthy donors. The fact that patients gave extra blood for our studies could have been a burden. However, we attempted to live up to the principle of nonmaleficence ("First, do no harm"). In these studies, it was impossible to do no harm at all. Yet we were obliged to minimalize the potential damages, so that they did not outweigh the potential usefulness of the studies.

As much as possible, extra blood samples for research purposes were taken at time points when patients anyway came to the hospital for a doctor's visit for normal follow-up or check-up. This meant that patients usually did not need to come to the hospital to undergo venipuncture for our research on extra occasions. In this way, we tried to minimize the harm we did, even if the additional sampling per se could have caused additional distress. One could argue that it was a burden for patients to let more blood be drawn than clinically necessary. However, the amount of extra blood that was drawn is small enough (max. 40 ml) to not significantly damage the patient.

4.6.2 To use individual clinical information

Results were frequently related to clinical parameters to get more information about which subgroups of patients reacted to the drugs in a certain way. This caused ethical considerations regarding the patients' privacy.

The doctors and nurses that collaborated with us in these studies received informed consent from the patients and sent deidentified information to us about gender, age, diagnostic subtype, other clinical parameters, and laboratory results. All the patients that were included in these studies gave informed consent for the use of their clinical information for research purposes as well.

4.6.3 Informed consent in observational studies

Based on the principle of autonomy, it is only ethical to use material or information from patients if they have given informed consent. An ethically acceptable informed consent should be based on information that is understandable and complete, and a decision that is made voluntarily and independently. Additionally, the research subjects should have the possibility to withdraw their consent at any time without providing a reason for it.

One could argue that it was challenging for the patients that are included in these studies to take a voluntary and independent decision. It was often the treating physician who informed patients about the study and received consent. A patient is always dependent on the physician and might therefore be inclined to agree on participating in research studies. Furthermore, there was often a lack of time and patients might have worried about their diagnosis and prognosis, which could have made it difficult for them to decide independently, consciously and without pressure.

4.6.4 Ethical considerations of an experimental treatment schedule

In **paper II**, we described an alternative treatment policy that is being tested. Based on the literature, we hypothesized that equal disease control is possible with our experimental treatment policy, while the risk of adverse events and resistance to treatment as well as the costs would be reduced. However, we simply did not know at the start of the study if the intermittent dosing of ibrutinib would be safe and non-inferiorly efficacious compared to the continuous dosing of the drug.

The potential harm to the individual patients was therefore bigger in this study than it was in the observational studies. On the other hand, the potential benefits for the patients in this study and future patients were also greater. When the stakes are this high, an ethically acceptable informed consent is even more important. Therefore, a long time was taken to thoroughly inform the patients and given to the patients to contemplate and make a voluntary and independent decision.

5 RESULTS AND DISCUSSION

5.1 PAPER I

Ibrutinib's On- and Off-target Effects on Plasma Biomarkers and Immune Cells in CLL

To advance our knowledge about the on- and off target effects of ibrutinib, we performed PEA using 3 different panels to measure 265 unique plasma biomarkers, and flow cytometry to investigate immune cells in a cohort of 13 CLL patients (11 of which were follow beyond the time point of 1 month) during up to 5 years of ibrutinib treatment. These biomarkers were also measured in 8 patients with XLA, which served as a negative control for alterations that might depend on BTK or the presence of mature B cells, and in 9 healthy donors, which served as a negative control for alterations that might be disease related. Previously published RNA sequencing data was used as a reference for protein expression in normal immune cells (264, 375).

In the patients with CLL, 32% (n=86) of the measured proteins was altered at some point during treatment, mostly decreasing. Of these, 58 (67%) were classified as CLL-associated because they significantly differed between healthy donors and CLL patients at baseline. The majority (91%) of these 58 biomarkers were elevated in CLL and declined during ibrutinib (**Figure 8**).

On the other hand, we classified 24 (28%) of the 86 altered biomarkers as "CLL non-associated" because these proteins had similar levels in healthy donors and CLL patients at baseline. Furthermore, almost all these biomarkers had equal levels in plasma from CLL patients at baseline and XLA patients, which implies that the production of these

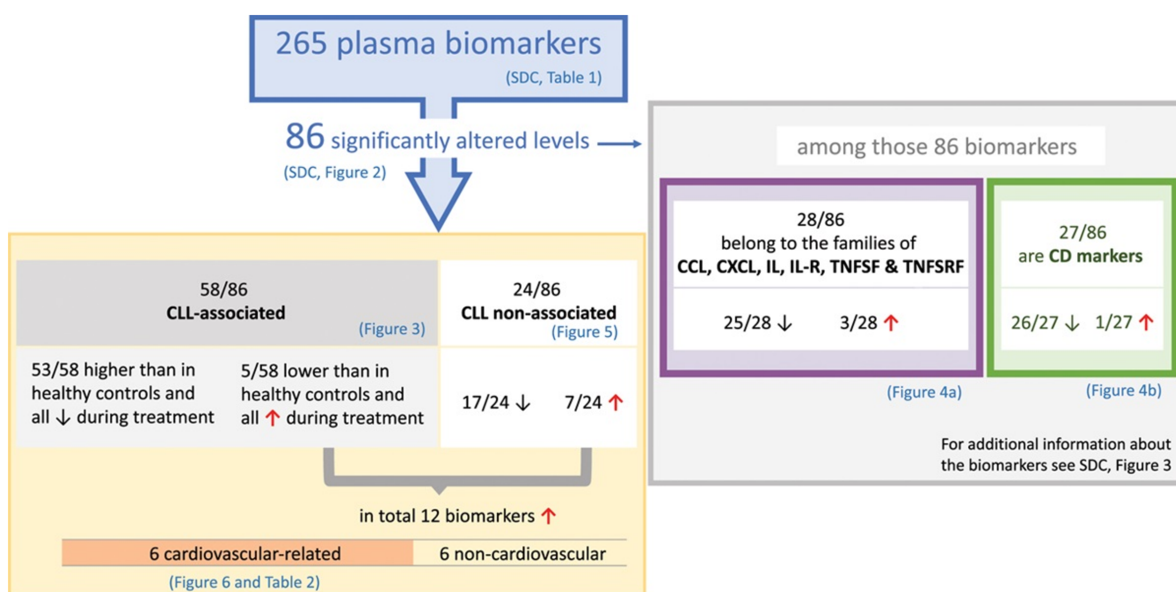

Figure 8 – Schematic overview of the results of the analyzed plasma biomarkers. From: Mulder TA, Peña-Pérez L, Berglöf A, Meinke S, Estupiñán HY, Heimersson K, Zain R, Månsson R, Smith CIE, Palma M. Ibrutinib Has Time-dependent On- and Off-target Effects on Plasma Biomarkers and Immune Cells in Chronic Lymphocytic Leukemia. *HemaSphere*. 2021 Apr 26;5(5):e564. This image was redistributed under the Attribution-NonCommercial-ShareAlike 4.0 International (CC BY-NC-SA 4.0) license. No changes were made.

biomarkers did not rely on BTK, but that it was rather a consequence of off-target binding of ibrutinib.

We found that a total of 12 (14%) of the 86 biomarkers that changed, increased during ibrutinib treatment (**Figure 8**). Therefore, we suspected that these could be involved in the development of any of ibrutinib's adverse events. As it turned out, half of these 12 biomarkers have been linked to AF (or cardiovascular diseases in general) in previous studies by others. These are: amphiregulin (AREG), CXCL12, epidermal growth factor (EGF), plexin-A4 (PLXNA4), TNFSF13 and wingless-type (Wnt) inhibitory factor 1 (WIF-1). Of these, only CXCL12 and WIF-1, whose levels normalized during treatment, could be classified as CLL-associated in our study, making them unlikely mediators of cardiovascular adverse events. Apart from CXCL12, these biomarkers are normally not produced by non-malignant B cells or CLL cells according to reference RNA sequencing data (264, 375). Therefore, these biomarkers could be involved in ibrutinib-induced AF.

No statistically significant differences were found in the expression of the 265 biomarkers between XLA patients and healthy donors, suggesting that non-malignant B cells are not major producers of any of them and that they are all largely BTK-independent.

When we looked at different immune cells during up to 5 years of ibrutinib treatment, we found that all T and NK cells decreased, paralleling a declining amount of CLL cells. Even though ibrutinib can reduce Th2 differentiation by inhibiting ITK (284), we found a strong reduction in Th1 cells, while Th2 cells remained unchanged. This essentially reduced the Th1/Th2 ratio after 1 year. All typical Th-cytokines remained relatively stable during treatment. The sharp reduction in Th1 cells, which aid cellular antitumor immunity, is most likely a consequence of the disappearing CLL cells. Although not evident from our data, Th2 differentiation could still be hampered by ibrutinib's ITK-blockade.

We also found that the numbers of Th17 cells and Tregs dropped during treatment. After 1 year of ibrutinib, the numbers of CTLA-4$^+$ T cells, PD-1$^+$ T cells and CD4$^+$ T_{EM}, CD8$^+$ T_{EM} and CD8$^+$ T_{EMRA} had all decreased. This suggests that successful treatment with ibrutinib (i.e., a disappearing tumor burden) reduces the amount of exhausted and terminally differentiated T cells.

Th1 cells and CTLs are abundant in CLL, and this excess is most likely caused by tumor-specific clones. Furthermore, immunosuppressive cells are known to be recruited by CLL cells to counteract these tumor-specific CTLs and NK cells. These facts raise the question if all these cells simply subside because of the declining tumor load or because of an ibrutinib-specific effect.

5.2 PAPER II

Intermittent Ibrutinib Treatment in Responding CLL Patients

A total of 22 patients were enrolled at three sites in an early-phase clinical trial that we conducted to investigate the safety and feasibility of intermittent ibrutinib treatment in patients that have a sustained response on this drug. The median age was 73,5 years, del(17p)/*TP53*-mutation occurred in 41% of the patients, and 36% of them were on ibrutinib as a first-line treatment. Half of the patients had PR and the other half CR at the start of this study.

Comparing the last year prior to the first interruption and the first year on the study, we observed that fragile nails and skin eruptions on fingertips were present in 10 patients before they entered the study, which disappeared after one year on the study. Bruising and bleeding was present in 12 out of 13 evaluable subjects before inclusion and disappeared as well. Two patients had developed AF during the last year before inclusion, but no such cases arose during the first year of this study. The rate of grade ≥ 3 infection was lower in patients during the first year within this study than during the preceding year.

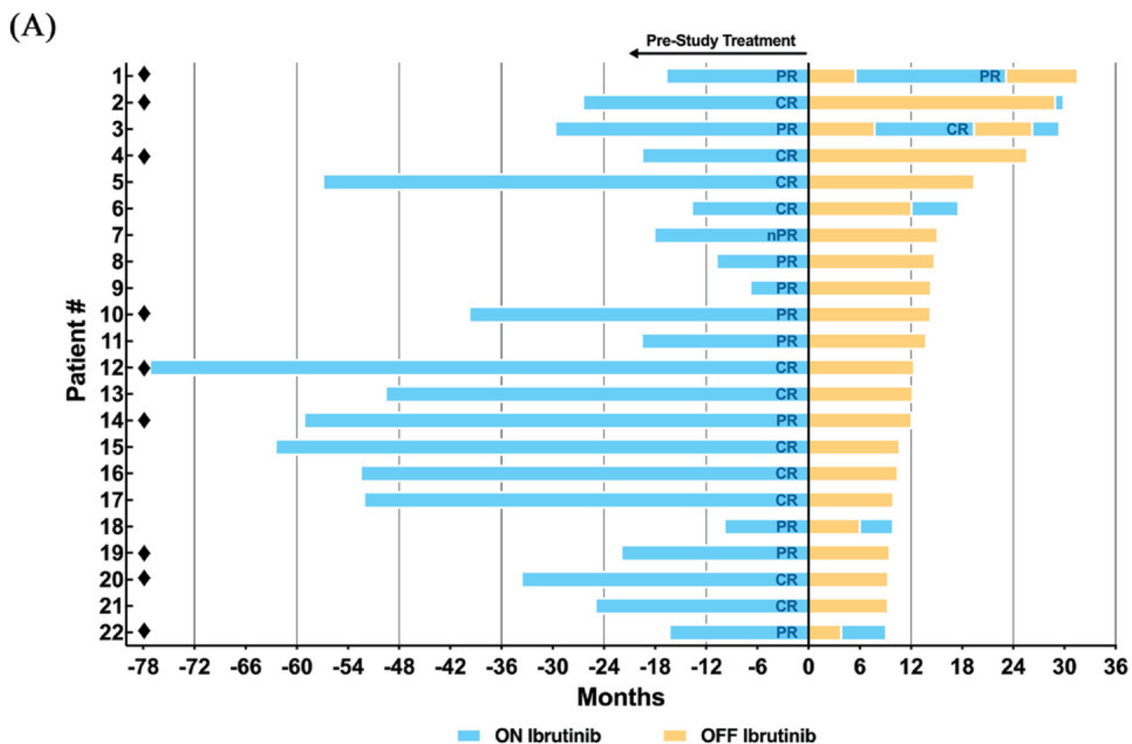

Figure 9 – Clinical responses during intermittent ibrutinib dosing. From: Lundin J, Mulder TA, Kättström M, Wästerlid T, Uddevik A, Mellstedt H, Heimersson K, Hansson L, Palma M and Österborg A. Temporary cessation of ibrutinib results in reduced grade 3-4 infections and durable remissions—Interim analysis of an on-off-repeat Phase 1b/2 study in patients with chronic lymphocytic leukemia. *eJHaem*. 2021 Aug;2(3):525-529. This image was redistributed under the Creative Commons Attribution 4.0 International (CC BY 4.0) license. The original image was cropped.

After a median follow-up of 13 months (range, 9 to 32), all patients were still being managed according to the intermittent treatment strategy. No disease flare, rebound phenomenon, non-tolerability or non-responsiveness had occurred in any of the patients. Sixteen (73%) of the patients were still in remission during their first interruption (**Figure 9**). However, the other 6 (27%) patients resumed ibrutinib after early signs of PD occurred after a median interruption of 7 months (range 4-29). Among these, 4 had entered the study with a PR and 2 with a minimal residual disease (MRD)-positive CR.

Two of these patients (both PR at study entry) had achieved a new response after 12 and 18 months of resuming ibrutinib and interrupted the drug again. Intriguingly, this second response was deeper (MRD-positive CR) in 1 patient. However, this patient experienced a second PD after 9 months off-drug and resumed the treatment for a second time. The 4 patients that had resumed ibrutinib without having achieved a response at the time of data cut-off, had been back on the drug for only 1-3 months.

We showed in **Paper I** that PB T-cell numbers drop during up to 5 years of ibrutinib treatment and that this correlates with a decreasing tumor burden. Accordingly, no change in T-cell numbers was observed during the first 6 months of interrupting ibrutinib, when the disease burden also did not seem to change in most patients. During this first interruption, NK cells first dropped and then increased again. The amount of Th1 and Th2 cells and their ratio, did not change when ibrutinib was interrupted. This means that the proposed block in Th2 differentiation by ibrutinib's inhibition of ITK (284), which we could not confirm in **Paper I**, does not translate into the clinical setting of ibrutinib interruption in responding CLL patients either. Interestingly, however, the number of PD-1$^+$TIM-3$^+$ T cells, which should be regarded as exhausted T cells, and Tregs did grow steadily during interruption. This could either reflect a subclinical rise in tumor burden or a recovery of mechanisms that depend on BTK or other kinases that are no longer inhibited.

5.3 PAPER III

Circulating Lymphocytes in Classical Hodgkin Lymphoma

To investigate disease-related changes in PB lymphocytes in cHL, we performed immunophenotyping in a cohort of 48 treatment-naïve patients and 20 healthy donors. These changes were then analyzed in relation to the cHL TME, inflammatory status, tumor burden and standard first-line treatment.

We regarded all statistically significant differences between cHL patients and healthy donors as disease-related changes. In this way, we found that cHL was associated with low numbers of circulating B cells and advanced-stage cHL with low numbers of NK cells. As expected, TARC levels in plasma were elevated and correlated with the frequency of Th cells in the lymph nodes and a high tumor burden (defined by the presence of any of the following characteristics: bulky tumor, > 2 nodal sites involved, stage IV). The numbers of all T-cell populations were similar in the PB of cHL patients and healthy donors.

However, judging by the increased frequencies of some T cells that are positive for Ki-67, CD69, CTLA-4 and PD-1, cHL seems to be associated with a chronically activated T-cell compartment that shows signs of exhaustion. CTLA-4^+ T cells, Th2 cells and Tregs were more abundant in patients with a high tumor burden.

Our findings partly contradict a recent report of disease-related alterations in early-stage unfavorable cHL, where they found low frequencies of T cells in general, and $CD4^+$ T cells and Tregs in particular, as well as normal levels of CD69 on T cells (370). However, matching our findings, they and others described a normal frequency of $CD4^+$ T_N cells and a larger portion of $CD4^+$ T_{EM} cells, as well as a decrease in $CD8^+$ T_N cells together with an increase in CD8+ T_{EM} cells, in cHL patients (370, 371).

The standard first-line treatment with ABVD and BEACOPPesc chemotherapy-based regimens that these patients were subjected to, lowered the $Ki67^+$ and CTLA-4^+ fractions of Th cells and CTLs, and the frequencies of $CD69^+$ and PD-1^+ CTLs. Thus, standard chemo-/radiotherapy seems to reverse T-cell exhaustion, just like PD-1 blockade does (370, 371). One could argue that rescuing T-cell exhaustion does not seem to be specific to the type of treatment. Instead, T-cell exhaustion seems to be related to the presence of cHL in general, and the continuous exposure to antigens and inflammation in particular.

Besides having low numbers of NK cells in the PB, advanced-stage cHL patients had activated NK cells, as high fractions were positive for CD69 and HLA-DR. However, these seemed functionally impaired since the frequency of $NKG2D^+$ DNAM-1^+ double-positive NK cells was reduced. NK-cell suppression seems to associate with high inflammation. After treatment, the numbers of total NK cells and $CD56^{bright}$ NK cells had normalized.

Interestingly, we found that patients who received radiotherapy had lower ALCs and T cells, but not B cells, after treatment. T cells were especially low in patients that underwent radiotherapy involving the mediastinum. Among the most affected T-cell subsets in these patients were Th2 cells, $CD4^+$ T_N cells, Tregs, and $CD8^+$ T_N cells, which led us to hypothesize that radiation of the thymus could be the cause of these low numbers. Theoretically, the low number of Th cells up to 6 months after the end of treatment could make cHL patients more susceptible to *Pneumocystis jirovecii* pneumonia (PJP), which might justify prescribing PJP prophylaxis to these patients for an extended period.

6 CONCLUSIONS

From our study on the on- and off-target effects of up to 5 years of ibrutinib treatment on plasma biomarkers and immune cells in CLL patients, which is described in **Paper I**, we conclude that this drug has clear immunomodulatory properties. This was exemplified by the biomarkers that changed and additionally could be classified as CLL non-associated. We identified several proteins that are potentially involved in ibrutinib-induced AF. This finding could initiate further studies into the mechanisms behind this common adverse event. Furthermore, we show that T cells, including exhausted T cells and Tregs, decrease in parallel with the disappearance of CLL cells. Th1 cells decreased sharply as well, while Th2 cells stayed relative stable, causing Th2-skewing.

From our clinical trial testing a novel intermittent dosing strategy of ibrutinib in responding CLL patients who sustain a durable objective response, which is described in **Paper II**, we conclude that it is safe to interrupt ibrutinib therapy in these patients and that new objective responses can be achieved by restarting ibrutinib in patients who progress while being off the drug. Moreover, patients seem to have a reduced risk of severe infections during the first interruption and the absolute numbers of exhausted T cells and Tregs increased after cessation.

From our investigation into the disease-related alterations in circulating lymphocytes in cHL, which is described in **Paper III**, we conclude that cHL patients have higher frequencies of terminally differentiated and exhausted T cells, lower amounts of B cells, and functionally compromised and less abundant NK cells. NK-cell suppression seemed to coincide with high inflammation, while a high tumor burden seemed to be associated with the concentration of TARC and the numbers of Th2 cells, Tregs, CTLA-4^{+} T cells. Standard first-line treatment reversed most of these alterations. Furthermore, radiotherapy involving the mediastinum was associated with low numbers of T cells after treatment, which might form an indication for longer PJP prophylaxis in these patients.

Overall, treatment with ibrutinib in CLL influences the immune system and strategic pauses of this treatment in patients who are in remission are safe and seem to reduce the risk of serious infections. Like CLL, cHL also has systemic influences on immune cells and treatment will normalize most of the immunological changes. A fading tumor burden seems to play a role in the immunomodulatory effects of successful treatment in both diseases.

7 POINTS OF PERSPECTIVE

Additional research is needed to clarify the mechanisms behind the adverse events of ibrutinib. This knowledge could help us to manage these in patients today and guide the further development of more selective and irreversibly binding BTK inhibitors that are already in clinical trials for the future treatment of CLL patients.

Until these new drugs become available, and even beyond, continued studies of intermittent ibrutinib in CLL patients remains highly relevant. Longer follow-up is needed to reveal if this alternative dosing strategy reduces the incidence of adverse events and the risk of non-tolerability over time. Furthermore, the measurement of resistance-inducing mutations in this trial is highly anticipated. If indeed intermittent dosing of ibrutinib would reduce the risk of resistance and subsequently delay the need of alternative treatment because of non-responsiveness to this drug, this would be of great benefit to CLL patients. Longer follow-up is also required to exclude the possibility that intermittent dosing of ibrutinib compromises disease control in any way. Finally, the cost-saving potential of this treatment strategy is another potential benefit that remains to be determined.

Further research is warranted into the disease-related systemic immune status of cHL patients, also regarding the potential use of PD-1 blockade as a first-line treatment option for these patients. Such knowledge could help clinicians to select patients that are most likely to benefit from this treatment in this setting and patients that are at high risk for autoimmune-related adverse events. Furthermore, knowledge about the pathophysiology of cHL could eventually foster the development of novel therapies.

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