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EXPLORING T CELL MEDIATED IMMUNOTHERAPY AGAINST THERAPY RESISTANT LEUKEMIC STEM CELLS

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Exploring T Cell Mediated Immunotherapy against Therapy Resistant Leukemic Stem Cells

Thesis for Doctoral Degree (Ph.D.)

By

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Dear reader, thank you and enjoy!

Popular science summary

Cancer stem cells are distinct cells that have the unique potential to initiate and sustain a cancer. Most cancer treatments target cells that are rapidly dividing but as cancer stem cells are rare and mostly not dividing, they escape treatment. Their persistence is therefore the cause of relapse. Elimination of cancer stem cells is therefore required, and potentially sufficient to achieve a cure. There is however still no treatment option that has been shown to specifically eliminate cancer stem cells.

Patients with blood cancer, also known as leukemia, have few curative treatment options with highly invasive stem cell transplantation being the only cure for many. Yet, relapse is common, and the prognosis is then very poor. In this thesis, we showed that targeted screening for remaining cancer stem cells enhanced the detection of remaining leukemic cells after stem cell transplantation (**study I**). This is clinically important because it might allow earlier, and more reliable prediction of relapse compared to previous methods. It also highlights the need for targeted therapies against the cancer stem cells to prevent relapse.

An emerging cancer treatment is immunotherapy, where the patient's own immune system is boosted or modified to eliminate the cancer. T cells are part of the human immune defence which protect us against diseases caused by virus and bacteria by killing infected cells. However, certain T cells have also been shown to efficiently kill cancer cells. T cells have a receptor on their surface which guides them to the cells they are supposed to kill. Today it is possible to take out healthy T cells from a patient, modify their receptor in the laboratory to specifically find and kill cancer cells, and then reintroduce them into the same patient. This type of treatment has proven to completely wipe out certain types of leukemia which shows how potent modified T cells are as an anti-cancer treatment. However, for most leukemias it has not been possible to find cancer-specific targets that allow T cells to eliminate the cancer, including the cancer stem cells, without affecting any vital healthy cells.

Herein, we established two new T cell immunotherapies which showed very efficient elimination of human cancer cells in mice without affecting healthy cells for two different types of leukemias that currently lack any available immunotherapy options (**study II** and **III**). Lastly, we propose that T cell immunotherapy targeted specifically towards the cancer stem cells can not only be a new therapeutic option, but also a new potent tool that can be used to

eliminate only the cancer stem cells to study what happens to the remaining leukemia and through that provide definitive evidence for cancers dependency on cancer stem cells. (**study IV**).

All studies are summarised in **Figure 1**.

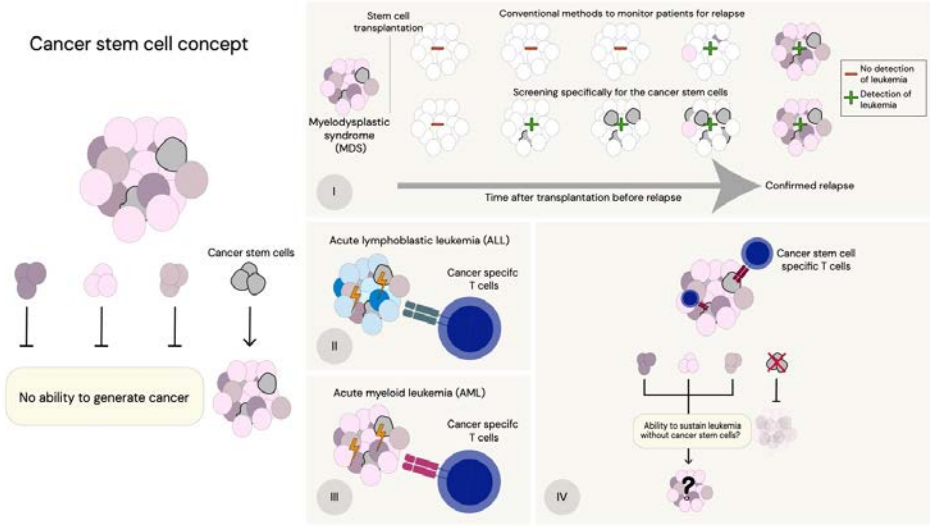

Figure 1. Graphical abstract summarising the content of this thesis (study I-IV) with the cancer stem cell concept as foundation.

Abstract

The cancer stem cell (CSC) model suggests that cancers depend on continuous replenishment from rare and distinct CSCs, but their existence has been challenging to prove for many malignancies. The CSCs in myeloid leukemias, known as leukemic stem cells (LSCs) have been well characterised and shown to selectively escape treatment, thus representing the cellular source of relapse. Relapse following initial periods of clinical remission represents the most significant threat to leukemic patients as the available treatment options at this stage are sparse due to the development of resistance. There is therefore a need for strategies facilitating earlier detection of an impending relapse to initiate pre-emptive treatments early, as well as for new targeted therapies to eliminate LSCs.

The cellular identity of LSCs was recently assigned to the hematopoietic stem cell (HSC) compartment in patients with low to intermediate risk myelodysplastic syndromes (MDS), for which the only curative treatment option is allogeneic stem cell transplantation (allo-HSCT). Detailed characterisation of LSCs in terms of phenotype, function, and treatment resistance is thus now possible, with the aim to ultimately identify novel targets allowing for targeted elimination of LSCs. In **study I**, we performed LSC-directed screening for measurable residual disease (MRD) in patients with MDS after allo-HSCT. By flow cytometric purification of the hematopoietic stem and progenitor cells, the MRD-sensitivity was enhanced 97-fold compared to conventional screening methods on unfractionated bone marrow cells. Consequently, in our patient cohort, targeted screening of the leukemia initiating cells led to the detection of impending relapses on average ten months before clinical diagnosis. In the clinic, this would allow for prescribing preventative treatments earlier while disease burden remains low which could improve outcome.

A potentially effective cancer treatment option is adoptive T cell therapy, using T cells that carry cancer-specific T cell receptors (TCR). TCR T cells can, unlike chimeric antigen receptor (CAR) T cells, recognise intracellular antigens but despite the theoretical large range of targetable antigens, very few are immunogenic. Consequently, no TCR T cell therapy is yet clinically approved for treatment against hematological malignancies, highlighting the need for new potential targets. In **study II** we showed that terminal deoxynucleotidyl transferase (TdT), which is normally transiently expressed during early B and T cell development and overexpressed in >80% of patients with B and T acute

lymphoblastic leukemia (ALL), is a promising target for TCR based immunotherapy in context of HLA-A*02:01 (HLA-A2). T cells targeting TdT, identified through the blood of healthy donors, showed very efficient elimination of TdT^+ leukemic cells, while sparing healthy hematopoietic stem and progenitor cells and mature lymphocytes in clinically relevant mouse models. Thus, TdT TCR T cells are a novel and promising immunotherapy option for patients with B- and T-ALL.

Myeloid leukemias are, to a greater extent than the lymphoid leukemias, characterised by somatic recurrent mutations which generate cancer-specific neoantigens representing a group of unique attractive therapeutic targets. In **study III**, an HLA-A2 restricted TCR with high specificity against a recurrent neoantigen generated from the *FLT3*-D835Y mutation in acute myeloid leukemia (AML) was identified and shown to exhibit great anti-leukemic effect restricted to the *FLT3*-D835Y mutated cells while non-mutated cells were spared. Interestingly, the FLT3-D835Y TCR T cells also had the potential to eliminate the LSCs in vitro as shown by loss of leukemia initiating formation in mice following co-cultures.

The results from **study II** and **III** revealed the therapeutic potential of TCR T cells, therefore, in **study IV** we proposed TCR T cells as a targeted therapy against LSCs. Identification of antigens that mediate specific elimination of all LSCs across many patients has been challenging due to large inter- and intra-patient heterogeneity. Myeloproliferative leukemia protein (MPL) has an important role in lifelong maintenance of HSCs and is therefore expressed on all HSCs and consequently also all LSCs in patients with low to intermediate risk MDS. We suggest TCR T cells targeting MPL presented on HLA-A2 as an immunotherapeutic approach with clinical relevancy to treat an impending relapse following an haploidentical allo-HSCT. MPL TCR T cells would potentially also represent a novel research tool to study the dependency of HSCs and LSCs in normal and malignant hematopoiesis.

List of scientific papers

I. **Identification and surveillance of rare relapse-initiating stem cells during complete remission post-transplantation**
M. Dimitriou, T. Mortera-Blanco, M. Tobiasson[#], S. Mazzi[#], **M. Lehander**[#], K. Högstrand, M. Karimi, G. Walldin, M. Jansson, S. Vonlanthen, P. Ljungman, S. Langermeijer, T. Yoshizato, E. Hellström-Lindberg, P.S. Woll and SE.W. Jacobsen[*] **Blood** (2024) 143 (11): 953–966. doi: https://doi.org/10.1182/blood.2023022851

II. **T cells targeted to TdT kill leukemic lymphoblasts while sparing normal lymphocytes**
M. Ali[#], E. Giannakopoulou[#], Y. Li, **M. Lehander,** S. Virding Culleton, W. Yang, C. Knetter, M. Odabasi, R. Chand Bollineni, X. Yang, Zs. Foldvari, M-L. Böschen, E. Taraldsrud, E. Strønen, M. Toebes, A. Hillen, S. Mazzi, A. de Ru, G. Janssen, A. Kolstad, G. Tjønnfjord, B. Lie, M. Griffioen, S. Lehmann, L. Osnes, J. Buechner, KG. Garcia, T. Schumacher, P. van Veelen, M. Leisegang, SE. W. Jacobsen[#], P. S. Woll[#] and J. Olweus[*]. **Nature Biotechnology**, 2022 Apr;40(4):488-498. doi: 10.1038/s41587-021-01089-x. Epub 2021 Dec 6.

III. **A T-cell receptor targeting a recurrent driver mutation in FLT3 mediates elimination of primary human acute myeloid leukemia *in vivo***
E. Giannakopoulou, **M. Lehander**, S. Virding Culleton, W. Yang, Y. Li, T. Karpanen, T. Yoshizato, E. Rustad, M. Milek Nielsen, R. Chand Bollineni, T. Tran, M. Delic-Sarac, TJ. Gjerdingen, K. Douvlataniotis, M. Laos, M. Ali, A. Hillen, S. Mazzi, D. Chin, A. Mehta, J. Sejerø Holm, A. Kai Bentzen, M. Bill, M. Griffioen, T. Gedde-Dahl, S. Lehmann, SE. W. Jacobsen[*,#], P. S. Woll[*,#] and J. Olweus[*]. **Nature Cancer**, 2023 Oct;4(10):1474-1490. doi: 10.1038/s43018-023-00642-8. Epub 2023 Oct 2.

IV. **MPL as a target antigen for T cell receptor mediated elimination of leukemic stem cells**
M. Lehander[*], S. Virding Culleton[#], Z. Földvári[#], A. Titov[#] C. Knetter, O. Chowdhury, A. Hillen, E. Chari, F. Grasso, W. Yang, M. Brennan, J. Zeun, J. Olweus, and SE.W. Jacobsen and P.S. Woll[*].
Manuscript

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[#] Equal contribution

List of scientific papers not included in the thesis

I. **Platelet and myeloid lineage biases of transplanted single perinatal mouse hematopoietic stem cell**
K. Belander Strålin[#], J. Carrelha[#], A. Winroth, C. Ziegenhain, M. Hagemann-Jensen, L. Kettyle, A. Hillen, K. Högstrand, E. Markljung, F. Grasso, M. Seki, S. Mazzi, Y. Meng, B. Wu, E. Chari, **M. Lehander**, R. Sandberg, P.S. Woll, SE.W.Jacobsen[*].
Cell Res *33, 883–886 (2023). https://doi.org/10.1038/s41422-023-00866-4*

II. **Alternative platelet differentiation pathways initiated by non-hierarchically related hematopoietic stem cells**
J. Carrelha[#*], S. Mazzi[#], A. Winroth[#], M. Hagemann-Jensen, C. Ziegenhain, K. Högstrand, M. Seki, M. S. Brennan, **M. Lehander**, B. Wu, Y. Meng, E. Markljung, R. Norfo, H. Ishida, K. Belander Strålin, F. Grasso, C. Simoglou Karali, A. Aliouat, A. Hillen, E. Chari, K. ' Siletti, S. Thongjuea, A. J. Mead, S. Linnarsson, C. Nerlov, R. Sandberg, T. Yoshizato, P.S. Woll, SE.W. Jacobsen[*].
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Contents

List of abbreviations

ALL	Acute lymphoblastic leukemia
Allo-HSCT	Allogeneic hematopoietic stem cell transplantation
AML	Acute myeloid leukemia
AML-MRC	AML with myelodysplasia-related changes
BiTE	Bispecific T cell engagers
BM	Bone marrow
CAR	Chimeric antigen receptor
CB	Cord blood
CD	Cluster of differentiation
CFC	Colony forming cell
CHIP	Clonal hematopoiesis of indeterminate potential
CML	Chronic myeloid leukemia
CMML	Chronic myelomonocytic leukemia
CMPs	Common myeloid progenitors
CR	Complete remission
CSCs	Cancer stem cells
ddPCR	Droplet digital PCR
ELN	European LeukemiaNet
FACS	Fluorescence-activated cell sorting
FLT3	Fms like tyrosine kinase 3
GMPs	Granulocyte-monocyte progenitors
GvHD	Graft versus host disease
GvL	Graft versus leukemia
HLA	Human leukocyte antigen
HLA-A2	HLA-A*02:01
HSCs	Hematopoietic stem cells
HSPCs	Hematopoietic stem and progenitor cells
ITD	Internal tandem duplications
Lin	Lineage
LMPPs	Lymphoid-myeloid primed progenitors

LSCs	Leukemic stem cells
LTC-CFC	Long-term culture colony forming cell
MAC	Myeloablative conditioning
MDS	Myelodysplastic syndromes
MDS del(5q)	MDS with isolated del(5q)
MDS-EB	MDS with excess blast
MDS-MLD	MDS with multilineage dysplasia
MDS-RS	MDS with ring sideroblasts
MDS-SLD	MDS with single lineage dysplasia
MDS-U	MDS unclassifiable
MEPs	Megakaryocyte-Erythroid progenitors
MHC	Major histocompatibility complexes
MkPs	Megakaryocyte progenitors
MNCs	Mononuclear cells
MPL	Myeloproliferative Leukemia Protein / TPO-receptor
MPN	Myeloproliferative neoplasm
MPPs	Multipotent progenitors
MRD	Measurable residual disease
NY-ESO-1	New York Esophageal Squamous Cell Carcinoma-1
PB	Peripheral blood
PDX	Patient derived xenograft
RIC	Reduced intensity conditioning
TAA	Tumour associated antigen
TCR	T cell receptor
TdT	Terminal deoxynucleotidyl transferase
TKD	Tyrosine kinase domain
TKI	Tyrosine kinase inhibitor
TPO	Thrombopoietin
VAF	Variant Allele Frequency
WHO	World health organisation

1 Introduction

1.1 Healthy hematopoiesis and the hematopoietic stem cells

1.1.1 A brief history of hematopoietic stem cell research

All the billions of mature blood cells that needs to be replaced daily in humans are generated through a tightly regulated process called hematopoiesis, originating from the hematopoietic stem cells (HSCs) (**Figure 2**). The first definitive experimental evidence for the existence of multipotent self-renewing HSCs was provided by Till and McCulloch in 1961[1,2]. Since then, the HSCs and the hematopoietic system has been the most well studied regenerative tissue. With several vital functions which include, but are not limited to, blood clotting, immune defence, oxygen transport, and wound healing[3], the hematopoietic system is highly conserved among vertebrates ranging from zebrafish, frogs, and birds to mice, humans, and all mammals allowing for a broad range of hematopoietic model systems[4]. The adult hematopoietic system in mammals is hierarchical organised with a rare population of bone marrow (BM) resident HSCs which sit at the apex of the hierarchy[5] (**Figure 2**)**.** This clear hierarchical organisation, combined with easy access to hematopoietic cells, experimental models, and assays, along with cell surface markers that can distinguish blood cell lineages, makes the hematopoietic system ideal for studies of stem cell biology. The HSCs are uniquely characterised by a combination of two functional features: multipotency and self-renewal. This describes the capacity to differentiate into all mature blood cell lineages through a series of short lived, increasingly myeloid or lymphoid lineage restricted hematopoietic progenitors and the ability to self-renew during cell division giving rise to yet another HSC, respectively (**Figure 2**). Combined, these two features ensures a functional HSC pool throughout life and ultimately allows for the continuous replacement of the mature blood cells[4–7].

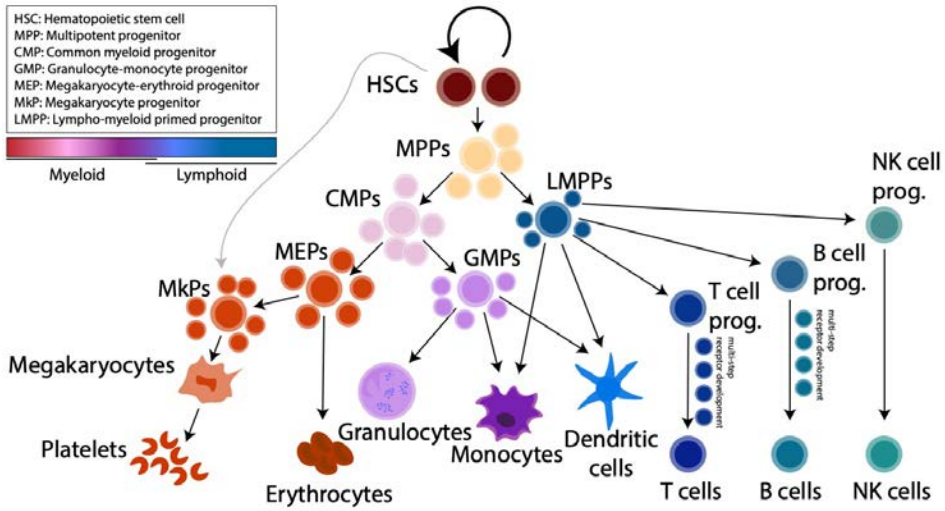

Figure 2. Hierarchical organisation of the human hematopoietic system with hematopoietic stem cells at the apex. Arrows show differentiation trajectories leading to the production of the mature cells where HSCs can self-renew, as indicated by circular arrow. Gray arrow indicate a direct pathway to the platelet lineage initiated from HSCs that has recently been identified in mice but yet to be determined in humans.

Till and McCulloch's research aimed to find therapeutic interventions against damage caused by ionizing radiation from atomic weaponry. A few years earlier, the first successful bone marrow transplantation was performed in humans[1,8,9] which might have inspired Till and McCulloch's attempt to rescue a myeloablative irradiated mouse with a bone marrow transplantation. Indeed, their study showed that a hematopoietic restoration generated from the donor mouse took place in the recipient with all mature blood cells represented, proving multipotency of the transplanted cells. Upon termination of the transplanted mice, analysis of the spleens unveiled clusters of donor cells, referred to as colonies. Each colony, distinguished by chromosomal aberrations, was identified as the progeny of a single cell. It was shown that the cells from these colonies were able to reconstitute hematopoiesis in a secondary irradiated recipient which proves self-renewal of the reconstituting cell. Combining the multipotency and the self-renewing capacity, cells with functional HSC properties had been discovered in the BM. This also marked the beginning for the golden standard HSC-transplantation assay, which still to this date is the most reliable assay for functional studies of HSCs[1,2].

In the years that followed, Spangrude and colleagues succeeded with isolating the long-term repopulating cells from the BM[10] through advances in flow cytometry and fluorescence-activated cell sorting (FACS) which allowed for separation of cell types based on their surface expression of various cluster of differentiation

(CD) antigens. Different phenotypic populations could thus be purified and functionally evaluated both through in vivo competitive transplantation assays and in vitro culture assays[11,12]. It was eventually determined that all cells with stem cell functions are confined to the rare Lineage$^-$(Lin$^-$)Sca1$^+$c-Kit$^+$Flt3$^-$CD150$^+$CD48$^-$ compartment in mice, representing less than 0.05% of total BM cells[12,13]. This identification allowed for single cell analysis of these cells, which revealed striking heterogeneity in the HSC pool, mainly in terms of lineage output[14].

Transplantation assays have also been fundamental to establish the human hematopoietic hierarchy, which resembles that of mice. The generation of immunocompromised mice lacking large portions of the adaptive immunity allowed for engraftment across species, also known as xenografts. Consequently, transplantation studies assessing long-term multipotent potential and self-renewal capacity of human cells was now possible[15,16]. In these studies, Lin$^-$CD34$^+$CD38$^-$CD90$^+$CD45RA$^-$ cells demonstrated prolonged self-renewal and multilineage capacity thus representing the purest human HSC population[17,18]. In vivo transplantation assays also allows for distinguishing HSCs from cells with multipotent engraftment potential which diminishes over time (multipotent progenitors; MPPs), as well as cells with short-term bi/uni-lineage engraftment potential or no engraftment potential (progenitors/mature cells)[19,17].

The in vitro assays used to study HSC biology are also grounded on long-term self-renewal capacity and multilineage colony-formation, and allows for more easily controlled studies on HSC functions and their relationship to other hematopoietic cells in both mouse and human[2]. In vitro assays have particularly played a crucial role in studying the influence of individual cytokines on HSC regulation where Stem cell factor (SCF) and Thrombopoietin (TPO) have been described as two main regulators[20]. There are also short-term in vitro culture assays that have been fundamental for characterisation of the down-stream progenitors. However, these now traditional hematopoietic assays have limitations (**Table 1**). The in vitro systems underestimate the complexity of native hematopoiesis and the interactions with the surrounding BM cells and do not necessarily support single progenitors to adopt all potential lineage fates[21]. Of note, the cytokine stimulation required for generating myeloid/erythroid cells differs from those necessary for lymphoid development which often also require support from stromal cells[22]. This reduces the range of potential assays available for examination of multilineage potential of hematopoietic stem and progenitor cells (HSPCs) in same in vitro assay. The in vivo transplantation assays also preclude from studying HSCs native behaviour. The myeloablation of the recipient induces an abnormal pressure on, and possibility for, the transplanted HSCs to regenerate the hematopoietic system. Thus, those assays rather assess the

potential of HSCs rather than their function in unperturbed hematopoiesis. For these reasons, genetically modified mouse models have been fundamental, as these have not only substantiated many findings about extrinsic factors regulating HSCs, but also offered a platform to investigate the intrinsic factors crucial for effective HSC maintenance and regulation, which includes various transcription factors and epigenetic modulators[23]. Our understanding of mammalian hematopoiesis has thus been significantly shaped by the utilisation of genetically modified mice.

A fundamental challenge with hematopoietic research lies in the fact that pure HSCs cannot be prospectively isolated, and cellular identity of an HSC can still only be defined based on functionality where the functional interpretations are assay dependent.

1.1.2 Recent advances in hematopoietic stem cell research

Through development of advanced FACS and detailed characterisation of HSCs using cell surface markers, functional HSCs in mice can now be purified by immunophenotype down to more than one in two, determined by competitive transplantation assays of single HSCs[25,26]. With a more pure HSC population, the data variability is reduced, and greater scientific interpretations of results generated using the phenotypic HSC population can be made. For single cell sequencing and transplantation analyses, advances in HSC purity have also attributed to ethical benefits as fewer recipient mice and donor cells are needed to achieve the same output. In humans, the highest reported HSC purity is 30%, as shown by repopulating transplantations into immunodeficient mice[18,27] but this data cannot be as robustly validated as for mouse HSCs likely due to the assay limitations. The absence of supporting human cells in the mice for interaction with the engrafted HSCs, coupled with the limitations in species cross-reactivity within the BM niche and the considerable size difference between human and mice, renders the xenograft transplantation assays even more conditional than mouse-to-mouse transplantations. Nevertheless, the power of the transplantation assays to assess HSC function cannot be denied. Those studies have given us definitive evidence that HSCs are crucial for hematopoiesis under stressed conditions, and that they are the only cells with the capacity to regenerate and sustain a full hematopoietic system long term and that even a single HSC can reconstitute a fully functional hematopoietic hierarchy. However, we have recently entered a new era for hematopoietic research where advances in sequencing and genetic fate mapping strategies now allows for studies on unperturbed hematopoiesis originating from single HSCs in both mouse and human[28,14].

Table 1. Common methods for studies of HSCs used for both human and mouse. Adapted from Clarke and Holyoake[24].

Assay	Description	Advantages	Limitations
In vitro colony forming cell (CFC) culture assay	Assessing proliferation and differentiation capacity of HSPCs based on the ability to generate colonies in semi-solid media	• Based on functional properties • Allows for harvesting of colonies for subsequent analysis • Time efficient • Easily manipulated	• Does not allow for distinction of HSCs from downstream progenitors • Underscores the complexity of BM environment
In vitro long-term culture colony forming cell (LTC-CF) / long-term cell initiating cell (LTC-IC) assay	Assessing long-term self-renewing capacity through a 6-week culture followed by colony generation in semi-solid media	• Based on functional properties to distinguish HSCs from progenitors • Allows for harvesting of colonies for subsequent analysis • Easily manipulated	• Time consuming • Risk for contaminations • Underscores the complexity of BM environment
In vivo transplantation assays	Assessing long-term repopulating capacity of the transplanted cells which can be quantified (for mouse) in competition with a CD45 syngeneic mouse. Can be followed by a secondary transplantation to assess self-renewal capacity	• Based on functional properties • Physiologically relevant in vivo model • Most reliable assay to define an HSC	• Time consuming, long-term experiments (>16 weeks for HSCs + secondary transplantations) • Assessing HSC capacity over function
Flow cytometry	Fluorescently conjugated monoclonal antibodies with affinity for cell type specific antigens allows for separation of distinct cell types through detection by various lasers	• Time efficient • Can be used for both analysis and cell sorting • Well characterised antigens in the hematopoietic system	• Not based on functional properties
Genetic tracing	Assessing HSCs contribution to hematopoiesis by tracing of molecular barcodes, either induced into the HSCs or naturally occurring as somatic mutations	• Allows for studies of HSCs in steady state	• Sensitivity and specificity issues in detection of rare HSC clones

Several genetic fate mapping strategies in mice are now available, but the principal remains the same; single HSCs are labelled with unique non-functional DNA-altering barcodes, either in utero, at steady state or before transplantation. The genetic alternations will be inherited to the progeny, which permits for tracking of differentiation trajectory of single HSCs. The mouse genetic fate mapping techniques have confirmed that although many trackable HSC clones

produce mature blood cells of all lineages thus falling into the traditional multipotent HSC definition, oligolineage and unilineage contributing HSC clones are observed[29–31]. These data compliments nicely existing phenotypic fate mapping studies through single cell transplantations which also showed that a fraction of long-term repopulating HSCs have a strong bias or even restriction in generating certain mature blood types. Together this work provides strong evidence for heterogeneity of individual HSC[32–34].

Genetic fate mapping has for the first time allowed for studies investigating the relative contribution of HSCs compared to the downstream progenitors to long-term steady state hematopoiesis. The first reports in mice indicated very little contribution from HSCs in steady state[35,36] which was later challenged by multiple studies showing that HSCs are active contributors even in native hematopoiesis[37–39]. The conflicting results from various studies from different research groups derived from a range of experimental techniques highlights the limitations in data interpretation from genetic fate mapping studies where the clone size of the most quiescent HSCs might be below the detection limit, thus not reflecting lack of existence of the cells, but rather be a question of method sensitivity. The lineage output of HSCs can also fluctuate overtime which might falsely assign an HSCs as lineage biased or non-contributing at a given time point. The limitation of genetic fate mapping is augmented by the restricted diversity in the number of possible unique genetic marks introduced in one system. This is nevertheless starting to be improved by, for instance, inclusion of DNA polymerases in the mouse constructs to increase the frequency of insertions thereby compensating for the loss of barcode diversity that occurs when using the deletion-prone clustered regulatory interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9) (CRISPR/Cas9) driven fate mapping mouse models[40]. Moreover, there is currently no conclusive evidence regarding the contribution of HSCs to maintain steady-state hematopoiesis. It remains uncertain whether long-lived progenitors can partially or entirely sustain steady-state hematopoiesis in the absence of HSCs, given that the long-term selective elimination of HSCs has not been accomplished so far.

Interestingly, genetic fate mapping approaches have also been applied to study human hematopoiesis both in terms of clonal contribution of HSCs and lineage output. The detection of clonal somatic mutations in healthy individuals, referred to as clonal hematopoiesis, can be used as naturally occurring barcodes and virally-corrected HSPCs used clinically for autologous stem cell transplantation can be utilised for fate mapping post transplantation[41]. Lee-Six and colleagues tracked somatic mutations acquired in human HSCs and estimated that the

number of HSCs contributing to human adult steady state hematopoiesis is between 50.000-200.000 cells in a representative 59-year old man[42]. This was followed up by another study from the same group where they assessed clonal contribution of HSCs in 10 adults over time through whole genome sequencing of single HSC/MPP derived colonies and found that adults below the age of 65 have a high degree of polyclonal contribution to hematopoiesis that reduces with age[43]. A similar approach was used by Chapman and colleagues on a stem cell transplantation cohort of paired sibling donors and recipients. They showed that the clonal diversity declined after transplantation and that less than 100.000 donor HSCs were responsible for the long-term multilineage contribution to hematopoiesis post-transplantation[44].

Taken together, the transplantation assays, the in vitro culture systems and more recently the genetic fate mapping techniques described above, in conjunction with genetically modified mouse models and other mammalian systems, have revealed the complexity of hematopoiesis. Genetically modified mouse models and in vitro assays has been particular important for establishing the regulatory mechanisms, consisting of both intrinsic and extrinsic cues, that allows for an HSC pool to maintain steady state hematopoiesis throughout life[45–47]. Although the detailed regulation of HSCs is outside of the main scope of this thesis, there are some regulatory mechanisms that are central for the work presented herein which are described in the following sections.

1.1.3 Hematopoietic stem cell quiescence

During embryonic development, the fetal liver serves as the primary site for highly proliferative HSCs to establish the hematopoiesis sytem[48–50]. On the contrary, by the time mammals reach adulthood, the HSCs have entered a quiescent state and predominantly reside in the G_0 phase of the cell cycle which they can reversibly exit when required[51,52]. Cellular quiescence is one of the most defining properties distinguishing adult HSCs from downstream progenitors and many studies have provided evidence for this[53]. Ki67-cell cycle assays have revealed that the majority (~70%) of the phenotypic adult mouse HSCs are in the G_0-phase of the cell cycle at any given time and through the usage of BrdU label retention assays, it has been proposed that a fraction of these remain dormant for >300 days[51,54,55]. However, the Ki67 assay is merely a snapshot in time of cell cycle state, the BrdU assay has relatively low resolution of division history and both assays are based on the phenotypic analysis of HSCs which do not allow for subsequent functional HSC assays[53]. Thus, notably, it has been demonstrated that HSC function is confined and enriched to the most quiescent compartment using long-term and serial transplantation assays of a H2B-GFP label retention mouse model, however,

this is also associated with low division resolution[51]. Same principles apply to human HSCs as shown by, for instance, Laurenti and colleagues using xenotransplantation of human cord blood (CB) HSCs. They demonstrated that the long-term engrafting HSCs divided less frequently compared to the short-term engrafting HSCs which were not capable of secondary transplantation[52]. However, transplantation of HSCs disrupts cellular dormancy, making cell cycle analysis following transplantation unlikely to reflect the physiological state accurately. Mathematical modelling of X-chromosome inactivation in females have generated an estimated division rate of adult human HSCs to once every 25-50 weeks[56] and Lee-Six and colleagues estimated that HSCs self-renew once in a range of 2-20 months[42]. Although the absolute division rate of a human HSC is still to be determined, these estimations support that HSCs divide less frequently than progenitors.

Cellular quiescence provides an essential protection for the HSCs making them more resistant to external and internal stressors such as reactive oxygen species, ionizing and ultraviolet radiation, chemical compounds, and error-prone DNA repair, all of which can induce irreversible damage to DNA during replication, ultimately leading to apoptosis, necrosis, or cancer transformation[57]. Purified mouse HSCs can repopulate a recipient mouse across the allogeneic barrier[58–61], indicative of HSCs ability to also escape immune recognition. In line with this, recent studies have reported that quiescence is a mechanism for immune escape[62,63], further supporting that the most quiescent HSCs are immune-privileged. Dysregulation of HSC quiescence results in exhaustion and hematopoietic failure which has been shown by multiple knock-out mouse models of various genes related to transcription and cell cycle regulation, DNA damage response, metabolism, and autophagy to mention a few[47,57,64,65]. The many different pathways involved highlights that regulation of HSC quiescence is a highly complex and not fully understood network of cell intrinsic and extrinsic mechanisms which all interplay to maintain the equilibrium. One of the key regulators for maintaining adult quiescent HSCs is the TPO receptor Myeloproliferative leukemia protein (MPL) expressed on the surface of HSCs. TPO is produced systemically in the liver and kidney, as well as by stromal cells and osteoblastic cells in the BM niche, around which HSCs have been shown to localise[66]. TPO signalling is mediated by dimerization of MPL upon binding of TPO and subsequent activation of the Janus kinase (JAK)-mediated signalling pathways. Initially recognised and investigated for its important role in megakaryocyte differentiation, MPL was subsequently found to exert crucial regulatory effects on HSCs[66–73], where MPL expression highly correlates with quiescence and the functional stem cell properties[66]. The biological relevance and importance of MPL signalling to HSCs has been well established in both mice and

human. Mice with a complete loss of *Mpl* have a reduced HSC pool and are unable to compete with normal HSCs in transplantation assays[69,71,74,75]. Patients with inherited genetic conditions with mutations in MPL or other genes in the downstream signalling pathways show clinical signs of various degrees of disrupted hematopoiesis[76]. Homozygous loss-of-function mutations in MPL causes congenital amegakaryocytic thrombocytopenia and onset of aplastic amenia and BM failure early in life due to a reduction in functional HSCs, which can only be rescued by an allogeneic HSC transplantation (allo-HSCT)[77–79]. In contrast, activating mutations in MPL or other components of the MPL-signalling pathway can lead to development of hematological malignancies such as myeloproliferative neoplasm (MPN), myelodysplastic syndromes (MDS) and leukemia[80,81].

1.2 Hematological malignancies

Hematological malignancies encompass a group of cancers that affect blood cells and originate from blood-forming tissues[82]. Cancer refers to abnormal and uncontrollable proliferation of cells. If not addressed through treatment, it leads to suppression of healthy tissues ultimately culminating in death. The world health organisation (WHO) through the International Agency for Research on Cancer estimated around 20 million new cancer cases and 9.7 million cancer related deaths in 2022[83]. While cancer encompasses various diseases, the common characteristics among them were conceptualised as the hallmarks of cancer by Hanahan and Weinberg in 2000[84,85]. Initially, these hallmarks consisted of six traits: 1) Self-sufficiency in growth signalling, 2) Insensitivity to anti-growth signalling, 3) Tissue invasion and metastasis, 4) Limitless replicative potential, 5) Sustained angiogenesis and 6) Evading apoptosis. As the knowledge of cancer has progressed since then, Hanahan revised the hallmarks in 2022 to now include up to 14 cancer characteristics that also covers genomic and epigenomic instabilities and the interplay between cancer cells and its surrounding tissues and cells[86]. However, the complexity of cancers goes beyond the hallmarks of cancer with great inter-and intra-patient heterogeneity.

1.2.1 The cancer stem cell concept

Cancer stem cells (CSCs) are characterised by a unique functional ability to initiate and propagate a tumour, and it is postulated that cancers depend on continuous replenishment from the CSCs (**Figure 3**). Thus, elimination of the CSCs is required and potentially sufficient to achieve a cure. Just as normal stem cells, the CSCs are mainly quiescent which represents a clinical challenge as conventional chemo- and radiotherapy target cells that are rapidly dividing[87]. The

CSCs are therefore particular challenging to target which promotes their persistence after treatment, contributing to relapse[88]. Because strategies to selectively eliminate CSCs are missing, it has been challenging to experimentally prove the existence and clinical implication of CSCs. Some of the most well characterised CSCs are the leukemic stem cells (LSCs) representing the CSCs for hematological cancers. The first experimental evidence for existence of any CSCs was provided in acute myeloid leukemia (AML) by Dick, Bonnet, Lapidot, and colleagues who showed that not all leukemic cells had the functional capacity to generate a leukemia in xenografts. The rare population of cells that could were assigned as the LSCs, and they were found in same broadly defined immunophenotypic compartment as normal HSCs ($CD34^+CD38^-$)[89,90]. Subsequent studies on LSCs in AML have provided evidence of LSCs in the $CD34^-$ compartment in patients lacking $CD34^+$ leukemic cells[91,92] and that two molecularly and immunophenotypically distinct progenitor populations with LSC capacities can co-exist[93], which underscores the heterogeneity of the disease.

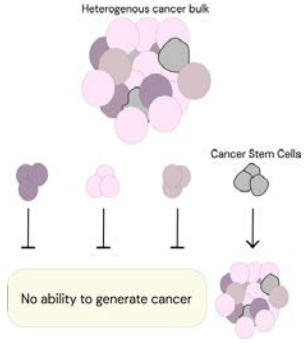

Figure 3. The cancer stem cell concept stipulates that a unique cancer cell type is needed to generate and sustain the disease.

The absence of robust reliable CSC assays is one of the main challenges preventing identification of CSCs for many cancers, together with the broad cancer heterogeneity and the estimated low frequency of proposed CSCs. While being a primary CSC assay well suited for the hematological malignancies, xenograft transplantations are hindered by the observation that cells from many patients do not successfully engraft into mice for reasons that largely remain unclear, although the level of severity of the disease has shown to matter for engraftment potential[94]. Due to the species variation in the BM environment, neither is it definitive that the cell type that propagates human disease in mice is the same population that sustains the cancer in humans[87,95,96,97,98]. The NOD/Scid/IL2R-gamma^{null} (NSG™) mice, or equivalent, with knock-out of the *Prkdc* and the X-linked *Il2rg* genes, resulting in a functional loss of mature B, T and NK cells and an impaired function of antigen presenting cells and phagocytes[99], are often used as recipients. These mice favour human lymphoid reconstitution, and leukemic cells from patients with myeloid diseases therefore show inferior engraftment potential[100–102]. Alterations to the immunodeficient mouse strains to include knock-in of genes encoding human cytokines and antigen presentation molecules, or knock-out of genes encoding mouse cytokines has notably increased engraftment potential of myeloid malignancies[103–106]. The introduced

modification to the BM environment might nevertheless alter the function of putative LSCs and might therefore obstruct interpretations of the results.

Additional means to traditionally study LSC function include the long-term colony forming assays as also used for studies on healthy HSCs but the differentiation blockade at the progenitor stages observed in patients with leukemia might influence their ability to properly generate colonies that can be scored as the read out. For this reason, co-culture systems with human BM stromal cells, called cobblestone area assays have been developed to provide a supportive microenvironment for the LSCs in vitro[107]. Further, both the xenograft transplantations and the in vitro cultures are exposing the proposed LSCs to abnormal external pressure thus not allowing for studies on LSCs in their physiological state. New assays and strategies are therefore warranted. Through recent developments which will be discussed below, LSCs has today also been identified and characterised for myeloid malignancies outside of AML, including chronic myelomonocytic leukemia (CMML), chronic myeloid leukemia (CML) and MDS.

1.2.2 Myeloid malignancies

The myeloid malignancies are characterised by several known recurrent somatic mutations and other genomic alternations that results in defective hematopoiesis affecting various stages of the myeloid differentiation branch[108–111]. Myeloid malignancies are frequently described as stem cell disorders as the disease emerges following initiating oncogenic lesions that target the HSCs[112]. The chance of acquiring a genetic lesion is likely to be equally distributed among the different hematopoietic compartments[113] but due to the short lifespan of the progenitors and most mature cells, those clones are probably eliminated before additional lesions that cause leukemic transformation, unless the cells also gain the capacity to indefinitely self-renew (**Figure 4**). Somatic mutations are continuously accumulated in HSCs at an estimated rate of approximately 14-17 mutations per year[42,113] and although most do not confer higher risk for leukemic development, some mutations do. Clonal hematopoiesis of indeterminate potential (CHIP) is an age-related detection of hematopoietic clones with a variant allele frequency (VAF) of at least 2% of leukemia associated mutations, without manifestation of disease. Around 10% of healthy individuals over the age of 70 have detectable CHIP[114], but by applying more sensitive sequencing techniques that allows for detection of mutations below 2%, CHIP clones could be detected in almost all elderly adults[115]. The presence of an expanded CHIP clone with a VAF exceeding 2% is conferred with a yearly 0.5-1% increased risk for leukemic development[114]. This is likely explained by the increased risk of

acquiring a secondary transformative oncogenic lesion in a pre-expanded clone but the exact mechanisms by which these mutations confer clonal advantage is still largely unknown. The presence of CHIP is therefore at times referred to as a pre-leukemic state. Nevertheless, it is apparent that most individuals with CHIP will not develop leukemia throughout their lives, which underscores that while single genomic events may be sufficient for leukemic initiation, they typically fall short of facilitating full leukemic transformation. When CHIP progresses into malignancy, it frequently manifests as MDS, MPN or AML.

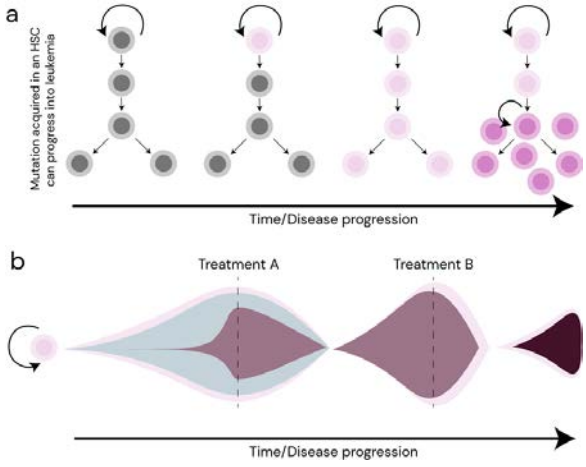

Figure 4. Schematic illustration of cellular and molecular dynamics of myeloid malignancies originating from HSCs. (a) Cellular composition of a heterogenous leukemia showing initiating mutation in HSCs (light pink) and acquisition of additional oncogenic mutations in downstream progenitors (dark pink) can confer self-renewal capacity and leukemic progression. **(b)** Genetic composition of a heterogenous leukemia showing mutational clonal outgrowth over time. Each colour indicates one clone and width indicate clone size following Treatment A and B.

MDS represents a heterogenous group of chronic BM disorders characterised by ineffective hematopoiesis resulting in diverse cytopenia and it is the most common chronic hematological malignancy in the elderly[116]. As of 2016, WHO has established six distinct classifications for MDS, some of which are further divided into sub-entities. These are: MDS with single lineage dysplasia (MDS-SLD), MDS with ring sideroblasts (MDS-RS), MDS with multilineage dysplasia (MDS-MLD), MDS with excess blast (MDS-EB), MDS with isolated del(5q) and MDS unclassifiable (MDS-U). MDS is also classified based on prognosis using the International Prognostic Scoring System (IPSS) ranging from low to intermediate and high risk MDS, where the high-risk patients have greater risk of AML transformation, occurring in about 30% of cases[116,117]. The median overall survival ranges from 1-10 years in the high risk to the low risk patients, and mortality is closely correlated to leukemic transformation[118]. AML is the most prevalent leukemia in adults affecting about 350 people in Sweden yearly and the incidence drastically increases with age with a median age at diagnosis of 68 years. The prognosis is dismal with an estimated 5 year survival of around 30%, but worse (<10%) for patients over 60 years old, and better (~50%) for younger patients[119]. Just like MDS, it is a biologically and clinically heterogenous disease.

One fourth of AML cases emerge as a secondary disease from other hematopoietic disorders, such as MDS, and are then referred to as acute myeloid leukemia with myelodysplasia-related changes (AML-MRC). The remaining cases are either de-novo or evolve as a result of treatment for other malignancies[120]. AML and MDS share many characteristics where the pathologic involvement of several somatic mutations causing an arrest in myeloid differentiation and clonal expansion of faulty HSPCs is central. The most common somatic driver mutations are found in genes related to transcriptional regulation, signal transduction, DNA methylation, chromatin modification and RNA splicing[108,109]. The multi-hit landscape of MDS and AML is clearly demonstrated by the high prevalence of *ASXL1*, *TET2* and *DNMT3A* mutations, which are also the most common mutations found in individuals with CHIP[114]. In AML, driver mutations in FMS-like Tyrosine Kinase 3 (*FLT3*) stands out as the most common as they are found among all defined risk groups[109]. The curative treatment options for patients with AML and MDS are very limited, but the identification of LSCs in AML and MDS has opened new avenues for the development of novel treatments.

1.2.3 Leukemic stem cells in myeloid malignancies

Although the xenograft transplantations remain the golden standard for studying the function of LSCs, molecular tracing of the pathogenic genetic alternations has allowed for assessing the leukemic cell of origin. By tracing the oncogenic lesions found in the leukemic blasts back to the $CD34^{+}CD38^{-}$ compartment, it can be concluded that these lesions must originate from within that compartment and that those cells therefore likely represent the LSCs. However, the heterogenous antigen expression of HSPCs and acquisition of mutations conferring self-renewal capacity to transform cells outside of the $CD34^{+}CD38^{-}$ compartment limits definitive conclusions of LSC-identify using this technique unless the genomic tracing is coupled with functional assessment and a retained hierarchical organisation of the leukemic compartments. Using techniques such as polymerase chain reaction (PCR), targeted DNA sequencing and fluorescent in situ hybridisation, it has been shown for the BCR-ABL fusion protein (central in the pathogenesis of CML)[121,122], mutations frequently found in CMML[123] and the common 5q deletion in MDS[124], that these oncogenic lesions are found in the $CD34^{+}CD38^{-}$ compartment. The 5q deletion could also be detected in B cell progenitors, which further strengthens the evidence of multilineage potential of the initial target cell[124]. Functional studies have validated that cells within the $CD34^{+}CD38^{-}$ compartment exhibited classical LSC-characteristics by xenograft repopulation ability and long-term colony formation capacity across all three malignancies[125–128]. These studies nevertheless failed to fully exclude LSC

potential outside of the HSC compartment. Woll and colleagues later provided definitive evidence of selective stem cell origin of low to intermediate risk MDS. They observed conserved phenotypic antigen expression and hierarchical relationship between HSCs and functional lineage-restricted granulocyte-monocyte progenitors (GMPs) and megakaryocyte-erythrocyte progenitors (MEPs) in the MDS patients. Through genetic fate mapping of the oncogenic lesions all mutations present in the patients could be backtracked to the most primitive HSC compartment. The purified HSCs were also the only HSPCs that could reconstitute long term myelopoiesis in xenografts with the same malignant features as the primary patient[124,129], thus for the first time assigning the cellular identify of LSCs in low to intermediate risk MDS to the phenotypic HSC compartment.

1.2.4 Lymphoid malignancies

The lymphoid branch of the hematopoietic system is fundamental for the adaptive immunity consisting mainly of T lymphocytes (T cells), B lymphocytes (B cells) and natural killer (NK) cells with anti-pathogenic and anti-tumour effects. A lymphoid cancer manifest in faulty differentiation, resulting in an accumulation of immature lymphoid progenitors at different stages[130]. Unlike myeloid cancers, many lymphoid cancers are not as clearly hierarchal organised, and the cells of origin are often still unidentified[131,132]. Through xenograft transplantations it has nevertheless been suggested that heterogenous LSCs are present in lymphoid malignancies such as acute lymphoblastic leukemia (ALL) as well[133,134]. However, cells with comparable LSC-function have been reported across several investigated phenotypic compartments within and among patients, thereby hindering the identification of the specific LSC cellular identity. ALL is just like its myeloid counterpart AML, a very aggressive disease in need of immediate treatment. On the contrary to AML, it is more common in children who have good curative prognosis with an 5-year survival of about 90%[135]. The prognosis deteriorates with age of onset, and the 5-year survival decreases to 30-40% in adults. For patients over 60, the long-term survival is as low as 5-15%[136,137]. ALL is divided into two main types: B-ALL and T-ALL affecting the progenitor stages of B and T cell lineages respectively[138]. Updated guidelines for classification of ALL now includes several sub-entities of B- and T-ALL taking into account the molecular status[139]. Like all leukemia, ALL arises in the BM, and chromosomal abnormalities are frequently pathogenic drivers. The type of chromosomal abnormality and age of onset are decisive factors for treatment strategy and prognosis[140]. Although not as prominent as for the myeloid cancers, mutations in presumptive driver genes have also been reported for both B- and T-ALL[141,142,139]. Because of the differences in genomic and phenotypic landscapes

of the myeloid and lymphoid leukemias, different treatment options for these patient groups are available. Further, older patients typically have lower tolerance for toxic therapies, compared to younger patients, which also guides the treatment choice.

1.3 Treatment strategies against hematological malignancies

1.3.1 Pharmacological therapies

Chemotherapy has been the long-standing standard therapy for hematological malignancies as for many other cancer types. Through a range of different mechanisms of action, conventional chemotherapy target cells that are actively and rapidly dividing and is therefore effective against the highly proliferative leukemic blasts but less effective against the quiescent LSCs[143,144]. Moreover, the available chemotherapies do not distinguish between leukemic and normal cells causing toxicity problems, especially in older frail patients who constitute the majority of individuals with MDS and AML. It is therefore often preferred to use more targeted therapies for treating these patients. More commonly used for treatment of MDS patients are hypomethylating agents such as 5-Azacitatine or Decitabine[145,146] or immunomodulating agents such as Lenalodomide[147] . In MDS, it is also common to administer long-term treatments to alleviate symptoms and address the cytopenia. This may involve stimulation of healthy blood cell production or even transfusions of healthy blood cells, including red blood cells and platelets.

Due to the severe side effects of cancer treatment and the lack of treatment response for some patients, there is a large clinical need for better predictive and prognostic markers that can help guide treatment. Knowledge of the molecular landscape of the patients creates potential for personalised therapies, however, currently there are limited available options for personalised treatments. A clinical example where the molecular genotype of the leukemia guides the treatment is using targeted therapies like Tyrosine Kinase Inhibitors (TKI) against *FLT3*-mutated AML or BCR-ABL$^+$ CML, but resistance is still common[148]. Until new targeted therapies are available, other means to predict treatment response and clinical outcome is needed. In a recently published model, Zeng and colleagues combined single cell gene expression analysis with functional LSC transplantation assays and performed drug-screens where they determined that the hierarchical composition of the leukemia has predictive value of the AML cells responsiveness to treatment[149]. Together with previous studies showing that high frequency of proposed LSCs and LSC-like cells correlate with worse outcome[150,151], this highlights the prognostic and predictive

value of LSC identification. However, as the LSC assays are technically challenging and demanding, the clinical implementation of such approaches is difficult. More clinically implementable approaches have shown that prognostication of outcome in patient with MDS was improved when detailed genomic profiling of the patients was conducted[111]. In MDS, actions like these are particularly important to improve outcome, because the only curative treatment option available is an allo-HSCT but many patients are not eligible.

1.3.2 Allogeneic hematopoietic stem cell transplantation

Allo-HSCT underscores the pivotal role of HSCs in a clinical context. It serves as a therapeutic modality for both myeloid and lymphoid malignancies; however, its applicability relies on the general health status of the patient due to the highly invasive nature of the procedure. Prior to the transplantation, the patient is conditioned with chemo- and/or radiotherapy to reduce the leukemic burden and make space for the incoming graft. The patient's hematopoietic system is then replaced through transplantation of HSCs collected from CB, BM or mobilised peripheral blood (PB) of healthy donors. The two main conditioning regimens used to bridge patients to allo-HSCT is reduced-intensity conditioning (RIC) and myeloablative conditioning (MAC), the former being less invasive using lower doses of chemotherapy thereby relying on graft versus leukemia (GvL) effect for leukemic clearance. MAC treatment on the other hand aims to clear the leukemia before allo-HSCT. Thus, the optimisation of RIC regimes has been fundamental to enable allo-HSCT treatment for older and more frail patients. As the general condition of the patient is decisive for preconditioning with RIC or MAC, few randomised clinical trials have been designed to directly compare the clinical outcomes following the two regimens for patients with MDS[152]. However, based on current available data, it appears that MAC is linked to increased treatment-related mortality, whereas relapse-related mortality is more prevalent after RIC[153,154], highlighting the need for more efficient RIC strategies and less toxic MAC-strategies. It nevertheless remains to be determined which of the two ultimately results in best long-term survival as the results are so far conflicting.

Healthy donor selection for allo-HSCT is based on matching of major histocompatibility complexes (MHC), referred to as human leukocyte antigens (HLA) in humans, between the donor and patient. The *HLA*-genes are however highly polymorphic resulting in a diverse HLA-repertoire among the population hindering identification of a suitable donor for many. Further, over one million $CD34^+$ cells/kg body weight is needed for a transplantation, which is not always

possible to obtain. This calls for optimisation of protocols which allows for ex-vivo expansion of HSC prior to transplantation[155].

Another type of stem cell transplantation used clinically is autologous HSCT where HSCs are isolated from the patient before start of heavy chemotherapies and later re-infused into the patient. Some genetic inherited disorders can be cured by ex-vivo manipulation of the autologous HSPCs prior to re-infusion to compensate for mutated HSPCs causing the disease[156], but apart from those instances, the curative potential of autologous transplantation is mediated by the high doses of chemotherapy, made tolerable due to the rescue transplant's ability to reinstate normal hematopoiesis. The therapeutic potential of an allo-HSCT is also partly mediated by the preconditioning chemotherapy, but also mainly by GvL effect. GvL is when alloreactive T cells in the donor graft elicit an immune reaction against the remaining leukemic cells[157] evidently shown to be important by the worse outcome of patients receiving donation from an identical twin[158–160]. An intentional minor HLA-mismatch between donor and patient can therefore be favourable to enhance the GvL effect[161]. This must nevertheless be well balanced as the same HLA-mismatched mechanism also mediates the potentially lethal graft versus host disease (GvHD) where donor T cells targets healthy tissues. The concept of GvL and how it is mediated by donor graft T cells has resulted in emergence of a new type of therapy for the hematological malignancies where the aim is to reprogramme the endogenous immune system to eliminate the cancer.

1.3.3 Immunotherapy

Individuals with an immunodeficiency disorder or who are on immunosuppressive treatment following an organ donation have an increased life-time risk of developing cancer[162,163] underscoring the anti-tumour potential of the immune system. The ability of cancer cells to evade the immune system has emerged as one of the recent hallmarks of cancer[85,86], prompting the development of treatments that exploit or boost the immune system to eradicate cancer. Today, immunotherapy is used in combination with standard therapies, such as chemotherapy, radiation, and surgery for certain cancers such as B-cell leukemia, melanoma and lung cancer, although it is still most often used as a second or third line of treatment[164,165]. The most broadly used immunotherapy is antibody mediated blockage of immune checkpoint molecules PD-1/PD-L1 and CTLA-4, a discovery that was awarded the Nobel Prize in Physiology or Medicine in 2018. This treatment reverts the immune dampening effect that tumour cells can have on the T cells, thus providing the endogenous T cells an opportunity to eliminate the tumour[166]. Clinically, immune checkpoint inhibitors have proven most effective

against solid cancers as indicated by FDA approvals of checkpoint inhibitors for treatment of 14 solid tumour types, but only two hematological malignancies[167]. Other emerging immunotherapies include bispecific T cell engagers (BiTE) which act by bringing cancer cells and T cells together[168] and cancer vaccines where an immunity is induced by a classical vaccination with tumour antigens[169] and adoptive T cell therapy, which so far has shown the most potent effect against hematological malignancies.

1.3.4 Adoptive T cell therapy

Adoptive T cell therapies, which include genetic modification and expansion of patient autologous T cells, revolves around the cytotoxic mechanisms by which T cells identify and eliminate tumour cells. Briefly, conventional $CD8^+$ cytotoxic α/β T cells have clonal T cell receptors (TCRs) on their surface that specifically recognise a given peptide presented on the surface through HLA class I-molecules. The HLA class-I molecule consists of an α-chain with three extracellular domains ($\alpha1$, $\alpha2$, $\alpha3$) folding into a structure containing a peptide binding cleft fitting peptides that are typically between eight to ten residues long, and is non-covalently associated with the $\beta2$-microglobulin chain[170]. All nucleated cells in the body can present peptides on HLA-I and the main function of this system is to present foreign peptides for detection by circulating T cells. Upon encounter of a peptide-HLA complex "classified" as foreign by the given T cell, a signalling cascade mediated by CD3 is initiated, eventually resulting in release of perforin and granzyme which induces cell death of the target cell (**Figure 5a**). The tremendous power of T cell mediated cytotoxicity goes far beyond any simplistic explanation and the $CD8^+$ T cells collaborate in a close and complex interplay with other immune cells, including the $CD4^+$ helper T cells and antigen presenting cells, and the effect is mediated by a constant balance between activating and inhibitory signals. Central for T cells role in immunity is their capacity to discriminate between self and non-self, necessary for T cells to spare endogenous healthy tissue. This distinction is named tolerance and takes place in the thymus in a stepwise process during T cell development where endogenous peptides are presented to the immature T cell pool. Positive selection ensures that T cells can recognise self-HLA molecules, while negative selection eliminates T cells with high affinity for self-antigens. This results in a diverse T cell repertoire with an estimation of $>10^{18}$ possible TCR rearrangements[171] without self-reactivity to presented peptides[157,172]. After thymic education, the T cell repertoire is, however, reduced to between 10^7-10^8 unique T-cell receptors that together should recognise all possible foreign antigens.

In chimeric antigen receptor (CAR) T cell therapy, the endogenous TCR on the T cells is exchanged to an artificial receptor built up by an intracellular signalling domain from a TCR, a short transmembrane domain and an extracellular antigen binding domain with a single-chain fragment variant from an antibody[173]. With the extracellular antibody domain linked to a functional intracellular signalling domain, the CAR T cells can identify specific surface antigens and elicit a cytotoxic immune response. CAR T cell therapy has successfully been used to treat lymphoid malignancies, and especially B cell malignancies targeting the lymphoid specific surface antigen CD19 and to some degree CD20. Both CD19 and CD20 are highly expressed on B-ALL cells and on many lymphoblastic lymphomas, however as these antigens are also expressed on normal B cells, treatment with CD19/CD20 CAR T cells results in B cell depletion[174–176]. Unlike depletion of T cells, which is associated with life threatening infections upon encounter of opportunistic pathogens[177], B cell depletion can be treated with immunoglobulins to compensate for the loss of B cells and is thus generally tolerable. This is demonstrated in patients with primary immunodeficient B cell aplasia who have manageable disease with continuous immunoglobulin treatment[178]. Unfortunately, CAR T cells against the progenitor and myeloid antigens CD33, CD123, KIT and FLT3 have not shown the same great clinical utility as the CD19/CD20 CAR T cells[179–183], likely due to the unacceptable prolonged myeloablation of the healthy myeloid lineages which must be overcome. For instance, limiting the CAR T cells persistence in vivo, applying a dual-target approach or epitope editing of the healthy cells are some approaches being explored for allowing CAR T cells to target surface antigens shared between cancer and healthy myeloid cells[184–187]. Until those approaches have been clinically established, most CAR T cell therapies targeting myeloid antigens are clinically evaluated as a preconditioning regimen for allo-HSCT[188].

In contrast to CAR T cells, TCR T cells, which are explored in this thesis, are genetically modified with an intact TCR of choice and can thus recognise intracellular antigens which increases the number of possible targets[189] (**Figure 5b**). Despite this, therapies with TCR T cells have so far not advanced as much as certain CAR T cells and there is currently no clinically approved TCR T cell therapy[190–192]. There are however several ongoing clinical trials with TCR T cells against both solid tumours[193] and hematological malignancies[194]. The results from initial Phase I studies of TCR T cells for patients with myeloid malignancies targeting wilms tumour 1 (WT1), preferentially expressed antigen in melanoma (PRAME) and minor histocompatibility antigens (MiHA) have shown that the antigen-specific TCR T cell can persist in the patient and that the response rates varied, but the treatment was generally tolerable with some reports of cytokine

release syndrome as an adverse event[194,195], as also seen following CAR T cell treatment[196]. Importantly, Chapuis and colleagues showed that prophylactic treatment of patients with AML post allo-HSCT with donor derived WT1 TCR T cells showed prolonged relapse free survival where none of the 12 treated patients experienced relapse during the median follow up of 44 months post allo-HSCT, compared to 46% observed relapses in the control group[197]. This indicates the potential clinical efficacy of TCR T cell treatment, but new targets and TCRs are needed to allow for more efficient treatment of additional patient groups.

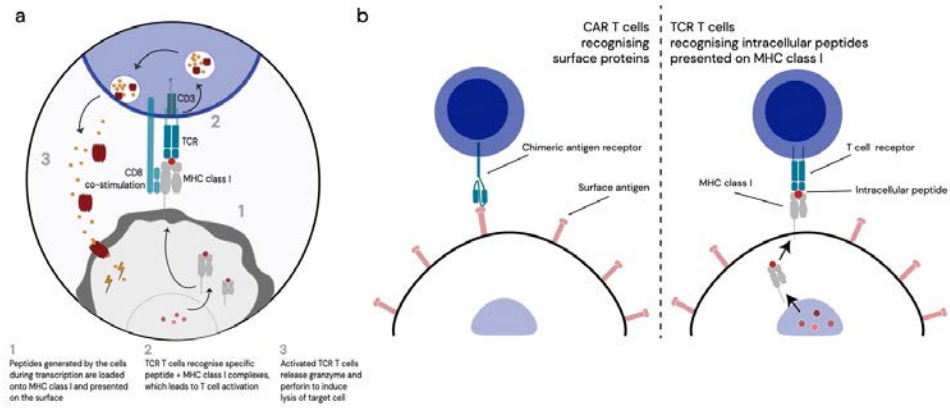

Figure 5. T cell activation is mediated through T cell receptor (TCR) and peptide-MHC class I interaction (a) Conventional cytotoxic T cell activation through intracellular peptides presented on restricted MHC class I (HLA) results in CD3 and CD8 mediated release of granzyme and perforin and lysis of target cell. **(b)** Chimeric antigen receptor (CAR) T cells recognising surface antigens and TCR T cells recognising intracellular peptides.

1.3.5 Antigen selection for adoptive T cell therapy

A target for adoptive T cell therapy needs to be visible to the T cells either as a surface antigen or peptide presented on the HLA-molecules. Ideally, a cancer antigen would be highly expressed by all cancer cells in all patients, with no expression in healthy cells. No currently known antigen meets these criteria. Instead, identification of antigens highly expressed by cancer cells in as many patients as possible with minimal expression in healthy cells and no expression in any vital organ is the aim. Further, an antigen with a function that is indispensable for the cancer cells would be beneficial as it reduces the risk of antigen loss as a resistance mechanism. Cancer antigens can roughly be classified into four categories; viral oncoproteins (not reviewed herein), tumour associated antigens (TAA), cancer/testis antigens and neoantigens[189,198]. TAA are antigens expressed by both normal and malignant cells but often to a higher

degree by the malignant cells[198]. A TAA serves as a potential treatment target if depletion of the normal cells expressing the antigen is tolerable or can be compensated for with additional treatments, exemplified by CD19 depletion by CAR T cells. Another example of a TAA is Terminal deoxynucleotidyl transferase (TdT) which is overexpressed on 80-94 % of lymphoblastic leukemias[199,200]. TdT is involved in V(D)J recombination of the B and T cell receptors, restricting its expression in normal tissues to early stages of B and T cell development[201,202]. Cancer/testis antigens are a type of TAA but normal expression is restricted to germ cells[203] which have limited expression of HLA-class I[204], making them attractive targets for TCR T cell based therapies in particular. New York Esophageal Squamous Cell Carcinoma-1 (NY-ESO-1), expressed by a wide range of solid tumour types is one of the cancer/testis antigens that has been widely adopted as a target for the development of immunotherapies[205]. Neoantigens are perhaps the most attractive targets as they are generated by mutated or abnormal genes that can be presented to the T cells by self-HLA and recognised as foreign[206]. Toxicities on healthy non-mutated cells are therefore limited with a neoantigen-specific T cell. However, very few of the candidate neoantigens generate an immunogenic HLA-presented peptide, and it has been shown for solid tumours that patient T cells recognise less than 2% of predicted neoantigens[206]. For therapeutic purposes, recurrent neoantigens shared between many patients covering as large fraction of the clones as possible are preferred targets. This excludes the majority of patient-specific neoantigens. As the myeloid malignancies are characterised by the presence of multiple recurrent genomic events and molecular profiling is used routinely in clinical diagnosis, these patients serve as source for identification of potential targetable neoantigens. In AML, *FLT3* mutations provide mutated cells with a proliferative advantage and are frequently (~30%) detected as a part of routine diagnostics[109,207]. Consequently, *FLT3* mutations constitute a potential set of neoantigens for adoptive T cell therapy. As the mutations or chromosomal abnormalities generating neoantigens are often somatically acquired later in life, T cells with a TCR targeting those have avoided the tolerance process. Thus, TCR T cells against such antigens might be present in the T cell repertoire from the patients or healthy donors both of which can be used as source for identification of antigen-specific TCR T cells for later clinical use[189,208,209].

1.3.6 Preclinical evaluation of adoptive T cell therapy

Following identification of a suitable antigen and a corresponding TCR for adoptive T cell therapy, the treatment needs to be carefully evaluated in terms of safety and efficacy in advanced preclinical models[189,210,211]. This involves extensive antigen-specificity screens of putative TCRs on the peptide level, bioinformatic

screens for potential cross-reactive peptides and functional testing against a wide range of cell lines of different tissue, and HLA-origins[211]. Although cell lines are a great preclinical tool to perform initial screens on, they lack physiological relevance, in particular if selected due to high expression of target antigen and relevant HLA. Further, cell lines do not reflect the heterogeneity found in primary cells. An important preclinical evaluation is therefore to test safety and efficacy of T cells on primary cells, but this does not come without challenge. As discussed above, the available in vitro assays suitable for studies on malignant hematopoiesis are few and is not applicable for all patient types, and access to patient material is limited. This might explain the limited reporting on effect and safety on primary cells in preclinical studies. The most physiological model system available for preclinical testing of T cell therapies targeting hematopoietic malignancies is humanised mouse models where the normal hematopoietic system is derived from healthy human HSCs for safety, and patient derived xenograft (PDX) models for efficacy. It is also possible to create models that permit concurrent investigations of safety and efficacy in the same system. This may involve engrafting $CD34^+$ humanised mice with a leukemic cell line before the engrafted $CD34^+$ HSPCs has had time to generate endogenous T cells which would likely mediate rejection of the cell line[212] or transducing HSPCs to induce leukemia subsequentially allowing for engraftment of both transduced and non-transduced fractions from the same donor[213]. In some cases of myeloid leukemia, residual normal HSCs can contribute to the graft thus generating both healthy and malignant hematopoiesis in the same mouse. This also enables the examination of T cell effects in the presence of other human immune components, a feature absent in conventional PDX models utilising immunodeficient mice.

A drawback with the in vivo models is the short follow up time it commonly allows for due to either xenoreactivity, where the infused T cells target the endogenous mouse cells, or alloreactivity where the infused T cells target the engrafted leukemia, confounding the therapeutic effect[214]. In these cases, the endogenous TCR repertoires of the genetically modified TCRs are causing xeno- and allo-reactivity. This can be mitigated by knocking out the endogenous TCRs in conjunction with introduction of the therapeutic TCRs. Utilisation of humanised mice where the healthy HSCs engrafted into the mice generates human T cells allows for isolation and transduction of those for testing of safety, which limits risk for alloreactivity thus allowing for longer follow up. Further it also mimics the clinically relevant setting of autologous T cell use for adoptive T cell therapy.

The short follow up in PDX models makes it particularly challenging to evaluate if resistance mechanisms develop over time, including downregulation or modulation of target antigen, downregulation of any essential proteins in the

antigen presentation machinery, upregulation of immune checkpoint inhibitors or non-persistent T cells, mediating escape from the cytotoxic T cells[215,216]. Preclinical evaluation of LSC elimination in vivo is also hindered by the short follow up as LSCs mediated relapse can occur long after initial treatment efficacy. Relapse in humans caused by therapy resistant LSCs can occur several years after initial remission although most relapses occur within five years[217,218]. A lack of observed relapse in PDX-treated mice might also reflect persisting T cells continuously suppressing the leukemia, rather than a complete elimination of LSCs, which could be assessed by secondary transplantation.

1.3.7 Measurable residual disease assessment after treatment

Regardless of treatment strategy, but particularly following an allo-HSCT the primary concern for patients with hematological malignancies is the occurrence of relapse after initial successful treatment[219–221]. Early detection of an impending relapse is thus aspirational so pre-emptive treatment can be initiated. A clinically used approach to monitor patients after treatment is analysis of measurable residual disease (MRD). MRD refers to the detection of leukemic cells above a certain set threshold based on lowest limit of detection, most often 0.1% with current assays[222]. Numerous clinical studies, mostly in AML, have shown that presence of MRD in patients with myeloid malignancies before allo-HSCT is associated with worst outcome, highlighting the importance of effective pre-transplantation conditioning[223–225]. MRD-positivity after treatment has also been associated with worse outcome[223,224,226]. The most widely used methods for MRD-analysis is flow cytometry, $CD34^+$ donor chimerism or molecular analysis using PCR or next-generation sequencing (NGS)[222,227]. Molecular monitoring of MRD is particularly relevant for patients with myeloid malignancies where the relapse is often mediated by a similar mutational landscape as the original disease[228,229], allowing for personalised targeted screening for the disease specific mutations. Although an MRD-positive finding after treatment is indicative of an impending relapse and worse outcome, there are patients with MRD-positivity who do not progress and vice versa[230,231] highlighting the need for improved strategies. Molecular MRD-analysis is commonly performed on unfractionated BM mononuclear cells (MNCs) but has recently been explored in purified $CD34^+$ cells from BM and PB which showed improved sensitivity down to 0.01%[232].

With emerging new MRD-assays with higher specificity there is also an imminent need for improved therapeutic options targeting the relapse initiating cells. With the identification of LSCs that selectively escape treatment and subsequently cause the relapse in AML, low to intermediate risk MDS and CML patients[233–237] targeted therapies against LSCs can be explored. However, the heterogeneity of

the diseases remains a challenge towards development of standardised therapies targeting LSCs.

1.3.8 Strategies targeting LSCs

The concept to specifically target LSCs in myeloid malignancies has become a scientific focal point in recent years, as highlighted by the many reviews published on the matter[217,238–241]. Some of the strategies that have been explored are induction of cell cycle entry to enhance susceptibility to chemotherapy, targeting of self-renewal pathways and perturbation of oxidative phosphorylation, which is suggested to the main energy source for LSCs[87,231,242,243]. Identification of surface antigens that would allow for distinction between LSCs and HSCs is much desired as it would facilitate studies of LSCs and importantly allow for targeted therapies against the antigens without affecting healthy HSCs. To this end, a broad range of surface antigens aberrantly expressed on $CD34^{+}CD38^{-}$ cells have been suggested to distinguish healthy HSCs from LSCs, including CD33[244], CD44[245], CD47[246], CD123[247], CD45RA[247], CLL-1[248], TIM-3[249] and IL1RAP[250] to mention a few. It however remains elusive if any of these antigens cover the entire stem cell population. The clinical relevancy to target these proposed LSC-antigens is yet to be determine as clinical trials are still ongoing with monoclonal antibodies, BiTEs and CAR T cells targeting these antigens as reviewed by Valent and colleagues[240]. All these antigens are also expressed on normal cells, and some even on HSCs but at lower levels (CD33 and CD123), which means that hematopoietic toxicities must be rescued by an allo-HSCT as shown recently through the results from the first in human Phase I CAR T cell therapy trial against CLL-1. Some patients showed promising response, but this was associated with severe cytokine release syndrome and pan-cytopenia which needed to be rescued with an allo-HSCT[240,251]. Thus, there is still no LSC targeted therapy that can be used to treat an impending relapse post allo-HSCT.

The presence of oncogenic driver mutations in the LSC compartment of the myeloid malignancies opens an avenue of targeting the neoantigen to eliminate the malignant clone while sparing healthy hematopoiesis. However, since expression of these antigens in LSCs often is linked with co-expression in the blasts, and that all LSCs might not harbour the targetable neoantigen, none of these antigens facilitate the study of exploring the dependency of LSCs in leukemic propagation.

1.4 Summary

Taken together, there is substantial evidence emphasising the importance of eradicating LSCs during cancer treatment, as they have been shown to

selectively escape treatment and cause relapse. Yet, as selective elimination of LSCs has not been accomplished, full clinical and biological relevancy of the LSC concept remains elusive. T cell mediated immunotherapy is emerging as a new pilar of cancer treatment and has shown immense effects on the treatment of hematological malignancies and is now starting to be explored against LSC driven myeloid leukemias. However, none of the suggested LSC-targets are widely and homogenously expressed on all patients which limits the clinical use and warrants for identification of new targets. To achieve this end, a continued detailed characterisation of the LSCs and their implication for pathogenesis is needed.

2 Research aims

The overall aim of this thesis is to explore if antigen specific T cells can be an efficient means to target therapy resistant LSCs, responsible for driving myeloid leukemia and the subsequent relapse after treatment. This thesis investigates the efficacy, safety, and effects of TCR based immunotherapy against different hematological malignancies. The specific aims for the four included studies are:

Study I – Investigate if purification of rare LSCs post allo-HSCT in patients with MDS can enhance sensitivity of mutational MRD-screening to allow for earlier prediction of relapse. In particular, I investigated if LSC-specific mutational screening would be a more reliable MRD-method compared to currently available flow cytometric analysis.

Study II - Investigate if TCR T cells targeting TdT could serve as an effective adoptive T cell therapy against patients with TdT^+ ALL. Specifically, I studied the safety and efficacy of TdT-TCR T cells against primary ALL in PDX and humanised mouse models.

Study III – Investigate if targeting of a shared neoantigen in AML using TCR T cells would serve as a potential therapeutic option. As in **study II**, I specifically investigated safety and efficacy of the neoantigen-specific TCR T cells in primary PDX models, as well as the effect on the proposed LSCs.

Study IV – Investigate if MPL is a suitable target for T cell mediated specific elimination of therapy resistant LSCs in myeloid malignancies.

3 Materials and methods

Detailed methods are described in the four individual studies. Below is a summary of the most relevant methods for the work of this thesis.

3.1 Ethical consideration

All studies involving human samples were approved by Swedish Ethical Review Authority, Stockholm (EPN 2018/901-31 and EPN 2017/1090-31/4) and performed according to the Declaration of Helsinki. All animal studies were approved by Stockholms Djurförsöksetiska nämnd (Dnr 17978-2018 with amendments 18539-2021). Other research groups at Karolinska Institutet, the University of Oslo and Aarhus University Hospital have performed experiments included in this thesis with separate original ethical approvals (see **study II-IV**).

Contributing to the preparation of a new ethical application for animal studies, as well as amendments, stimulated reflection of refinement, reduction, and replacement of animal experiments, which has been incorporated into the work included in this thesis. This includes for instance sequential blood and bone marrow sampling from the mice instead of using unique mice for each timepoint, training animals to handling before the use in experiments and adding aids to enhance mouse welfare during procedures. Mouse models are still the most useful tool for studies of HSCs as the in-situ interactions between HSCs and the niche has so far not been well recapitulated in vitro, but alternative strategies have been used in this thesis when possible. A continuous ethical reflection related to animal experiments has been upheld through participation in educational seminars, both theoretic and practical, and close contact with trained staff at the animal facilities, as well as with veterinarians. Further, according to ethical responsibility towards reproducibility and documentation of experimental results, all experimental data (including raw data, analysed data and experimental protocols) included in this thesis and the correlated studies have been carefully double checked by at least one other scientist.

3.2 Mouse models

The golden standard assay to evaluate human HSC/LSC function is xenotransplantation into mice. PDX models and humanised mice were generated by transplantation of leukemic or normal BM/CB cells into the tail vein or the femur of immunodeficient mice, either NSG (NOD.Cg-Prkdc scid Il2rg tm1Wjl /SzJ) or NSG-SGM3 (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl} Tg(CMV-IL3,CSF2,KITLG)1Eav/MloySzJ), where the latter strain specifically supports engraftment of human myeloid lineages by expression of human IL-3, GM-CSF and SCF. Stock mice were

obtained from the Jackson Laboratory and bred in-house. For some experiments evaluating TCR T cells effect in vivo on either healthy and malignant hematopoiesis, mice engrafted with HLA-$A2^+$ healthy $CD34^+$ CB cells (**study II**) or *FLT3*-D835Y mutated BM AML cells (**study III**) respectively were obtained from the Jackson Laboratory. All mice were housed 2-5 mice per cage in IVC-Mouse GM500 cages in 21°C with 45-50 % humidity and a 12-hour light cycle at Karolinska Institutet animal facilities KM-A, KM-B, KM-W and AFL.

3.3 Isolation of hematopoietic tissues

3.3.1 Human

PB and BM aspirations were collected by treating physicians and processed into MNCs by Ficoll separation by biobank scientists. Cells were then cryopreserved for later use. Prior to analysis, human MNCs were thawed in 37°C water bath and dropwise diluted in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 20 % (vol/vol) fetal calf serum (FCS) and DNase (0.1 mg/mL) followed by a gentle centrifugation at 200 *g* for 15 minutes at room temperature.

3.3.2 Mouse

PB was collected by tail vein bleeding or cardiac puncture into Ethylenediaminetetraacetic acid (EDTA) coated tubes to prevent clotting. The PB was diluted 1:1 in Dulbecco's phosphate buffered saline (DPBS) and mixed 1:1 with 2% dextran and incubated for 20-25 minutes in 37°C water bath to allow for separation between erythrocytes and leukocytes. The leukocyte fraction was collected and exposed to ammonium chloride (NH_4Cl) for 2 minutes to lyse the remaining erythrocytes.

BM was collected on live anaesthetised mice through aspirates of the femur to analyse BM composition over time or BM leukemic burden pre-TCR T cell treatment. At termination, BM from both hind legs (femur, tibia, and pelvic bone) was collected for analysis. BM aspirates were exposed to ammonium chloride for 2 minutes to lyse erythrocytes. Whole bones were crushed with mortar and pestle into DPBS supplemented with 2-5% FCS and 2 mM EDTA (FACS buffer) and filtered through a 40 µM pore cell strainer. Spleen and thymus were collected and gently crushed through a 40 µM pore cell strainer in FACS buffer.

Cell counts were determined on a Sysmex hematology analyser XP-300.

3.4 Flow cytometry

Flow cytometry was a central method for generating the data presented in this thesis. The hematopoietic hierarchy has been well defined in both mouse and

human using flow cytometry, where surface markers can separate the different cell types with high specificity. Flow cytometry was therefore used to phenotypically analyse HSPCs from normal donors and patients, to identify antigen specific T cells, to sort various hematopoietic cell types for further analysis and as a readout for functional tests of T cells. Although flow cytometry is very useful tool, it cannot be used as a definitive method to study HSCs, as HSCs can only be accurately defined based on functionality. Therefore, phenotypic flow cytometry analysis was accompanied with functional stem cell assays.

For phenotypic analysis and cell sorting, hematopoietic tissues from mouse and human were collected as described above and incubated with Fc receptor (FcR) blocking antibody to prevent binding of the Fc region of monoclonal antibodies to the FcR expressed on various hematopoietic cells. Following 10-15 minutes FcR-block, the cells were incubated with different combinations of fluorescent conjugated monoclonal antibodies for 15-30 minutes in dark, 4°C to allow for identification of different cell types using CD-proteins as surface markers. Samples were then subjected to multiparametric analysis on a BD LSRFortessa or sorted on a BD FACSAria Fusion. Most sorts were performed with aseptic technique to reduce risk of sample contamination. Compensation controls and fluorescence minus one (FMO) controls, and for some experiments also isotype controls, were used, and data was analysed using FlowJo™ software. Gating was performed using a combination of FMOs, a known antigen-negative cell population, isotype controls, back-gating, and population-based gating. Phenotypic definitions of human HSPCs and mature PB lineages mostly utilised in this thesis are outlined in **Table 2.** The detailed information about all monoclonal antibodies is collected in the respective manuscripts of **study I-IV**.

3.5 TCR T cell identification

A close collaboration with Professor Johanna Olweus and her group at the University of Oslo was established to identify the therapeutic T cells used in this thesis according to previously published methods with minor modifications[209]. The T cell repertoire from healthy donors is extremely diverse (10^7-10^8 unique sequences[252,253]) and can thus be used as a source to identify antigen specific TCRs for therapeutic purposes, but it can be compared to identifying a needle in a haystack. To allow for identification of T cells against self-antigens (TdT and MPL) restricted to one of the most frequent HLA-alleles (HLA-A*02:01 hereon referred to as HLA-A2), PB MNCs from HLA-$A2^-$ donors were used as T cell source. For discovery of T cells against a neoantigen (FLT3-D835Y), HLA-$A2^+$ donors could be used as the T cell repertoire has not undergone thymic selection against the neoantigens. Cultures were set up between donor T cells and target cells

presenting the antigen of choice, followed by flow cytometry screening using peptide-specific HLA-A2-tetramers. To increase specificity, each specific HLA-A2-tetramer was conjugated to two independent streptavidin-conjugates and only double positive T cells were considered positive[254] after applying Boolean gating. Identified T cells were then functionally tested by activation and killing assays using target cell lines either naturally expressing the target antigen or loaded with the target peptide onto the HLA-class I molecules. The antigen-negative cell lines without peptide loading were used as negative controls. The TCRs from the promising T cell clones were sequenced and later transduced into healthy donor T cells to generate a T cell pool with the TCR of interest to be used for further experiments. Preferential pairing of the introduced α- and β-chains of the TCR was achieved by inclusion of the constant mouse (m) TCRβ-chain, which also allowed for identification of the T cells expressing the introduced TCR through a commercially available monoclonal antibody against mTCRβ.

3.6 TCR T cells in vitro effect on target cell lines

The transduced TCR T cells were tested for efficacy and specificity using cancer cell lines as targets. Both naturally antigen expressing, and peptide loaded, as well as HLA-A2$^+$ and HLA-A2$^-$ cell lines were used. Prior to cultures, either the cell lines or the T cells were labelled with cell trace violet for 5 minutes in the dark to distinguish the two cell types during FACS analysis in combination with additional cell line specific (e.g. MPL, CD19) or T cell specific (CD8, CD3, CD137) surface antigens. T cells were cultured with the cell lines at different effector to target (E:T) ratios for 24-72 hours. As positive control, target cell lines were pre-loaded with the target peptides and cultured with the T cells as described above. After the culture, remaining cells were stained with fluorescent conjugated antibodies and subjected to flow cytometric analysis. Initial screens assessed T cell activation through upregulation of CD137 on CD8$^+$ T cell or IFN-γ production. However, it was noticed in some experiments that CD137 upregulation failed to reliably predict the killing capacity of the T cells, so T cell activation analysis was coupled to killing analysis by quantification of remaining target cells after co-cultures. To allow for exact quantification of remaining target cells, CountBright™ Absolute Counting Beads were added to each sample and equal number of bead events were acquired and cell numbers were calculated through (cell count/counting beads per sample) x counting beads count.

3.7 Evaluation of HSPC function in vitro

As HSPCs are defined based on function and not phenotype and that, unlike cell lines, access to primary material is limited, flow cytometry is not a suitable assay for evaluating HSPC functions or what effect TCR T cells have had on these

compartments. Instead, we utilised established hematopoietic assays and the unique functional properties of HSPCs generating colonies when cultured in semi-solid medium for the line of experiments evaluating both unique stem cell properties and what effect TCR T cells have on HSPCs. For evaluating long term stem cell self-renewal functionality in vitro, long-term culture colony forming cell (LTC-CFC) assays were performed where HSPCs from healthy donors or patients were sorted and placed into culture for 6 weeks (in which only cells with self-renewal capacity will survive) before transfer to methylcellulose to support colony generation. After 10-14 days in methylcellulose, the colonies generated from the surviving cells were scored under an inverted microscope as either myeloid or erythroid according to StemCell Technologies instructions.

For evaluating TCR T cells effect on primary HSPCs, short term colony forming cell (CFC) assay was performed. BM cells were sorted and placed into culture for 48-72 hours together with TCR T cells sorted as $CD4^{-}CD19^{-}$. As a positive control, the BM cells were externally loaded with 1 μM of target peptide for 2 hours, followed by a 48-hour co-culture with or without TCR T cells in the presence of 100 nM peptides. After the co-cultures, the remaining product was transferred to methylcellulose and colonies were scored as described above. This experimental set up allows us to quantify the number of viable functional HSPCs after co-culture with the TCR T cells (which do not generate any colonies) as one counted colony is a progeny of one functional HSPC. Culturing conditions for both assays were 37°C, 5% CO_2 and samples were kept in a humidity box within the incubator.

3.8 In vivo studies on primary normal and malignant cells

To investigate human HSC function and the TCR T cells effect in vivo on primary cells, both humanised mice engrafted with BM/CB from healthy donors and PDX mice engrafted with cells from patients with various types of leukemia were generated. Immunodeficient mice (NSG or NSG-SGM3) of different ages were non-myeloablative irradiated (2.5-3.3 Gy for 8–12-week-old mice and 1.25 Gy for 3–4-week-old mice, CIX2/CIX3 Xstrahl X-ray cabinets) to create space for the engrafting cells in the BM and transplanted with human BM and CB cells through the tail vein or intrafemorally. The transplanted cells were always depleted for $CD3^{+}$ T cells to prevent xenoreactivity either by CD3-depletion, CD34-enrichment by beads (Miltenyi) or sorting of purified HSPCs. Primary human engraftment was confirmed and monitored in PB or BM by flow cytometry. In experiments assessing TCR T cells effect, the mice were allocated to treatment groups based on engraftment level to ensure there were no significant differences between the experimental groups before treatment. Three days prior to treatment, TCR T cells were thawed and kept in culture prior to intravenously injections into the tail vein.

The TCR T cell dose varied between 5-10 x 10^6 cells between experiments and the mice were injected i.p daily with IL-2 to support the T cells. The hematopoietic engraftment and effect of the T cells was followed continuously through flow cytometry analysis of PB. As the transduction efficacy of the T cells was never 100% in our studies, some T cells maintained their endogenous TCR which could elicit an alloreactive response toward the leukemia and confound the results of the effect of the therapeutic TCR. Therefore, the experiments were ended either at signs of leukemia-related illness, or when signs of alloreactivity were observed measured by a leukemic reduction in the control treated group or an expansion of $CD4^+$ T cells. At endpoint of all type of in vivo experiments, PB and BM, and in some cases spleen and thymus, were subjected to detailed flow cytometric analysis. The in vivo studies performed herein are powerful tools to model human hematopoiesis in mice, however limitations lie in general poor engraftment capacities of cells from patients with myeloid malignancies, the challenge to follow TCR T cell treated mice long term due to xeno- and alloreactivity, and the lack of innate immunity in the immunodeficient mice, which precludes any observations on the potential interaction with the immune system as discussed in the introduction.

3.9 Droplet digital PCR

For mutational analysis we performed droplet digital PCR (ddPCR) according to manufacturer's instructions (Bio-Rad) on DNA isolated from sorted populations subjected to whole-genome DNA amplification using the REPLI-g Single Cell Kit (Qiagen). Mutation-specific primer-probes (Bio-Rad) were designed for each assay based on mutations identified through whole exome sequencing or targeted sequencing. The experimental samples were subjected to the PCR and compared to normal non-mutated bone marrow, non-template amplified samples and H_2O to minimise false positive interpretation of the results. For analysis, plates were read on a QX200 droplet reader (Bio-Rad) and QuantaSoft version 1.5.38.1118 (Bio-Rad) was used for numerical quantification of VAF as fractional abundance based on Poisson distribution.

3.10 Statistical analysis and data reproducibility

Most statistical analyses were performed using GraphPad Prism, version 6-9 generally using non-parametric two tailed Mann-Whitney test or Kruskal-Wallis one-way ANOVA by Dunn's multiple comparison test to assess differences based on ranks between two or three treatment groups respectively. Although the statistical power of parametric t-tests is greater, non-parametric tests were preferred to compare differences between treatment groups as normal distribution of the mean was not assumed nor tested due to the relatively small

sample size in each treatment group (n = 4–8). Further, the effect of the TCR T cells were in most cases so profound that the significant effects could clearly be observed even without statistical tests, motivating the usage of the less powerful non-parametric tests over the assumption-based parametric tests. Estimation of samples sizes for mouse studies was most often based on smaller pilot experiments, however a minimum of four mice per treatment group were always included. Most experiments were performed more than once to support reproducibility of the findings. Dr Tetsuichi Yoshizato supported with statistical analysis using multilevel linear regression with the R package 'lmerTest' in **study III**.

Table 2. Immunophenotypic definitions of human hematopoietic cells used throughout these studies. See **Figure 2** for hierarchical organisation.

Population	Phenotypic definition
HSCs	$Lineage^-CD34^+CD38^-CD90^+CD45RA^-$
MPPs	$Lineage^-CD34^+CD38^-CD90^-CD45RA^-$
LMPPs	$Lineage^-CD34^+CD38^-CD90^-CD45RA^+$
CMPs	$Lineage^-CD34^+CD38^+CD123^+CD45RA^-$
GMPs	$Lineage^-CD34^+CD38^+CD123^+CD45RA^+$
MEPs	$Lineage^-CD34^+CD38^+CD123^-CD45RA^-$
MkPs	$Lineage^-CD34^+CD38^+CD41^+$
Total HSPCs	$CD34^+$/$Lineage^-CD34^+$
Myeloid cells	$CD33^+CD19^-CD3^-$
B cells	$CD33^-CD19^+CD3^-$
T cells	$CD33^-CD19^-CD3^+$
CD4/CD8 T cells	$CD33^-CD19^-CD3^+CD8^+$ and/or $CD4^+$
Therapeutic TCR T cells	$CD33^-CD19^-CD3^+CD8^+mTCR\beta^+$
Lineage cocktail	CD2, CD3, CD4, CD7, CD8a, CD10, CD11b, CD14, CD19, CD20, CD56, CD235a,b

HSCs: Hematopoietic stem cells, MPPs: Multipotent progenitors, LMPPs: Lympho-myeloid primed progenitors, CMPs: Common myeloid progenitors, GMPs: Granulocyte-monocyte progenitors, MEPs: Megakaryocyte-Erythroid progenitors, MkP: Megakaryocyte progenitors.

4 Results and Discussion

Much of what has been produced in the scope of this thesis has been parts of published manuscripts (**study I-III**). **Study IV** is still ongoing, but the main findings are presented and discussed in the corresponding manuscript. Therefore, the following section will rather reflect a short rationalisation for each study, and a summary of the results, followed by a targeted discussion regarding outcomes and implications related to our reported findings. The referencing to figures in this section refers to the figures in the corresponding publications/manuscripts.

4.1 Study I - Identification and surveillance of rare relapse-initiating stem cells during complete remission post-transplantation

Patients with MDS can only be cured by an allo-HSCT, but relapse is nevertheless common, and subsequent prognosis is very poor[255]. Today, disease progression post allo-HSCT is usually monitored in clinical practice through donor chimerism analysis using short tandem repeats to distinguish donor and patient DNA in distinct populations where the most predictive measure for relapse is a decrease in donor $CD34^+$ chimerism over time[256]. Clinical interventional studies with pre-emptive Azacytidine treatment based on decreasing donor $CD34^+$ chimerism while in complete remission (CR) showed prolonged relapse free survival during the follow up period[257,258], which highlights the clinical relevancy of earlier detection of an impending relapse. Recently, presence of MRD at 30 days post allo-HSCT has been shown to correlate with progression of patients with MDS, using both flow cytometric and molecular based methods[230,259]. However, while using a sensitivity threshold of 0.1-0.5% on unfractionated BM in these studies, there were still both false-positive and false-negative results, highlighting the need for improved strategies.

Based on the rationales that LSCs are confided to the HSC compartment in low to intermediate risk MDS, that primitive mutated HSPCs are sustaining the leukemia in patients with MDS, and that they are the cellular source of relapse[129,234], we hypothesised that enhanced sensitivity and potentially also specificity could be achieved for molecular MRD-analysis post allo-HSCT in patients with MDS (MDS, MDS/MPN and AML-MRC) by purification of the HSPCs. To this end, oncogenic lesions were identified through targeted DNA sequencing in diagnostic samples from a cohort of 29 MDS patients that had previously undergone allo-HSCT (Fig. 1). Of these, 16 experienced relapses, while 13 remained in continuous-CR during the 66-101 months follow up period. In retrospective analyses, BM HSPCs from multiple timepoints post allo-HSCT were purified and sorted from the patients while they were in CR without clinical signs of disease. The sorted HSPCs were

then subjected to targeted ddPCR for the mutations identified at diagnosis, and through this approach the sensitivity increased 97-fold compared to performing molecular MRD-analysis on total BM MNCs (Fig. 3). The specificity was also pronounced, as we only observed one MRD-positive sample among all 54 samples analysed from the 13 patients who were in continuous complete-CR more than 6 years after transplantation (Fig. 4), and therefore unlikely to relapse. In the only MRD^+ case, subsequent samples were consistent MRD-negative, and the first MRD^+ sample might therefore reflect remaining leukemic cells early post allo-HSCT. This finding nevertheless showcases the importance of sequential sampling and analysis to prevent overtreatment.

As implementation of molecular MRD-analysis on sorted HSPCs in the clinical practice may prove to be cumbersome and costly, we also compared the sensitivity and specificity to alternative MRD-methods. This included analysis for patient-specific single nucleotide polymorphisms (SNPs) and flow cytometric analysis for aberrantly expressed antigens using panels recommended by European LeukemiaNet (ELN)[222,260,261], which is a is a cooperative network that gathers leading researchers within the field of leukemia. Through the SNP-analysis, the sensitivity remained high, but the leukemic-specificity was reduced as SNPs were detected in all six analysed patients that remained relapse-free during the follow up of >66 months, although at low levels (Fig. 4). The use of flow cytometric analysis for diagnostic and prognostic purposes for MDS is relatively new and recommendations have been delayed compared to AML, thus MDS flow cytometric analysis is still often conducted according to AML recommendations[227,262]. While flow cytometric MRD analysis can be useful for distinguishing leukemic aberrant phenotypes compared to normal blood cells, its effectiveness is constrained by the non-uniform expression of surface markers in leukemic patients. In some cases, one aberrant phenotype may not encompass all leukemic cells. Antigen expression varies greatly among patients, but the most common leukemic phenotypes are, expression of lymphoid-restricted antigens on non-lymphoid cells, overexpression of an antigen compared to healthy cells or loss of antigens[263,264]. It is, therefore, important to ensure purity as contaminating normal blood cells with expression of the antigen would lead to a false-positive MRD result. This is important as there is a significant need for high-quality and standardised workflow for detection of aberrant phenotypes, especially in the very rare LSCs[260,265,266]. The evidence for any prognostic or predictive value of flow cytometric MRD analysis for patients with MDS post allo-HSCT has therefore been lacking, but recent studies monitoring post-allo HSCT MRD by flow cytometry in both MDS and AML have shown that a positive MRD-finding correlates with worse outcome and can on a group level predict relapse[223,259,267,268]. Flow cytometry MRD

analysis was therefore performed using two separate panels, one focusing on detection of leukemic blasts and one for identification of the proposed LSCs[261,265], where the latter has recently been suggested to be superior in predicting outcome post allo-HSCT in AML[268]. In our study, neither of these panels could confidently distinguishing leukemic cells from normal cells, unless the leukemic burden was high (Fig. 5). Although the flow cytometry based MRD-analysis was performed with a limited number of markers on a small number of patients, all of which never experienced relapse, our consistent observation that the presence of the selected pre-defined leukemic phenotypes[269] could not be distinguished between normal donors and the patients pre and post allo-HSCT on a group level, was sufficient to conclude that it lacks coverage for many MDS patients. This is in line with previous studies using flow cytometry on patients with MDS[267,270]. We therefore did not extend that analysis further.

The data presented herein showcases several important aspects. First, it provides further molecular characterisation of LSCs in patients with MDS and gives evidence for the LSCs role in mediating the relapse post allo-HSCT. The role of clonally involved HSCs to mediate disease initiation and relapse have previously been established in low to intermediate risk MDS as discussed above. In this study, the data supports that clonally involved HSPCs selectively evade toxic preconditioning and immune-mediated cytotoxicity following allo-HSCT in the more advanced cases of MDS as well. Further, it showcases a platform that enables the confident detection of mutated HSPCs. Although this translated into a theoretical prediction of relapse on average ten months before clinical diagnosis (Fig. 3), absolute evidence for MRD-positivity being indicative of relapse is still lacking. Furthermore, it cannot provide strong predictive measures for which patients will not relapse, as the relapse in some cases are mediated by new somatic mutations or cytogenetic changes not dominating at diagnosis[228]. Therefore, further improvements are needed before clinical implementation. To avoid overtreatment, a possible measurement to guide clinicians could be an increase in VAF of the mutations in the purified HSPCs, as this would likely be a more reliable predictive indicator of an impending relapse compared to a positive result at a single timepoint. The enhanced MRD-sensitivity from purification of HSPCs for the mutational analysis presented herein is then useful. Our study was performed on a heterogenous and limited patient group in a retrospective manner where clinical outcome was already known with variability in follow-up time, and with samples collected at different intervals and timepoints for each patient, all of which hampers the translation of our findings into clinical practice. Our flow cytometric MRD-analysis, although limited, further contributes to the notion that HSCs and LSCs cannot be distinguished solely based on existing surface markers. So, although the clinical implications and relevance of the proposed approach is

yet to be determined by comprehensive clinical studies, this study provides an important proof of concept of the importance of directing MRD-analysis and treatment specifically against the relapse initiating cells. Currently, a clinical interventional study following a clinically adapted protocol is being conducted based on the findings presented herein and a subsequent clinical study[271]. This work aims to investigate if pre-emptive treatment of patients with MRD-positivity post allo-HSCT can improve outcome (NCT05788679). Donor lymphocyte infusions is one of the available post allo-HSCT treatment options that have shown to be effective, but most patients will still progress[272] highlighting the efficacy of, but also need for improved, immunotherapeutic options for targeted elimination of the LSCs. The results from Chapuis and colleagues showing that AML patients treated with WT1 TCR T cells post allo-HSCT had longer relapse free survival compared to controls[197] is indicative that targeted TCR therapies have a great clinical benefit in this setting.

4.2 Study II - T cells targeted to TdT kill leukemic lymphoblasts while sparing normal lymphocytes

T cell mediated immunotherapy with CAR T cells against $CD19^+$ B-ALL and lymphoma has been shown to provide a new potent treatment option for previously incurable patients. However, relapse is often mediated by downregulation of CD19, and is observed in almost half of the patients[273–275]. This necessitates the development of new treatment alternatives. We suggest TdT as a targetable TAA, overexpressed in almost all patients with B and T-ALL. TdT is localised intracellularly which makes it inaccessible to CAR T cells, but available for TCR T cell treatment.

Professor Johanna Olweus' group at the University of Oslo previously established a strategy for identification of neoantigen restricted TCR T cells from the blood of heathy donors[208,209]. Modifications to this protocol has now enabled identification of TCR T cells reactive to self-antigens, such as TdT, in context of foreign HLA-class I molecules. Through this approach, two TdT-reactive TCR T cells (T1 and T3) restricted to HLA-A2 were identified and carefully validated by the Olweus group (Fig. 1). Of note, there was no evidence of any other naturally occurring 9- or 11-mer peptides in the human proteome that could induce a T cell response mediated by either of the TdT TCRs (Fig. 2), which diminishes the risk for off-target reactivity of the proposed TCRs. The TdT TCR T cell effect was investigated in vivo on xenograft models of two $TdT^+HLA\text{-}A2^+$ leukemic cell lines (NALM6 and BV173) and in vitro on multiple primary ALL patients of both B and T cell origin. The T3 TCR T cells showed a particularly striking efficacy against both the aggressive leukemic cell lines (Fig. 3) and primary patient cells, while progenitor cells and mature B and

T cells were spared (Fig. 4). These results show strong support for safety and efficacy of TdT TCR T cells against TdT$^+$ B- and T-ALL. To provide further evidence for this in more clinically relevant models, we initiated a collaboration with Olweus group.

Through this collaboration, we established two in vivo models generated from a representative patient with B-ALL and from healthy human CB cells (obtained from The Jackson Laboratory) to study the efficacy and safety of the T3 TdT TCR T cells in vivo, respectively. Compared to the control groups – either untreated mice or mice treated with DMF5 TCR T cells (targeting the MART-1 antigen[276]) which had high BM leukemic burden (mean 42.9 ± 3.4%; and mean 40.1 ± 4.5% respectively), the PDX mice treated with T3 TdT TCR T cells had minimal levels of detectable leukemic cells remaining in the BM (0.009 ± 0.005%), blood, and spleen at time of termination eleven days post treatment (Fig. 5). Further, no concerning toxicities on healthy hematopoiesis in vitro or in vivo in the humanised mouse model were observed (Fig. 6). We investigated potential toxicities of the T cell lineages in depth, as potential ablation of T cells would lead to severe immune defects, and exposure to any pathogen would become life-threatening. Importantly we did not observe any significant difference in T cell distribution or the number of TdT$^+$ cells in the thymus after T3 TdT TCR treatment. This is likely explained by the mutually exclusive expression pattern of TdT and HLA-A2 that we observed in human thymocytes (Fig. 6).

Although very efficient elimination of leukemic cells was observed after only eleven days post treatment, the curative potential could not be investigated due to the short follow up the PDX model allowed for due to developing alloreactivity of the infused T cells as observed by the starting reduction of leukemic cells in the DMF5 TCR treated mice and expansion of mTCRβ^- CD4$^+$ T cells (data not shown). Alloreactivity is a previously reported challenge with models assessing human HLA-mismatched adoptive T cell therapy in xenografts[277]. The T cells used in our study were predominantly of a naïve phenotype (CD62L$^+$CD45RO$^-$) and although mediating efficient anti-tumour responses, the risk of alloreactivity increases concomitantly due to the broader T cell repertoire diversity of naïve as compared to memory T cells[278,279]. This is further exaggerated by the high leukemic burden in these mice where high expression of HLA class I, class II and co-stimulatory molecules led to a more rapid expansion of alloreactive T cells. Hence, the experimental approach of studying the eradication of already established leukemia, as opposed to suppression of leukemic growth, carries the disadvantage of potential confounding alloreactivity. In CAR T cell therapy, alloreactivity can be limited by CRISPR/Cas9 insertion of the construct into the T cell receptor alpha-chain (*TRAC*) locus, which suppresses the endogenous TCR of the T cells[280].

However, a recent study using CRISPR/Cas9 knock-out of the TCR in the T cell receptor beta-chain (*TRBC*) locus showed reduced CAR T cell persistence in vivo[281]. In our humanised model assessing the T cells potential off-target effect on healthy hematopoiesis, we could isolate T cells from the spleen, derived from the engrafted CD34⁺ HSPCs, transduce them with the TdT TCR, expand and infuse into the remaining humanised mice engrafted with the same donor CD34⁺ HSPCs (Fi. 6). This approach mimics the clinical autologous setting and limits alloreactivity. Despite the reduction in alloreactivity, the limited antigen-stimulation of T cells hindered their persistence and necessitated an experimental endpoint analysis on day 17 post-treatment, during which TCR T cells could still be reliably detected.

Taken together, the preclinical findings presented herein indicate that TCR T cells targeting TdT could serve as a new potent immunotherapy for patients with ALL who have very poor prognosis following relapse[220,282], while sparing their healthy lymphocytes. This approach is set to be investigated in a Phase I clinical trial currently in preparation. For patients with T-ALL, this represents the first TAA which has demonstrated elimination of the leukemia while sparing the healthy T cells. However, isolation of healthy T cells from a patient with T-ALL for transduction of the TCR into autologous T cells represents a significant clinical challenge. For patients with B-ALL this represents a promising alternative approach to CD19⁺ CAR T cell treatment as TdT expression was shown to be preserved at relapse when CD19-expression was lost. As our model did not allow for longer follow up time, it was not investigated whether the leukemic cells develop resistance against the TdT TCR T cells by downregulation of TdT over time. However, combinatorial treatments of different targets might limit the cancer cell's ability to escape by antigen downregulation which would be a potential interesting follow up study.

4.3 Study III - A T cell receptor targeting a recurrent driver mutation in FLT3 mediates elimination of primary human acute myeloid leukemia in vivo

In a second collaborative study with the Olweus group, we investigated if TCR T cells targeting a shared neoantigen expressed in approximately 5% of patients with AML could serve as a promising immunotherapeutic approach. AML is the most common leukemia in adults with few available curative treatment options, thereby emphasising a significant unmet medical need. The mutational landscape of AML, characterised by several recurrent driver mutations, offers a variety of potential targetable neoantigens, but identification of neoantigen specific TCR T cells is challenging due to the low immunogenicity of most neoantigens[208]. FLT3 is a receptor tyrosine kinase expressed on hematopoietic progenitor cells and has

important signalling functions in early hematopoiesis for myeloid and lymphoid differentiation[283]. *FLT3* mutations are frequently found in AML and result in FLT3-Ligand independent activation of the receptor, thus providing the mutated cells with a proliferative advantage. Internal tandem duplications (ITD) in the juxtamembrane domain are the dominating *FLT3* mutations followed by point mutations in the activation loop of the tyrosine kinase domain (TKD)[283,284], both generating intracellular aberrations inaccessible to CAR T cell therapies. In the context of TCR T cell therapy, *FLT3*-TKD mutations offers a more practical approach compared to ITDs, as the ITDs exhibit variable lengths and partly retain the natural sequence, consequently, they do not encode shared neoantigens. Among the *FLT3*-TKD mutations, the *FLT3*-D835Y substitution is most prevalent[285]. By screening the T cell repertoire from the blood of 16 healthy donors, the Olweus group identified a TCR with high specificity for the FLT3-D835Y peptide presented on HLA-A2 (Fig. 1). The specificity and efficacy of the TCR against FLT3-D835Y ($TCR^{FLT3D/Y}$) was carefully validated in vitro on both a wide range of cell lines (Fig. 1) as well as primary cells including the mutated AML cells and non-clonally involved B and T cells from eleven different patients (Fig. 2). Reactivity was consistently only observed against cells harbouring the *FLT3*-D835Y mutation and HLA-A2, with exception of three potential cross-reactive peptides (Fig. 1). However, there was no evidence that these peptides were being naturally processed and presented on HLA-A2, thus limiting the risk for off-target cross reactivity.

Following the in vitro validation of the $TCR^{FLT3D/Y}$ T cells, and promising in vivo elimination of cell line xenografts, we investigated the effect of the $TCR^{FLT3D/Y}$ cells in multiple PDX models derived from two independent patients, representing various clinical scenarios, including a model with high leukemic burden, a model with leukemia driven by $CD34^+$ LSCs and a model mimicking clinical MRD-levels. In all models (Figs. 3-5), we observed a striking elimination of the leukemic cells, as quantified by flow cytometry and ddPCR, in the mice treated with $TCR^{FLT3D/Y}$ T cells. Conversely, the leukemia remained high or at similar levels as at the start of treatment in the mice treated with control TCR T cells targeting NY-ESO-1 (TCR^{1G4})[286]. The greatest effect observed was a reduction from above 80% leukemic cells in the BM of control treated mice, down to below 1 % in the $TCR^{FLT3D/Y}$ T cell treated mice over the course of only 15 days (Fig. 3). The risk of confounding alloreactivity was likewise a concern in this study, but by generating PDX models with lower leukemic burden, we managed to follow certain cohorts of mice for up to 34 days post treatment (Fig. 4). However, as the T cells persisted in these mice for the entire duration of the experiment, data from these in vivo PDX models could not provide evidence to support any conclusions regarding potential elimination of leukemia propagating cells, as the remaining T cells would likely

prevent a secondary outgrowth of the leukemia. For this reason, an alternative approach was applied where *FLT3*-D835Y mutated primary AML cells were cultured in vitro with $TCR^{FLT3D/Y}$ T cells for 48 hours and then transplanted into NSG mice to assess leukemia development in the mice (Fig. 5). Any potential remaining T cells in the co-cultures did not persist to detectable levels in vivo up to 28 weeks post transplantation (Extended Data Fig. 10). None of the mice transplanted with co-cultures of AML cells with $TCR^{FLT3D/Y}$ T cells showed any detection of AML engraftment at any time point, which contrasts to the mice transplanted with co-cultures of AML cells with TCR^{1G4} T cells or without T cells who all developed leukemia with time (Fig. 5). Thus, the $TCR^{FLT3D/Y}$ cells were able to eradicate in vitro LSCs with the capacity to propagate leukemia in vivo. To address direct elimination of LSCs in vivo, secondary transplantations of T cell depleted BM from the treated PDX mice would be necessary. However, as $TCR^{FLT3D/Y}$ treated mice could not be followed for a long time due to alloreactivity, it is possible that a secondary leukemia would be generated from remaining leukemia cells in these marrows. Treatment with TCR T cells without endogenous TCRs (knock-out) might permit long enough treatment in absence of alloreactivity, facilitating serial transplantation.

Despite the virtual elimination of leukemic cells in our preclinical models, the clinical potential of $TCR^{FLT3D/Y}$ cells to mediate a cure for patients with *FLT3*-D835Y mutated AML can only be determined in patients. Conceptually, curative outcome should only be possible by targeting of the initiating or transforming mutation. Both *FLT3*-ITD and TKD mutations are frequently secondary events that confer transformation but not initiation of the disease[287,288]. However, clinical benefit can also come from reducing the leukemic burden which might prolong life, shown clinically by the broad usage of FLT3 TKIs which seldom eliminate the initiating clone. Prior to the development of the FLT3 TKIs, *FLT3* mutations correlated with worse outcome. However, the current prognostic significance of *FLT3* mutations, and *FLT3*-TKD mutations in particular, is not fully understood[289,290]. A major limitation of FLT3 TKIs is the transient anti-leukemic effect mediated by mechanisms of resistance[290]. Currently, there are two types of FLT3 TKIs where type I inhibitors have the potential to bind to both the inactive and active conformation of the FLT3 receptor. The type II FLT3 TKIs can, on the other hand, only bind to the inactive conformation of the receptor. All *FLT3*-TKD mutations result in receptor configuration into the active form, thus conferring resistance to all type II TKIs[290]. Acquisition of new, or selective expansion of existing *FLT3*-TKD mutations post TKI treatment thus represents a mechanism of escape[291,292]. In this situation, treatment with the $TCR^{FLT3D/Y}$ T cells could provide an interesting therapeutic opportunity, following treatment with, or in combination with, FLT3 TKIs. The $TCR^{FLT3D/Y}$ T cells could be prepared from autologous T cells, facilitating

immunotherapy in elderly individuals ineligible for toxic allo-HSCT or even chemotherapy, otherwise provided with very limited therapeutic options. Such T cells could also reduce leukemia burden in patients that are difficult to get into remission, required to qualify for allo-HSCT (bridge to transplant). In addition, $TCR^{FLT3D/Y}$ T cells can be manufactured from donor T cells following relapse from allo-HSCT in younger individuals.

Furthermore, targeting of secondary oncogenic events can potentially be curative if the *FLT3* mutation is only preceded by mutations associated with CHIP such as *DNMT3A*, *TET2* or *ASXL1*. By reanalysis of previously published datasets[109,292], we showed that this mutation pattern is not uncommon (Extended Data Fig. 5). There is also an emerging interesting phenomena of utilising "bystander" killing of antigen-negative target cells mediated by the antigen-specific T cells[293,294], which would serve as another scenario whereby targeting of later oncogenic events could have curative potential.

Taken together, we present with this study an important proof-of-concept with clinically relevant implications of targeting recurrent neoantigens with TCR T cells. Just as in **study II**, we show that well selected TCR T cells have specificity and efficacy to selectively eliminate primary leukemic cells in several independent preclinical models. Interestingly, $TCR^{FLT3D/Y}$ T cells also showed in vitro potency against the in vivo leukemia propagating cells. This observation stimulated further exploration of TCR T cells against LSC specific antigens as outlined in **study IV**.

4.4 Study IV - MPL as a target antigen for T cell receptor mediated elimination of leukemic stem cells

Through **study IV**, we aimed to explore if TCR T cells can mediate efficient and specific elimination of LSCs, and for the first time provide experimental evidence for the dependency of LSCs in hematological malignancies, independent of LSC transplantation assays. The expression pattern of the TCR target antigen is dependent on what the aim of the treatment is. For clinical targeting of LSCs, it is most important that no vital organs express the target and that all functional LSCs express the antigen to avoid escape. It would be advantageous for the leukemic blasts, not representing LSCs, to also exhibit high antigen expression, facilitating direct targeting of these cells as well, but it would not necessarily be a requirement to cure based on the CSC concept. Lack of antigen expression in healthy hematopoietic cells would also be clinically beneficial as it would allow for TCR treatment without a haplo-identical transplantation to rescue normal hematopoiesis. No such antigen has so far been discovered. In contrast, development of LSCs-specific TCR T cells as a novel preclinical tool to study the dependency of LSCs in hematological malignancies requires a selective

expression of the antigen in all LSCs without expression in other leukemic cells. Meeting both types of target criteria for clinical and preclinical purposes is challenging, but common for both is that the antigen needs to be expressed on all LSCs.

Given that some of the most well characterised LSCs are found in low to intermediate risk MDS[124,129,234] and that they are confined to the HSC compartment, we hypothesised that an antigen expressed in all HSCs might also be expressed in all LSCs. With this rational, TCR T cells against such a target would additionally provide a novel tool to study the dependency of HSCs in normal hematopoiesis.

In this study, we began to investigate MPL as a potential TCR target for specific elimination of LSCs and HSCs. MPL has been proposed as a LSCs target by others and a preclinical study proposing a TPO-CAR T cell targeting MPL as a potential pre-allo-HSCT treatment regimen was recently published[239,295]. Efficacy was nevertheless limited due to shedding of the TPO-CAR construct, potentially contributing to unwanted excessive TPO stimulus of MPL[295]. The benefit with a TCR T cell targeting MPL is that it can be used for treatment of impending relapse post haploidentical allo-HSCT if the therapy resistant LSCs express MPL. In this setting the HLA-restriction means the healthy donor HSCs, which also express MPL, will not be targeted. Our study therefore included mapping of MPL expression in the hematopoietic hierarchy in both healthy and malignant hematopoiesis through flow cytometry and molecular analysis (Fig. 1). This was combined with functional assays comparing MPL^+ and MPL^- cells (Fig. 2). Functional assessment of normal MPL^+ and MPL^- cells have been done by others, with conflicting results. In most studies, MPL^+ cells have shown superior engraftment capacities in immunodeficient mice, and enhanced LTC-CFC ability in vitro[66,68,296] in line with HSC function being restricted to the MPL^+ cells. However, there have been reports of rare stem cell activity of MPL^- CB[296,297], which motivated a new investigation of this. Our results showed that the stem cell function is confined to the MPL^+ compartment in both healthy and malignant hematopoiesis (Fig. 2). HSCs have the highest MPL-expression of all hematopoietic cells apart from MkPs, which will likely also be targeted by MPL TCR T cells. Although CMPs, and to some degree also GMPs and MEPs seem to retain some/low surface MPL expression in our flow cytometric analysis, previously published RNA-sequencing data show very low levels of MPL transcripts in CMPs and almost no detection in GMPs and MEPs[129], which indicates that few MPL peptides would be actively transcribed and presented to the T cells from these cells. Additionally, the effects of Romiplostim, which acts through the MPL receptor, seemed to be specific to HSCs in mice as shown in comparison to GMPs (Fig. 4), implying that levels of MPL is low or absent

on the GMPs. However, to what extent any downstream progenitors are expressing sufficient levels of MPL to be targeted remains to be investigated.

In parallel to the work carefully validating MPL as a target based on the expression and relevance for myeloid malignancies, we collaborated with the Olweus group who initiated identification of MPL:HLA-A2 restricted TCR T cells, using the same approach as for **study II** (TdT TCR) as MPL is a self-antigen. Initially, this screen had identified two potential MPL-restricted TCRs that were transduced into healthy donor T cells and subjected to detailed characterisation (Thesis of Zsofia Földvarí, University of Oslo, 2022). Both TCRs met all the initial safety and specificity criteria when assessing potential cross-reactive peptides and T cell activation as assessed by CD137 upregulation and IFN-γ production. However, one of the TCRs showed unexplained MPL-independent killing of various cell lines not expressing MPL, and the other did not show clear efficacy against an MPL$^+$ leukemic cell line in vivo or primary HSCs in vitro (data not shown). These observations stimulated new efforts to identify additional MPL-TCR T cells which is presented in **study IV** (Fig. 3). Additionally, we are now re-considering a previously investigated MPL TCR T cell initially not prioritised as it showed less efficient eradication of primary HSCs, however, importantly it displayed MPL restricted activation and elimination using cell lines. Compared to expression of MPL on leukemic cell lines (either natural, transduced or loaded with peptide), the expression on primary cells is much lower which might explain the efficient recognition of MPL$^+$ leukemic cell lines but failed elimination of primary HSCs in our assay. It is also compatible with the notion that HSCs have inherent immunomodulatory characteristics[298] making them less susceptible to targeting compared to leukemic cell lines. It is therefore important to establish whether the MPL expression on primary HSCs/LSCs is at sufficient levels and that the peptide presentation machinery is active in the HSC/LSCs for an MPL-TCR T cell to kill, and if not, to explore mechanisms to induce it. Our preliminary data (not shown) show that when the HSCs are externally loaded with the target MPL peptide, they are susceptible to elimination, which suggests that HSCs can be targeted by TCR T cells, but the endogenous peptide presentation might not be sufficient. In our study we show that stimulation of HSCs with Romiplostim leads to activation, internalisation of MPL, and enhanced MHC class I presentation in mice (Fig. 4). Thus, romiplostim treatment is a potential tool to enhance MPL peptide presentation and targeting, but this remains to be confirmed in conjunction with MPL TCR T cell therapy.

As discussed throughout this thesis, in vivo assays play a crucial role in HSC and LSC research. To this end, it is vital to examine the effect of MPL TCR T cells in relevant mouse models. The establishment of humanised mice with a human

hematopoietic hierarchy closely mimicking human primary HSPC compartments with preserved MPL expression on the HSCs as shown in **study IV** was therefore fundamental (Fig. 2). A unique foreseeable challenge with in vivo targeting of MPL with TCR T cells compared to the targeting of TdT and FLT3-D835Y in **study II** and **II** is the low frequency of the target cells. Although we did observe effective T cell elimination of leukemic cells composing about 0.1% of total BM cells in **study III**, MPL$^+$ HSCs/LSCs in the BM are even more rare which would lead to fewer potential interactions between T cells and targets. In a recent study, Goddard and colleagues demonstrated that low frequency of cells was a potential mechanism for immune escape in metastatic breast cancer, but that it could be overcome by continuous immunity boosts, either by vaccinations or adoptive T cell therapies[299]. This suggests the need for multiple doses of T cell infusions to eliminate rare MPL$^+$ cells in contrast to the single-dose effect observed in **studies II** and **III**. Moreover, HSCs/LSCs exhibit immune-privilege demonstrated by, for instance, their capacity to cross allogeneic barriers[58] and expression of immune-checkpoint molecules such as CD274 (PD-L1) and CD47[300,301] which mediates their ability to evade the GvL effect post allo-HSCT[219]. These inherent traits that likely confer resistance to TCR T cell therapy, would have to be overcome. It was recently shown in a study by Hernández-Malmierca and colleagues that HSPCs from both mice and human have the capacity to act as antigen presenting cells. Presentation of self-peptides to antigen specific CD4$^+$ T cells through MHC class II mediated an immunosuppressive effect, representing a protection mechanisms for HSCs[302]. However, when immunogenic antigens were presented by HSPCs, it led to HSC exhaustion. This phenomena uncovers a potential for antigen-specific CD4$^+$ T cells to participate in TCR T cell mediated elimination of HSCs/LSCs with CD8 co-receptor independent TCRs[303].

As discussed above, there are numerous known, and potential unknown, mechanisms that might influence the efficacy of MPL TCR T cells to eliminate the HSCs/LSCs. Before approaching those more in depth, a continued validation of the newly identified MPL-TCR, as well as re-evaluation of the previously discarded MPL-TCR that showed MPL restricted-activity but lacked efficacy in some of our assays, will be performed to investigate if the efficacy can be enhanced.

In our established preclinical settings, the expression of MPL on both healthy HSCs and malignant LSCs is a minor issue as separate models will primarily be used to assess the effect on these cell types. Additionally, the HLA-restriction of the TCR T cells excludes potential lethal elimination of mouse HSCs causing concerns in the in vivo models. However, in the clinical application, MPL TCR T cells will likely be restricted to use in conjunction with an HLA-mismatched (so-called haplo-identical) allo-HSCT due to the expression of MPL on normal HSCs. It is therefore

important to carefully validate the HLA-restriction of the proposed MPL TCR T cells before clinical use and to ensure that HLA-A mismatching is generally tolerable, in line with previous reports[304,305]. Whether treatment with HLA-restricted MPL TCR T cells would be most advantageous pre or post allo-HSCT requires further investigations. Adoptive T cell therapy as part of a preconditioning regimen might enable more eligible patients for transplant as it may allow for lower doses of chemotherapy. However, this is only possible if a therapeutic window can be achieved for LSC-directed therapy as it would not have direct effect on most of the blasts and would target healthy HSCs. Further, unless the T cells are harvested from the future donor, the T cells would be eliminated by the donor graft, thus not persist post-transplant. Using donor derived T cells in a preconditioning regimen, instead of autologous T cells, could however be associated with a risk of severe GvHD. It is therefore likely that MPL TCR T cells will be more efficient to use post allo-HSCT as treatment or prevention of relapse. Currently, our phenotypic characterisation of MPL on LSCs is restricted to diagnostic/pre allo-HSCT samples, consequently, an extended analysis is needed to characterise the role of MPL in post allo-HSCT LSCs as phenotype and function of LSCs can change post allo-HSCT[298]. Our group is currently conducting a new study where LSCs on single level at diagnosis, remission and relapse are subjected to RNA-sequencing to investigate a detailed characterisation of the relapse initiating LSC.

5 Conclusions and Points of Perspectives

Despite recent advances in cancer treatment, many patients still face very few treatment options due to old age, high disease burden and development of treatment resistance over time. This thesis makes contributions to the fields of hematopoiesis and cancer immunotherapies, bringing further attention to the therapy resistant LSCs. The primary outcomes of this thesis have been to provide evidence for fundamental concepts and to explore if T cell mediated immunotherapy is a potent therapeutic option for targeting of the therapy resistant LSCs. Summarised, in **study I**, we demonstrate the clinical relevancy of therapy resistant LSCs which escape treatment and subsequently cause relapse and how their elimination is essential for cure. Further, we showed that target molecular MRD-analysis of the LSCs may have important clinical implications. In **study II** and **III,** we demonstrated the potent effect of TCR T cell mediated immunotherapy against two different types of leukemias and antigens: the TAA TdT in **study II** and the neoantigen FLT3-D835Y in **study III**. In **study IV**, which is currently ongoing, we aim to utilise the power of TCR T cells for targeted elimination of the therapy resistant LSCs, but also for specific elimination of HSCs to study the dependency of stem cells in healthy and malignant hematopoiesis in preclinical models. The application of adoptive T cell therapy with genetically modified T cells in cancer treatment is relatively new. Notably, the first CAR T cell against a hematological malignancy received clinical approval in 2017[306], highlighting that we are still in the starting phase of utilising this new cornerstone of cancer therapy. An interesting aspect to explore in the future is combination treatment of two antigen-specific TCR T cells, where the MPL TCR T cells and the FLT3-D835Y TCR T cells might be particularly interesting to use in combination. The FLT3-D835Y TCR T cells efficiently eliminates the fraction of leukemic cells harbouring the mutation, but since it might not represent a founder mutation, the LSC specific TCR T cell could be a good complement to eradicate the remaining earlier clones.

Although this thesis is focused on human hematopoiesis and leukemia, there are interesting biological questions that are more suitable to address in mouse hematopoiesis. For instance, cellular quiescence is a main feature of HSCs and LSCs and recent studies have shown that quiescence is a main mechanism for immune escape[62,63]. Transplanting human HSCs/LSCs into mice disrupts the innate quiescent state and consequently does not allow for models to study the full impact of quiescence in mediating potential immune escape. The human HSCs/LSCs are also lacking interactions with their endogenous environment in the mice. In light of this we are currently exploring and establishing mouse models that

allow us to study immune mediated elimination of HSCs/LSCs in an unperturbed environment where the physiological state of the HSCs/LSCs is preserved.

6 Scientific outreach

Sharing results, knowledge, ideas, and points of perspectives is a fundamental aspect of being a scientist, and something I find great joy and excitement in doing. I was therefore pleased to be offered the opportunity to pen a Swedish piece summarising and discussing our findings from **study I**, published in "Onkologi i Sverige - Den oberoende tidningen för svensk cancervård".

https://www.onkologiisverige.se/ny-studie-ger-hopp-om-att-forutspa-aterfall-av-blodcancer-efter-en-stamcellstransplantation-tidigare/

Similarly, we were given the opportunity from the Nature Portfolio and Springer Nature Communities to share our deeper thoughts and discussions regarding our findings in **study III** in the "Behind the paper" format.

https://communities.springernature.com/posts/shared-neoantigens-as-targets-for-tcr-t-cell-adoptive-therapies

However, from my perspective, the most exciting form of scientific communication and outreach are oral presentations with room for discussions. For the use of humanised mouse models from the Jackson Laboratory in **study II**, I had the great pleasure of being invited to present our publication in the "Humanized mice journal club" organised by the Jackson Laboratory.

https://www.youtube.com/watch?v=NMv_2EWVCqA

All these opportunities generated new connections, discussions and ideas that would not have taken place solely through the published manuscripts. I take great pleasure in communicating science to a broader audience. I have learned a lot from these experiences and look forward to continuing to do so in the future.

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