From Department of Clinical science, Intervention and Technology
Karolinska Institutet, Stockholm, Sweden

CHARACTERIZATION OF HUMAN GAMMA DELTA T CELLS IN ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

Ahmed Gaballa

Karolinska Institutet

Stockholm 2020

Published by Karolinska Institutet.
Printed by Universitetsservice US-AB

ISBN 978-91-7831-874-2

CHARACTERIZATION OF HUMAN GAMMA DELTA T CELLS IN ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Ahmed Gaballa

Principal Supervisor:
Professor Michael Uhlin
Karolinska Institutet
Department of CLINTEC
Division of transplantationskirurgi

Co-supervisor(s):
Professor Moustapha Hassan
Karolinska Institutet
Department of Laboratory Medicine

Professor Jonas Mattsson
Karolinska Institutet
Department of Oncology and Pathology

Dr. Sarah Thunberg

Opponent:
Professor Dieter Kabelitz
University of Kiel
Institute of Immunology

Examination Board:
Associate professor Jakob Michaelsson
Karolinska Institutet
Department of Medicine
Center of Infection Medicine (CIM)

Associate professor Cecilia Götherström
Karolinska Institutet
Department of CLINTEC

Professor Jan Holgersson
Göteborgs Universitet
Department of Laboratory Medicine, Institute of Biomedicine

"نَرْفَعُ دَرَجَاتٍ مَنْ نَشَاءُ ۗ وَفَوْقَ كُلِّ ذِي عِلْمٍ عَلِيمٌ "

"We raise in degrees whom We will, but over every possessor of knowledge is one more knowing."

Quran surah Yusuf (12:76)

To my late father and mother, I dedicate this work….

ABSTRACT

Over the last five decades, allogeneic hematopoietic stem cell transplantation (HSCT) has evolved rapidly, continuing to offer a cure for several hematological diseases. Nevertheless, associated life-threatening complications remain an obstacle against exploiting its full therapeutic benefit. Among these complications, infection, relapse, and graft-versus-host disease (GVHD) represent not only the most common but also the most serious ones. Though commonly regarded as distinct clinical events, their underlying pathophysiology is firmly related from an immunological perspective.

T lymphocytes are key players in HSCT complications and their proper reconstitution following allogeneic HSCT is central for beneficial clinical outcome. The last two decades have witnessed a growing interest in a subset of T cells known as gamma delta (γδ) T cells. The immunological capabilities of these unconventional cells have been intensively explored. However, more efforts aimed at unraveling the immunobiological features of different γδ subsets are warranted to effectively exploit their full immunotherapeutic potential.

In the present work, I tried to tackle several immune-related aspects that directly influence allogeneic HSCT outcome with a special focus on γδ T cells. In **paper I**, the main objective was to address the impact of different GVHD prophylaxis regimens on *de novo* generation of T and B lymphocytes. Using PCR methods, T cell receptor recombination excision circle (TREC), kappa deleting recombination excision circle (KREC), and telomere length (TL) were quantified in the peripheral blood (PB) of transplanted patients at several time intervals. Although there was no significant difference between the two GVHD prophylaxis groups, we identified other transplant related factors that were associated with reduced TREC and/or KREC levels after HSCT. Furthermore, we showed that high levels of these excision circles correlated with favorable outcome post HSCT.

In **paper II-IV**, more attention was paid to explore the role of γδ T cells in donor grafts. Using multicolor flow cytometry together with other molecular and functional assays, we found a significant association between graft frequencies of CD8+γδ T cells and acute GVHD (aGVHD) grade II-III in **Paper II**. Additionally, we showed that higher frequencies of CD27+ γδ T cells in the stem cell grafts were correlated with both less relapse and CMV incidences.

The results from paper II highlighting a potential role of CD8+γδ T cells in donor grafts raised our interest to further investigate this subset to elucidate their immunological characteristics. In **paper III** we thoroughly analysed γδ T cells in BM grafts using multicolor flow cytometry and TCR repertoire analysis using next generation sequencing (NGS). We showed that grafts from CMV+ donors contained higher proportions of CD8+γδ T that preferentially expressed Vγ9- and differentiated towards terminal effector memory phenotype. Additionally, analysis of TCRγ chain revealed a clonally focused repertoire in CMV+ donor grafts. We also showed that CD8+γδ T cells differentially respond to TCR stimuli suggesting adaptive-like phenotype

In **paper IV**, we sought to address whether allogeneic HSCT outcome is influenced by γδ TCR repertoire composition in donor grafts. Immunosequencing of TCRγ chain by NGS revealed a more public repertoire and increased presence of long sequence clonotypes in graft given to non-relapsed patients. Further analysis of the amino acid sequences identified 12 public and 4 private sequences that were exclusively found in high frequencies in grafts given to non-relapsed patients.

Finally, in **paper V** we aimed to optimize a protocol for efficient *in-vitro* expansion of Vγ9Vδ2 T cells from umbilical cord blood (UCB). Phenotypical and functional characterization of expanded cells was comparable to PB and suggests that UCB can be a reliable source for Vγ9Vδ2 T cell expansion.

LIST OF SCIENTIFIC PAPERS

I. Törlén J*, **Gaballa A***, Remberger M, Mörk LM, Sundberg B, Mattsson J, Uhlin M. Effect of Graft-versus-Host Disease Prophylaxis Regimens on T and B Cell Reconstitution after Allogeneic Hematopoietic Stem Cell Transplantation. * contributed equally
Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation 2019 25;6 1260-1268

II. **Gaballa A**, Stikvoort A, Önfelt B, Mattsson J, Sundin M, Watz E, Uhlin M. T-cell frequencies of CD8+ γδ and CD27+ γδ cells in the stem cell graft predict the outcome after allogeneic hematopoietic cell transplantation.
Bone marrow transplantation 2019 54;10 1562-1574

III. **Gaballa A**, Arruda LCM, Radestad E, Uhlin M. CD8(+)gamma delta T Cells Are More Frequent in CMV Seropositive Bone Marrow Grafts and Display Phenotype of an Adaptive Immune Response.
STEM CELLS INTERNATIONAL 2019 2019; 6348060-

IV. Arruda LCM, **Gaballa A**, Uhlin M. Graft γδ TCR Sequencing Identifies Public Clonotypes Associated with Hematopoietic Stem Cell Transplantation Efficacy in Acute Myeloid Leukemia Patients and Unravels Cytomegalovirus Impact on Repertoire Distribution.
Journal of immunology (Baltimore, Md. : 1950) 2019 202;6 1859-1870

V. Berglund S, **Gaballa A**, Sawaisorn P, Sundberg B, Uhlin M. Expansion of Gammadelta T Cells from Cord Blood: A Therapeutical Possibility.
STEM CELLS INTERNATIONAL 2018 2018; 8529104-

OTHER RELATED SCIENTIFIC PAPERS NOT INCLUDED

I. **Gaballa A**, Clave E, Uhlin M, Toubert A, Arruda LCM. Evaluating Thymic Function After Human Hematopoietic Stem Cell Transplantation in the Personalized Medicine Era. Frontiers in Immunology. 2020;11(1341).

II. Takuya Sekine, André Perez-Potti, Son Nguyen, Jean-Baptiste Gorin, Vincent H. Wu, Emma Gostick, Sian Llewellyn-Lacey, Quirin Hammer, Sara Falck-Jones, Sindhu Vangeti, Meng Yu, Anna Smed-Sörensen, **Ahmed Gaballa**, Michael Uhlin, Johan K. Sandberg, Christian Brander, Piotr Nowak, Paul A. Goepfert, David A. Price, Michael R. Betts, Marcus Buggert. TOX is expressed by exhausted and polyfunctional human effector memory CD8+ T cells. Science Immunology. 03 Jul 2020: Vol. 5, Issue 49, eaba7918

III. Arruda LCM, **Gaballa A**, Uhlin M. Impact of γδ T cells on clinical outcome of hematopoietic stem cell transplantation: systematic review and meta-analysis. Blood advances 2019 3;21 3436-3448

IV. **Gaballa A**, Norberg A, Stikvoort A, Mattsson J, Sundberg B, Uzunel M, Remberger M, Uhlin M. Assessment of TREC, KREC and telomere length in long-term survivors after allogeneic HSCT: the role of GvHD and graft source and evidence for telomere homeostasis in young recipients. Bone marrow transplantation 2018 53;1 69-77

V. Sundin M, Uhlin M, **Gaballa A**, Ramme K, Kolios AG, Marits P, Nilsson J. Late presenting atypical severe combined immunodeficiency (SCID) associated with a novel missense mutation in DCLRE1C. Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology 2018 29;1 108-111

VI. Alnabhan R, **Gaballa A**, Mörk LM, Mattsson J, Uhlin M, Magalhaes I. Media evaluation for production and expansion of anti-CD19 chimeric antigen receptor T cells. Cytotherapy 2018 20;7 941-951

VII. Stikvoort A, **Gaballa A**, Solders M, Nederlof I, Önfelt B, Sundberg B, Remberger M, Sundin M, Mattsson J, Uhlin M. Risk Factors for Severe Acute Graft-versus-Host Disease in Donor Graft Composition. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation 2018 24;3 467-477

VIII. Berglund S, Magalhaes I, **Gaballa A**, Vanherberghen B, Uhlin M. Advances in umbilical cord blood cell therapy: the present and the future. Expert opinion on biological therapy 2017 17;6 691-699

IX. **Gaballa A**, Sundin M, Stikvoort A, Abumaree M, Uzunel M, Sairafi D, Uhlin M. T Cell Receptor Excision Circle (TREC) Monitoring after Allogeneic Stem Cell Transplantation; a Predictive Marker for Complications and Clinical Outcome. International journal of molecular sciences 2016 17;10

CONTENTS

LIST OF ABBREVIATIONS

αβ	Alpha beta
γδ	Gamma delta
ACT	Adoptive cell therapy
ADCC	Antibody-dependent cellular cytotoxicity
a(c)GVHD	Acute (chronic) graft versus host disease
AIRE	Autoimmune regulator
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APC	Antigen presenting cells
ATG	Anti-thymocyte globulin
ATP	Adenosine triphosphate
BTN3A1	Butyrophilin Subfamily 3 Member A1
BM	Bone marrow
CAST	Center for allogeneic stem cell transplantation
CCR	chemokine receptor
CCR7	Chemokine receptor 7
CD	Cluster of differentiation
CDR3	Complementary determining region 3
CLL	Chronic lymphatic leukemia
CML	Chronic myeloid leukemia
CMV	Cytomegalovirus
CsA	Cyclosporine A
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
DAMP	Danger associated molecular pattern
DC	Dendritic cell
DLI	Donor lymphocyte infusion
DNA	Deoxyribonucleic acid
DNAM1	DNAX accessory molecule 1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-CSF	Granulocyte colony stimulating factor

GM-CSF	Granulocyte macrophage colony stimulating factor
GVHD	Graft versus host disease
GVT	Graft versus tumor
HLA	Human leukocyte antigen
HMBPP	(E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate
HMGB-1	High-mobility group box-1
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
IFNγ	Interferon gamma
IL	Interleukin
iNKT cell	Invariant Natural Killer T cell
IPP	Isopentenyl pyrophosphate
KIR	Killer cell immunoglobulin like receptor
KREC	Kappa deleting recombination excision circle
MAC	Myeloablative conditioning
MAIT cell	Mucosal associated invariant T cells
mHAg	Minor histocompatibility antigen
MHC	Major histocompatibility antigen
MICA/B	MHC class I-related chain A/B
MNC	Mononuclear cells
MSC	Mesenchymal supporting cells
mTOR	Mammalian target of rapamycin
Mtx	Methotrexate
NGS	Next generation sequencing
NK	Natural killer
NKG2D	Natural killer group 2D receptor
OS	Overall survival
PAgs	Phosphorylated antigens
PAMP	Pathogen associated molecular pattern
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cell

PD	Programmed cell death protein
PDGFα	Platelet-derived growth factor α
PCR	Polymerase chain reaction
PRR	Pattern recognition receptor
RIC	Reduced intensity conditioning
RNA	Ribonucleic acid
SAA	Severe aplastic anemia
SCID	Severe combined immunodeficiency
Sir	Sirolimus
SNP	Single nucleotide polymorphism
Tac	Tacrolimus
TBI	Total body irradiation
TCR	T cell antigen receptor
TGFβ	Transforming growth factor β
TL	Telomer length
TNF	Tumor necrosis factor
TREC	T cell receptor recombination excision circle
TRD	T cell receptor δ gene
TRG	T cell receptor γ gene
Treg	T regulatory cell
TRM	Transplantation related mortality
UCB	Umbilical cord blood
ULBP	UL16 binding protein
V(D)J genes	Variable, diversity, joining

1 INTRODUCTION

1.1 ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

1.1.1 Background

Allogeneic hematopoietic stem cell transplantation (HSCT) is a well-established yet complex treatment for a variety of disorders of the hematopoietic system (Figure 1) (1). In principle, allogeneic HSCT is preceded by cytoreductive conditioning regimen, that aims at reducing malignant burden, avoiding graft rejection, and enhancing engraftment. The stem cell (SC) graft is then infused from a suitable donor selected based on human leukocyte antigen (HLA) matching. The SC grafts can be obtained from bone marrow (BM) or from peripheral blood (PBSC) after mobilization by G-CSF or plerixafor. Umbilical cord blood (UCB) units can also be used as graft source (2, 3).

Initially, allogeneic HSCT was limited to few indications such as acute leukemia, severe aplastic anemia (SAA), or severe combined immunodeficiency (SCID). The remarkable progress in HSCT as a consequence of the advancement in transplant technique and better understanding of the human immune system has led to a progressive increase in the number of HSCTs performed to a level that, in just half a century, over 1 million HSCTs were performed worldwide, of which about 40 % were allogeneic HSCTs (4). The current practice of HSCT is rapidly evolving and guidelines are continuously updated and modified reflecting the complexity of this procedure.

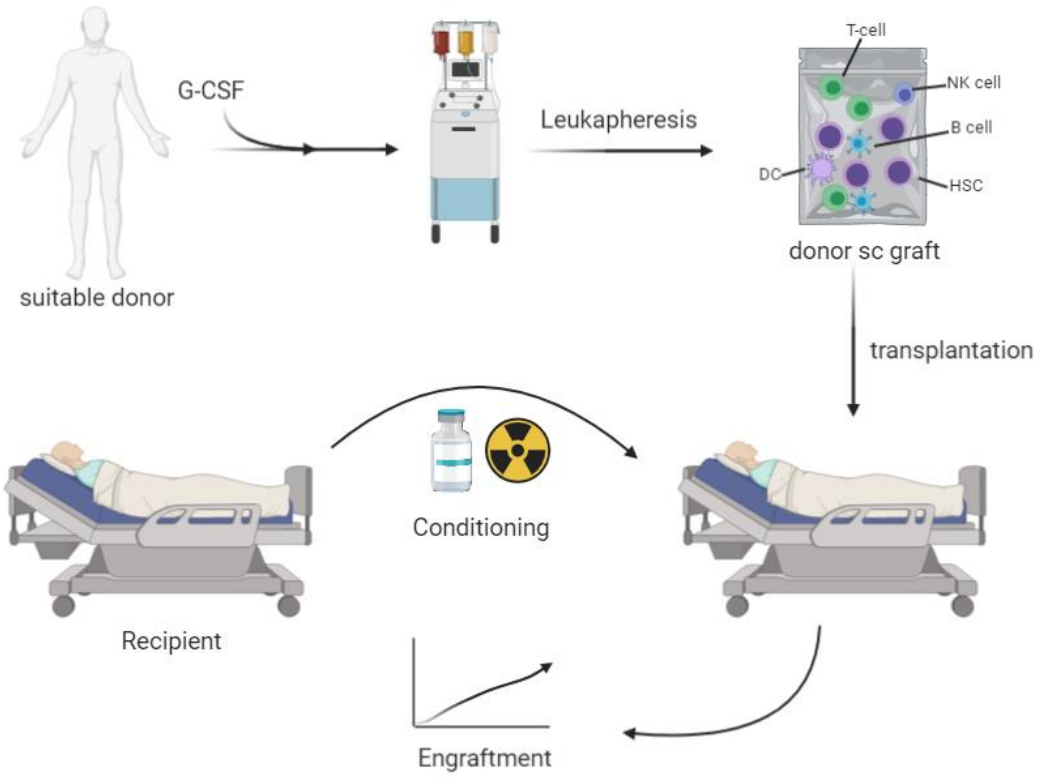

Figure 1. Illustration of the process of allogeneic HSCT.

Despite major improvements in allogeneic HSCT, it is still hampered by the associated complications which represent a major cause of morbidity and mortality among transplanted patients (5). These complications are due to several elements such as conditioning regimen associated toxicities, prolonged immune deficiency, and organ and tissue damage. Among these complications, graft-versus-host disease (GVHD) remains a major leading cause of non-relapse mortality following allogeneic HSCT (6). Since GVHD is a central outcome in my thesis (papers I, II, and IV), in the next section, I will discuss in more detail the pathophysiology and underlying immune mechanisms implicated in GVHD

1.1.2 Graft-Versus-Host Disease

Graft-versus-host disease (GVHD) is an unfavorable immune response mounted by donor immune cells that recognize recipient cells as foreign, resulting in organ and tissue damage (7). GVHD is a frequent event following allogeneic HSCT. It has been estimated that around 50 % of allogeneic HSCT patients develop some degree of GVHD (8). Morbidities caused by GVHD are due to several factors including organ/tissue damage, immune suppression due to the prolonged use of immunosuppressive drugs with the resulting increased risk of infection (9).

Despite the major advancements in the field, little progress has been achieved in GVHD treatment rendering it one of the leading causes of transplant-related morbidities and mortalities. GVHD is classically seen in both an acute and chronic form, with symptoms ranging from mild to devastating organ damage (10).

1.1.2.1 Acute GVHD

Acute GVHD (aGVHD) is the second leading cause of death after relapse in allogeneic HSCT patients. The main target organs involved are skin, intestine, and liver. aGVHD is classed into IV grades, with grade I indicating mild disease and grade IV for very severe disease (11).

Disparity of human leukocyte antigen (HLA) between donor and recipient is a common genetic trigger and is associated with increased risk of aGVHD(12). Nevertheless, not all HLA mismatches induce severe GVHD as some of these mismatches (permissive mismatches) are not associated with severe aGVHD (3, 9). Besides HLA allele mismatches, other peptide products of polymorphic genes, known as minor histocompatibility antigens (miHAgs), can be recognized by donor T cells as alloantigens and can initiate aGVHD. Multiple studies indicated that miHAgs encoded by the Y chromosome are significantly associated with severe aGVHD in male recipients that received allografts from female donors (13, 14).

Other non-genetic risk factors for GVHD include graft source, intensity of conditioning regimen, and age (15). While the role of stem cell source has been thoroughly investigated, the impact of conditioning regimen is still controversial. In this regard, several clinical studies showed increased risk and severity of GVHD, particularly cGVHD in patients receiving

PBSC as graft source compared to BM grafts. Moreover, co-morbidities prior to allogeneic HSCT are associated with GVHD incidence and severity (6).

The pathophysiology of aGVHD is classically described in two main stages: an initial inflammatory stage and an effector stage (Figure 2) (6). The inflammatory process is commonly initiated by trigger molecules in the extracellular matrix. Several endogenous molecules such as ATP, uric acid, High-mobility group box-1 (HGMB1), and heparan sulfate are known as danger-associated molecular patterns (DAMPs). These DAMPs are released in response to tissue damage and, thus, induce sterile inflammation (7). Alternatively, inflammation can be initiated by exogenous pathogenic components of microbes and bacteria that translocate through damaged intestinal mucosa or skin. These molecules are known as pathogen-associated molecular patterns (PAMPs) and include lipopolysaccharides and bacterial RNA or DNA molecules (16).

Both DAMPs and PAMPs serve as ligands for pattern recognition receptors (PRR) expressed by innate immune cells and professional antigen presenting cells (APCs) such as monocytes, dendritic cells, and macrophages. Once APCs are activated by these danger molecules, they upregulate HLA molecules and other costimulatory molecules necessary for T cell priming (17).

Another crucial event during this phase is the secretion of soluble pro-inflammatory mediators. The classical cytokines involved in aGVHD include the IL-1B, IL-6, and TNFα. Other cytokines such as IL-17 and IL-23 have been also described (18). These pro-inflammatory cytokines are pivotal for the inflammatory process and facilitate the recruitment of other immune cells (19).

Microbiota have also been involved in the pathophysiology of GVHD, particularly intestinal GVHD, in a way that involves various delicate homeostatic mechanisms (20). Paneth cells, present in the intestinal mucosa, secrete antimicrobial peptides that maintain microbiome homeostasis (21). Pro-inflammatory cytokines induce damage to Paneth cells resulting in dysbiosis. Alternatively, recent evidence showed that microbiota-derived metabolites are important for the regeneration of intestinal stem cells (ISCs) which are crucial for the integrity of the intestinal barrier (22). Additionally, gut microbiota can secrete butyrate that modulates immune cell response through indolamine-2,3-dioxygenase dependent mechanism. The use of broad-spectrum antibiotics was associated with severe GVHD probably due to dysregulation of the microbiota (23, 24).

The effector stage is initiated when donor T cells encounter alloantigens/mismatched HLA presented by host APCs. This results in activation and proliferation of donor T cells which, in turn, secrete inflammatory cytokines such as IL-2, TNFα and IFNγ further aggravating tissue damage (25). In contrast to host APCs, the role of donor APCs in GVHD is still unclear.

Host tissue damage in aGVHD can be mediated by CD4 or CD8 donor T cells (26). A key difference is that CD8 T cells require direct interaction with cognate antigen/HLA class I

complex on host tissue, whereas CD4 T cells can mediate tissue damage through cytokine release without requiring direct cognate recognition (7, 27).

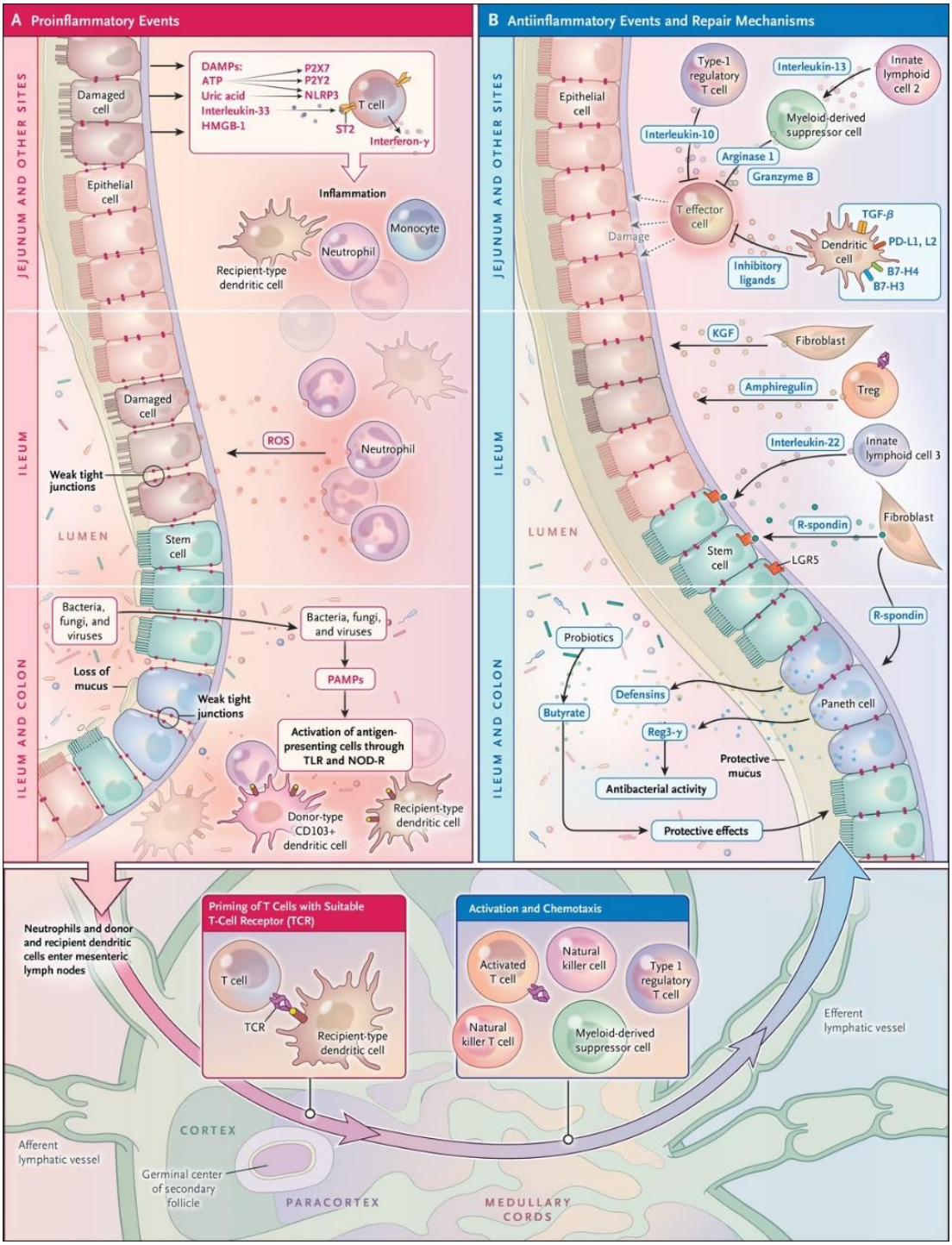

Figure 2. Immunological mechanisms in acute GVHD. (Reproduced with permission from Zeiser R et al. (11), Copyright Massachusetts Medical Society).

1.1.2.2 Chronic GVHD

Chronic GVHD (cGVHD) is also a common complication after allogeneic HSCT, with an incidence ranging between 30-70% (28). cGVHD is distinguished by aberrant tissue repair with characteristic formation of fibrosis. Patients developing cGVHD are at higher risk of long-term morbidity and low quality of life after allogeneic HSCT (29). The clinical features of cGVHD are heterogenous reflecting the involvement of different organ systems with pleiotropic manifestations. According to the 2014 National institute of health (NIH) consensus criteria (30), cGVHD is graded into mild, moderate, and severe (Table 1). This classification was based on a scoring system that assesses the severity of disease for each of the involved organs (skin, oral, ocular, GIT, genital, liver, lung, fascia, and joints). Several risk factors for cGVHD have been reported including, prior aGVHD, HLA mismatch, female donor for a male recipient, older age, high-intensity conditioning, and chronic myeloid leukemia (31)

cGVHD stage	Score
Mild	< 3 Organs involved with score 1 (Lung score 0)
Moderate	>=3 organs involved with score 1. OR At least 1 organ (not lung) with a score of 2 OR Lung score 1
Severe	At least 1 organ with a score 3. OR Lung score of 2 or 3

Table 1. Scoring system for chronic GVHD

Unlike aGVHD, the underpinning immune mechanisms of cGVHD is less understood and represents a complex interaction of several immune mechanisms. Recent studies in mice models have been crucial for establishing many of these underlying mechanisms.

The pathophysiology of cGVHD can be divided into 3 stages (Figure 3) (28). The first is the inflammatory stage which like aGVHD is initiated by DAMPS and PAMPS, released extracellularly as a result of tissue damage or as a component of microbial pathogens. The net result is the activation of the innate immune system and release of soluble pro-inflammatory mediators. Th17 cells have been implicated as they are induced to differentiate by IL-1β and IL-6 and can maintain inflammatory state through IL-17 (32, 33). Additionally, neutrophils can amplify tissue damage and inflammation through reactive oxygen species (ROS) and inflammasomes (34). Furthermore, APCs become activated and upregulate their HLA molecules, facilitating the activation of adaptive immune cells.

The next stage involves the activation of adaptive lymphocytes by APCs. In addition to T cells, B cell hyper-responsiveness and impaired immunoglobulin response occurs in cGVHD (35). Additionally, injury of cortical and medullary thymic epithelial cells (cTEC and mTEC) by conditioning drugs or as a result of aGVHD, leads to impaired central tolerance allowing export of autoreactive T cells to the circulation, further aggravating disease severity (36). Besides central tolerance, peripheral tolerance is also compromised as both T regulatory (Treg) and B regulatory (Breg) cells are dysfunctional or deficient in cGVHD patients (37).

The third stage is manifested by abnormal tissue repair. This has been attributed to the activation of fibroblasts by macrophages through TGFβ and PDGFα, the result of which is excess release of extracellular matrix collagen (38).

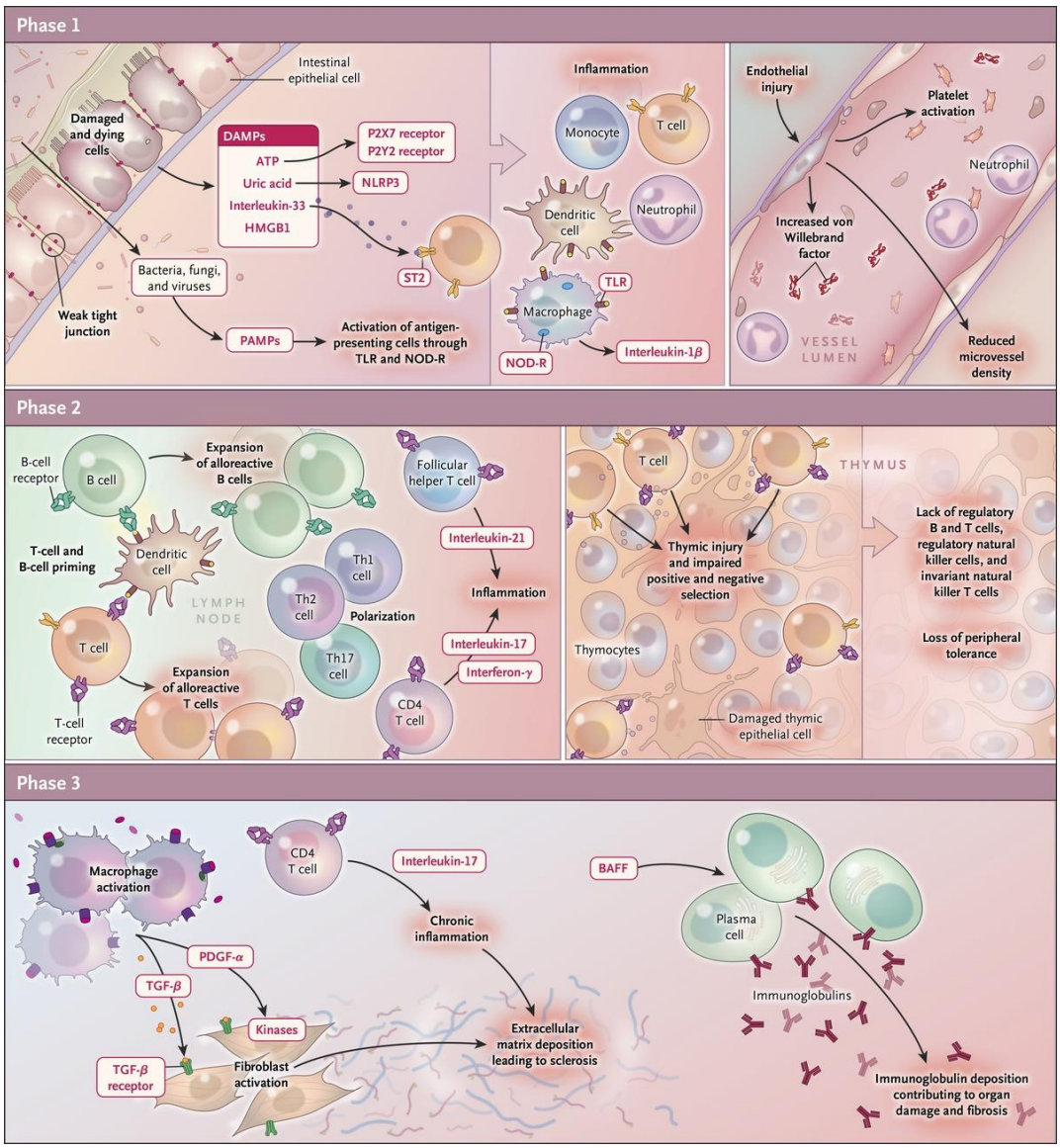

Figure 3. Immunological phases of chronic GVHD. (Reproduced with permission from Zeiser R et al. (28), Copyright Massachusetts Medical Society).

1.1.2.3 GVHD biomarkers

Several research efforts have been directed towards identifying reliable biomarkers for GVHD (39, 40). Biomarkers would allow early diagnosis before clinical signs and could be useful prognostic markers for the risk of GVHD progression and unresponsiveness to therapy (41). A reliable biomarker should be able to differentiate between GVHD and other inflammatory conditions. It should also be non-invasive and easily performed (simple) as well as inexpensive. Some of these biomarkers are summarized in table 2

Biomarker	Significance	Notes and references
Ratio of Treg: effector T cells	Associated with GVHD	(42, 43)
High serum level of IL-4, -6, -8, --10, -17, TNFα	Proinflammatory cytokines increased in the serum of GVHD patients	Can increase in other inflammatory conditions (41)
Elafin	Skin specific	(44)
REG3α	Gut specific	(39)
ST2, CXCL9, matrix metalloproteinase 3, osteopontin	Correlated with cGVHD severity	Can be used for risk assessment (45)
Serum levels of miRNAs (miRNA-155, miRNA 146)	Associated with aGVHD	(46)
SNPs in CCR5, CCR6, FGFR1, CTLA4 genes	Association with cGVHD	Requires further validation in multi-center cohorts (47, 48)

Table 2. Biomarkers for GVHD.

1.1.2.4 Role of T cells and other immune cells in GVHD

Donor-derived T cells are substantial in the pathogenesis of GVHD. That said, they also mediate beneficial effects. The alloreactive response between donor-derived lymphocytes and neoplastic cells is known as the graft-versus-leukemia (GVL) or graft-versus-tumor (GVT) reaction and represents a cornerstone for the effective eradication of malignant cells in allogeneic HSCT (49). The GVL effect is the basis for the immunotherapeutic effect of allogeneic HSCT, offering a survival advantage for patients with malignant disorders (50).

The dual face of alloreactive T cells has also been shown in the clinical setting. It was noticed that the risk of relapse after allogeneic HSCT was lower in patients who developed cGVHD compared to those who did not show any symptoms of GVHD (51-53). Furthermore, induction of GVHD by tapering immunosuppression has been shown to re-establish remission in leukemic relapse after allogeneic HSCT (54-56). It is worth mentioning that both GVHD and GVT responses are virtually absent in transplantations with grafts fully depleted of T cells (57).

Unlike donor memory T cells, naïve T cells plays a substantial role in GVHD initiation (58). This has been attributed to their abundant expression of CD62L and CCR7, allowing homing of naïve T cells to secondary lymphoid organs where they can be primed and activated by APCs. Additionally, naïve T cells possess higher T cell antigen receptor (TCR) repertoire diversity compared to memory cells. This feature could explain their ability to recognize a broader spectrum of alloantigens (59, 60).

B cells also have a significant role, particularly in cGVHD. By acting as professional APCs, as well as their ability to produce cytokines such as IL-21, they can facilitate T cell activation (61). Additionally, it has been demonstrated that rituximab (anti-CD20) containing conditioning regimen was associated with a reduced rate of GVHD (62). Conversely, B regulatory cells can improve GVHD severity in an IL-10 dependent manner (63).

Natural killer (NK) cells, like other effector cells, have been implicated in GVHD pathophysiology, though they are less likely involved in the direct GVHD initiation (64). In fact, clinical studies have shown that higher NK cell doses in allografts were associated with

less severe aGVHD, most probably due to the elimination of host APCs by donor NK cells (65).

1.1.2.5 GVHD Prophylactic and Therapeutic strategies

Most current prophylactic protocols center on suppression of alloreactive donor T cells (9). The standard GVHD prophylactic approach that is currently used in most HSCT recipients comprises a calcineurin inhibitor; cyclosporine A (CsA) or tacrolimus (Tac) combined with methotrexate (Mtx), a folate antagonist. Alternative regimens that include the use of mammalian target of rapamycin (mTOR) inhibitor such as sirolimus (Sir) instead of Mtx, have been used yet without superior advantages over the standard regimen (66).

Post-HSCT cyclophosphamide has been shown to selectively deplete proliferating alloreactive T cells with minimal or no effect on stem and Treg cells, probably because of the high expression of aldehyde dehydrogenase by these cells (67). This approach has permitted transplantation across major HLA barriers. In this regard, clinical studies have reported less severe GVHD in haploidentical transplants that received post-transplant cyclophosphamide (68).

Other novel pharmacological agents that act through targeting specific signaling pathways are currently under investigation in preclinical studies or clinical trials and could hold promise to treat GVHD in the future. Recently, Ibrutinib, a novel drug that targets the burton's tyrosine kinase (BTK) pathway, has been approved by FDA for the treatment of glucocorticoid-resistant cGVHD patients (28). The mechanisms of actions of these drugs are illustrated in figure 4.

In addition to current pharmacological drugs, cell-based therapies have been extensively investigated. Donor T cell depletion in allografts has been associated with less severe GVHD. T cell depletion can be achieved either through *ex vivo* manipulation of allografts (e.g. CD34+ positive selection, CD3+ depletion, αβ depletion) or *in vivo*, using neutralizing anti-T cell antibodies. While this approach remarkably reduced the incidence of GVHD, it is hampered by the associated increased risk of relapse, infections, and slower immune reconstitution (69).

Another cell-based approach is based on inducing peripheral tolerance. Multiple studies investigated the use of Treg cells to treat GVHD. Mesenchymal stromal cells (MSC) have been also investigated with promising results especially in steroid refractory GVHD. Importantly, the progress in HLA-typing has significantly improved outcome by reducing GVHD incidence and severity, while increasing the rate of engraftment (70-72).

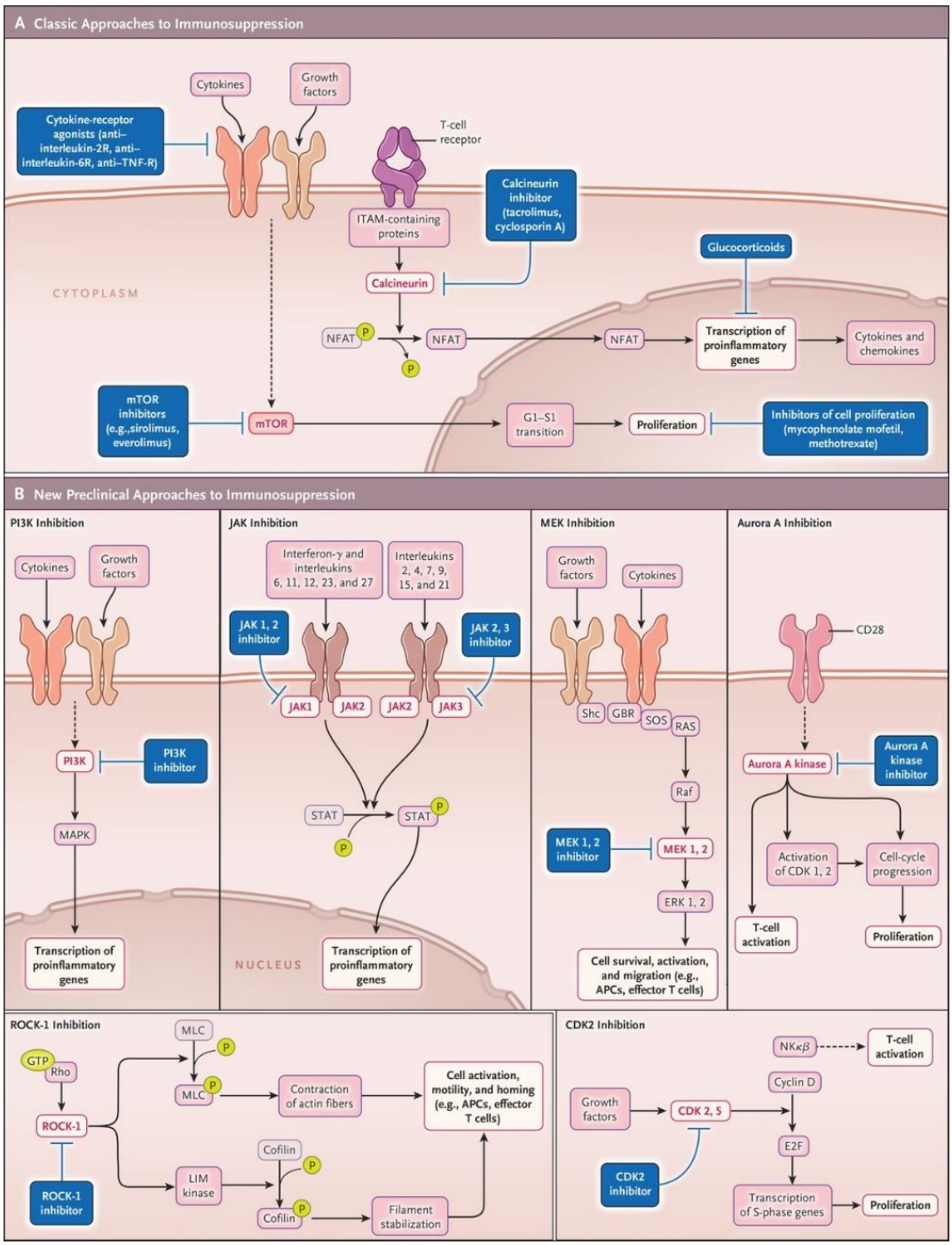

Figure 4. The mechanism of actions of classical and emerging drugs for GVHD prophylaxis Reproduced with permission from Zeiser R et al. (28), Copyright Massachusetts Medical Society).

1.2 T CELL RECONSTITUTION FOLLOWING ALLOGENEIC HSCT

Allogeneic HSCT is associated with a critical period of immune deficiency, predisposing patients to increased susceptibility of severe life-threatening bacterial, viral, and fungal infections. Therefore, efficient restoration of functional immune system is substantial for beneficial outcome (73).

The innate immune system recovers earlier during the first weeks following HSCT, providing essential protection against bacterial infection. The first innate cells to recover are monocytes,

followed by neutrophils (74). Regeneration of damaged mucosal barrier provides additional protection against pathogen penetration. Neutrophil engraftment is defined clinically as the first day of three successive days with absolute neutrophil count > 0.5 $\times 10^9$ cells/L. The time to engraftment is critical and varies between patients depending on several factors. It has been shown that neutrophils reach normal levels earlier in HSCT using Peripheral Blood (PB) compared to Bone marrow (BM) as graft source, while UCB is associated with prolonged time to engraftment (75). Since T cells are more relevant to my thesis, in the following section I will discuss different aspects related to T cell reconstitution following allogeneic HSCT.

1.2.1 Human Thymus: an overview

The development of the human thymus starts early in fetal life from the endoderm of the third and fourth pharyngeal pouches. By the 7th gestational week, stem cells populate the thymus which continues to descend until it reaches its final position in the superior mediastinum above the heart (76). Structurally, the thymus consists of bilateral lobes enclosed in mesenchymal capsule. The thymic parenchyma consists of an outer cortex and an inner medulla, together comprising the niche essential for supporting proper maturation of thymocytes (77).

The biological role of the thymus remained among the most intriguing mysteries in the history of medicine for decades until its immunological role was first discovered by J.F Miller in the early 60s (78). Within the thymus, T cell progenitors undergo multi-differentiation steps and phenotypic changes, ultimately leading to the generation of a diverse repertoire of self-tolerant naïve T cells (Figure 5). These steps entail complex interactions between T cell precursors and both thymic epithelial cells (TECs) and dendritic cells (DCs).

The rearrangement of the T cell antigen receptor (TCR) takes place in the thymus through somatic recombination of their variable (V), (diversity (D)), and joining (J) gene segments. This complex process is mediated by recombinase activating genes and is pivotal to generate a diverse repertoire of T cells, allowing recognition of a wide range of pathogens (79).

As a result of the randomly generated TCRs, some autoreactive clones are produced. Thus, a stringent process comprised of two consecutive selection steps is implemented to eliminate autoreactive clones. In the first step, thymocytes at the double-positive (DP) stage are tested for the ability of their TCR to recognize peptide/MHC complexes expressed by cTECs in a process referred to as positive selection. T lymphocytes that successfully recognize peptide/MHC complexes survive and differentiate into CD4+ or CD8+ single positive thymocytes (80).

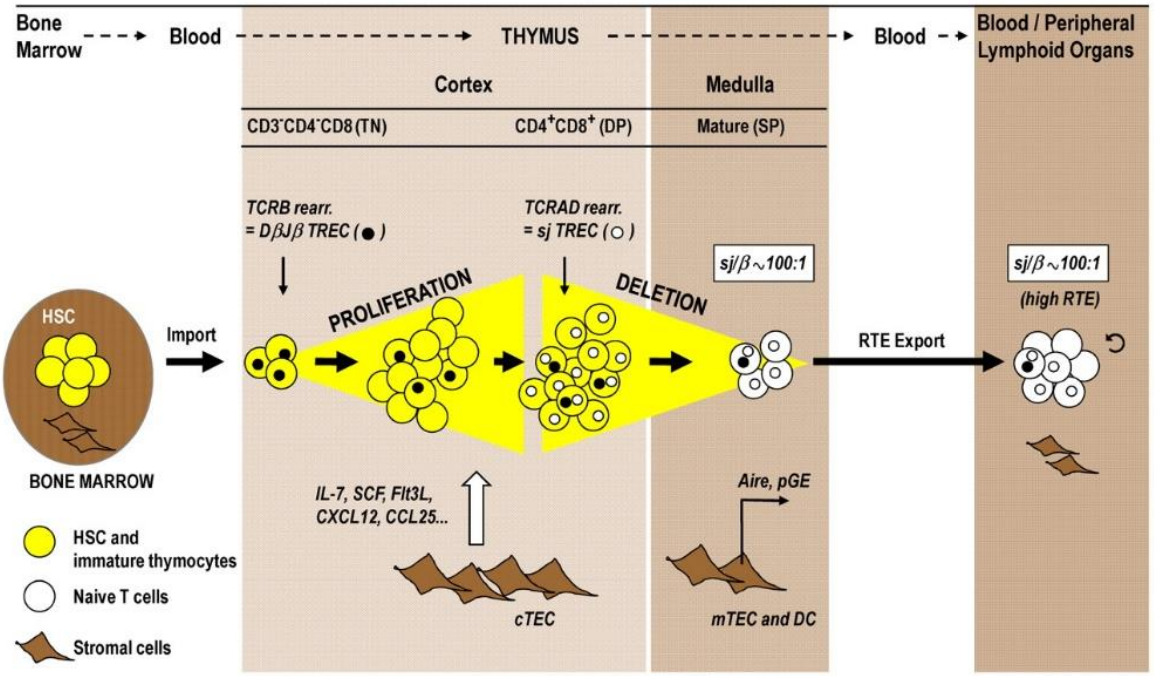

Figure 5. The process of thymocyte development in the thymus. (reproduced with permission from Krenger et al. (81))

Thymocytes upregulate CCR7 expression which allows them to migrate to the thymic medulla where the second stage takes place. Within the thymic medulla, clonal deletion of autoreactive T cells occurs in a process referred to as negative selection (77). Medullary thymic epithelial cells (mTECs) provide the microenvironment essential for negative selection. The transcription factor autoimmune regulator (Aire) and more recently described Fezf2 that regulate the expression of a large set of self-antigens including the tissue-restricted antigens (TRA) are selectively expressed by mTECs (80).

1.2.2 Reconstitution of T lymphocytes: two distinct pathways

The clinical outcome following allogeneic HSCT relies to large extent on optimal T cell reconstitution. Following allogeneic HSCT, the recovery of the T cell pool is accomplished through two distinct processes; A) thymic-independent homeostatic proliferation of donor-derived mature T cells, and B) thymic-dependent *de novo* generation of naïve T cells from donor HSCs (Figure 6) (74, 77, 82). In fact, in HSCT settings where donor T cells are intentionally depleted, the thymus represents the main source for T cell generation (83). Though the thymic-independent pathway is a short track for rapid replenishment of the virtually empty T cell pool, the extent to which it contributes to protection against infection is rather limited due to skewing of the TCR repertoire (84, 85). In this case, the TCR diversity depends on the initial diversity state of the donor-derived T cells in the SC graft (81). Additionally, T cells undergoing homeostatic proliferation are more prone to activation-induced cell death (86). Moreover, donor-derived T cells are potentially alloreactive, which can be beneficial through exerting a potent GVL effect, but also can be harmful as it has been associated with the development of GVHD (87). On the other hand, thymic-generated T cells

undergo TCR rearrangement and stringent selection steps resulting in a self-tolerant, highly diverse repertoire of T cells (82, 88).

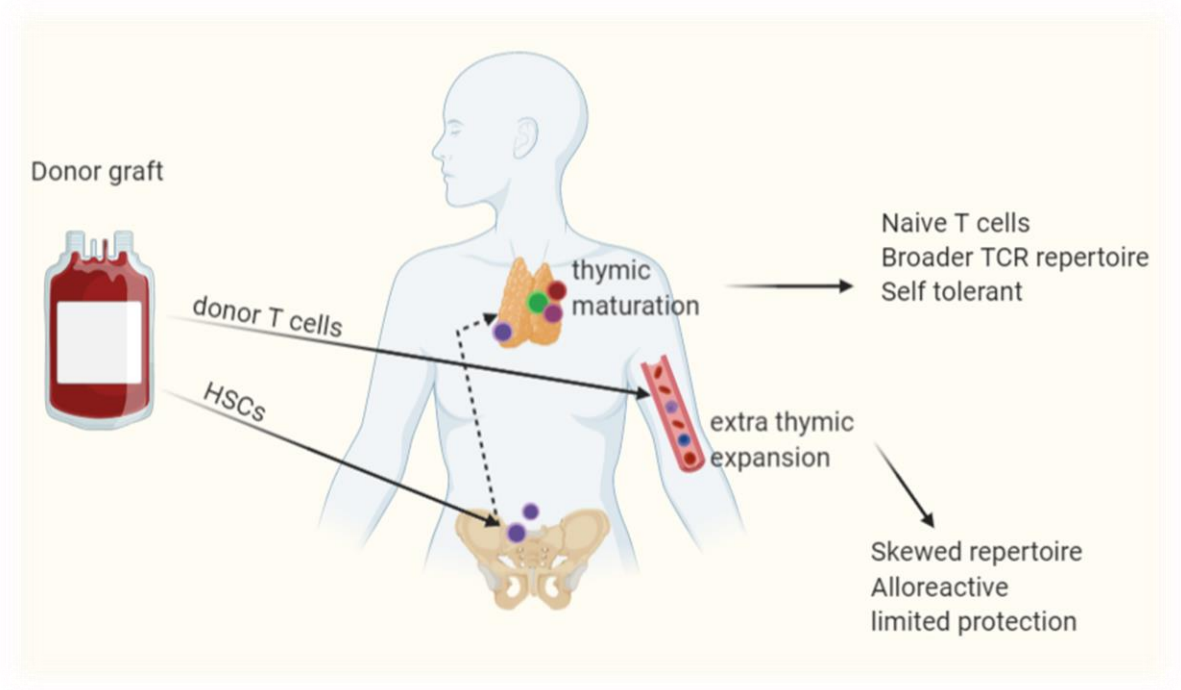

Figure 6. Reconstitution of T cells through thymic and extrathymic pathways.

1.3 GAMMA DELTA (γδ) T CELLS

1.3.1 Background

Gamma delta (γδ) T cells are unconventional T lymphocytes that express T cell antigen receptor (TCR) formed of γ and δ chains (89). Alongside B cells and αβ T cells, γδ T cells co-evolved in vertebrates for several million years and have been implicated in anti-microbial and anti-tumoral immune surveillance (90).

The proportion of γδ T cells varies depending on the type of tissue. While accounting for 1-10% of T lymphocytes in the peripheral blood of an adult human, they usually constitute 10-70% of resident T cells in other tissue compartments such as skin, intestinal mucosa, and the female reproductive system (91). In contrast to αβ T cells, most γδ T cells do not express CD4/CD8 co-receptors. Additionally, γδ T cells can be activated in both TCR-dependent and independent manners, acting as a bridge between innate and adaptive immunity (92).

1.3.2 Subsets of human γδ T cells

Though γδ T cells have co-evolved in rodents and mammals, human γδ T cells orthologically differ from their mouse counterparts due to evolutionary divergence of the γ and δ loci between the two species. While the γ chain is used to classify murine γδ T cells (e.g γ1, γ4, γ5, etc...), human γδ T cells are basically defined by their δ chain. Accordingly, human γδ T cells

can be grouped into two major subsets Vδ2+ subset and Vδ2- subset of which Vδ1+ cells are the predominant fraction (93).

1.3.2.1 Vδ2+ subset

In adults, γδ T cells expressing a semi-invariant TCR comprised of Vδ2 and Vγ9 domains (Vγ9Vδ2) are the predominant subset in peripheral blood (50-95% of γδ T cells) and are characterized by their reactivity to phosphoantigens (PAgs) (94, 95).

Vγ9Vδ2 T cells play a pivotal role in lymphoid immune stress surveillance. Their ability to recognize infected or transformed cells through a TCR-independent or TCR-dependent but HLA-unrestricted manner (Figure 7), has placed these cells in the first line of defense (96).

The Vγ9Vδ2 TCR can recognize a group of nonpeptidic phosphorylated antigens known as phosphoantigens (PAgs). These antigens can be produced endogenously as intermediates of the isoprenoid biosynthesis of the mevalonate pathway (e.g. isopentenyl pyrophosphate (IPP)), or exogenously, such as HMBPP produced by the microbial non mevalonate pathway (97). Of note, IPP is less potent than HMBPP and its physiological levels are below the activation threshold of Vγ9Vδ2 cells. Noteworthy, in tumor cells, the mevalonate pathway is upregulated resulting in accumulation of IPP, thus acting as a tumor-associated antigen (93).

Additionally, Vγ9Vδ2 T cells can recognize cells under stress, such as transformed or infected cells, through TCR-independent mechanisms. Besides pattern recognition receptors (98), Vγ9Vδ2 T cells are equipped with other receptors such as natural killer group 2 D (NKG2D) and DNAX accessory molecule 1 (DNAM1) receptors (89). The ligands of NKG2D receptors comprise the stress-inducible ligands MHC class I-related chain A/B (MICA/B) and UL16 binding protein (ULBP). These ligands are upregulated in transformed and stressed cells, allowing rapid recognition and elimination by Vγ9Vδ2 T cells (99).

Furthermore, Vγ9Vδ2 T cells express CD16, a low affinity receptor that can bind the constant region of immunoglobulin (FcγIII). Thus, they can mediate recognition and killing of opsonized target cells through antibody dependent cellular cytotoxicity (ADCC) without need for TCR engagement (100).

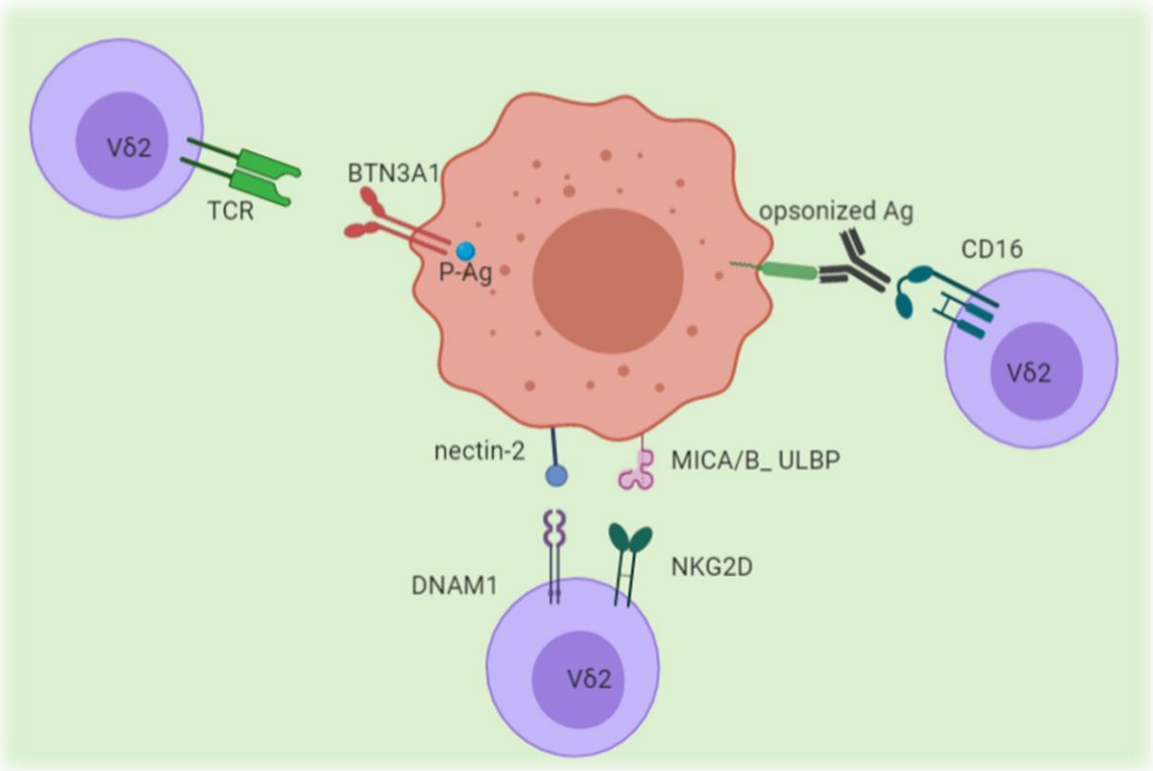

Figure 7. Tumor cell recognition by Vγ9Vδ2 T cells

1.3.2.2 Vδ2- subset

Vδ2- γδ T cells are dominated by a subset that express a TCR that contains a δ chain variable region 1 paired with various γ chains, known as Vδ1+ T cells and represent approximately 50% of γδ T cells in fetal blood. However, in adults they are predominantly found in epithelial tissues (101). Vδ1+ T cells can effectively eradicate tumor cells and display potent anti-viral response especially against CMV after transplantation.

In contrast to Vγ9Vδ2 T cells, Vδ1 TCRs recognize a wider range of antigens (Figure 8), including lipid antigens presented by MHC-like molecules, CD1c and CD1d (101). Additionally, MICA/B have been suggested as ligands for TCR of Vδ1+ cells, though NKG2D affinity to MICA/B was shown to be 1000-fold higher compared to TCR (102).

In addition to NKG2D and DNAM1 mediated recognition, Vδ1+ cells activated *in vitro* through the TCR can express natural cytotoxicity receptors such as NKp30 and NKp44. Moreover, downregulation of HLA class I as tumor immune evasion mechanism can enhance the cytotoxicity of Vδ1+ cells through activation of killer inhibitory receptors (KIR).

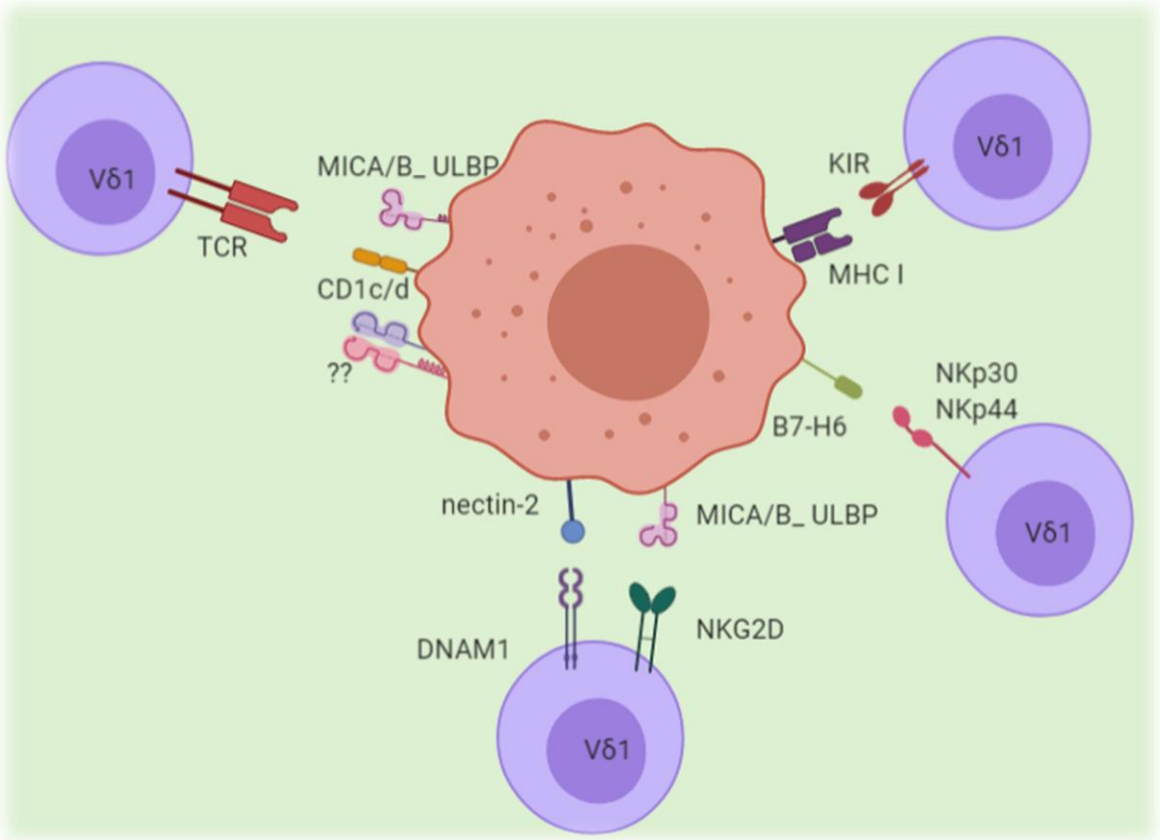

Figure 8. Tumor cell recognition by Vδ1 T cells

1.3.3 γδ TCR repertoire and ligands

T cells including γδ T cells differentiate from a common T cell progenitor in the thymus, where poorly defined mechanisms involving complex signaling pathways drives their lineage commitment (αβ or γδ) (101). However, unlike αβ T cells, antigen recognition by γδ TCR is fundamentally different in that it is major histocompatibility complex (MHC) unrestricted. Furthermore, the CDR3 length pattern of the δ chain is more analogous to that of antibodies than the conventional TCRs (103, 104).

Both γ and δ chains are encoded by two distinct sets of genes. The *TRD* locus is located on chromosome 14, embedded within the *TRA* gene, whereas *TRG* is found on chromosome 7 (105). An essential prerequisite for γδ T cell development and lineage commitment is the formation of a functional TCR. This entails somatic recombination of the variable (V), diversity (D), and joining (J) gene segments in the *TRD* locus and the V and J segments in the *TRG* locus. In theory, somatic recombination of the γδ TCR can yield up to 10^{17} possible unique clones, resulting in extensive diversity. Nevertheless, circulating γδ T cells are dominated by cells that express a semi-invariant TCR formed of Vγ9Vδ2, suggesting the potential of early neonatal exposure to PAgs that shapes the repertoire of γδ T cells throughout life (106).

Next generation sequencing (NGS) has enabled in-depth analysis of CDR3 of TCR δ and γ repertoires. Studies have indicated that the TCR δ repertoire is more diverse yet comprises highly private (less public) CDR3 sequences. Contrastingly, TCR γ repertoire displays limited diversity and contains CDR3 sequences that are commonly shared among individuals (public) (107).

Furthermore, the TCR repertoire displays fundamental differences between different subsets. While the TCR repertoire of Vδ1+ cells is initially highly diverse at birth and becomes more focused at adulthood. The repertoire of Vγ9Vδ2 T cells has intermediate diversity and does not considerably differ between adults and cord blood (Figure 9) (108).

The semi-invariant nature of Vγ9Vδ2 TCR has been further elucidated through exploring the γ9 repertoire. CDR3 sequences of γ9 are enriched in public amino acid sequences that persist throughout life. This has been attributed to the presence of multiple germ line encoded sequences with limited junctional diversity beside the effect of convergent recombination.

The responsiveness of Vγ9Vδ2 cells to PAgs has also been linked to the TCR molecular structure. The exclusive pairing of Vγ9 to JγP but not to other Jγ families, has been shown to mediate PAgs recognition. Likewise, CDR3 of δ2 is enriched in hydrophobic amino acid residues at position 5 that are crucial for PAgs recognition.

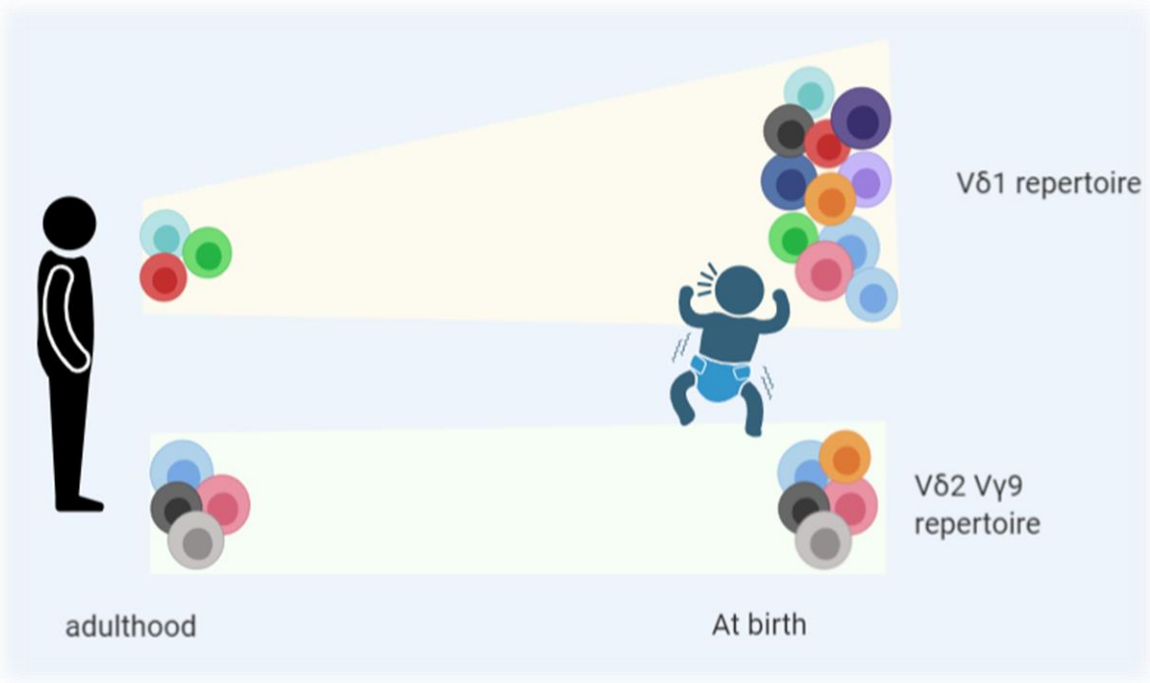

Figure 9. TCR repertoire of Vδ1 and Vδ2Vγ9 cells at birth and adulthood.

γδ TCR ligands are under extensive ongoing research. Immunotherapeutic strategies using γδ T cells have been hindered by poor understanding of the mode of TCR mediated antigen recognition (109). Thus, identifying such ligands would be of utmost importance to unleash the full immunological capabilities of γδ T cells and overcome the current limitations to

exploiting γδ T cells in immunotherapeutic strategies. Furthermore, distinguishing a specific ligand for a certain γδ clone could allow *in vitro* clonal expansion in a large clinical scale sufficient for use in adoptive immunotherapy.

The earliest defined antigens for γδ T cells are PAgs that specifically induce activation and proliferation of Vγ9Vδ2 T cells. Though Vγ9Vδ2 responsiveness to PAgs had been discovered in early 90s, their underpinning recognition mechanism remains unknown. Large body of evidence suggests a member of the B7 super family, BTN3A1, to function as an antigen presenting molecule though its exact mode of recognition remains elusive (110). In one model, BTN3A1 was proposed to capture and present PAgs directly to Vγ9Vδ2 TCR (111). More recently, this model was challenged by results from other groups supporting an inside-out signaling model where PAgs bind an intracellular domain of BTN3A1, known as B30.2. this binding induces conformational changes in the extracellular domain and results in TCR activation (112, 113). Sebestyen and colleagues, proposed a model that involves RhoB intrinsic GTPase activity. Suggesting that RhoB relocalizes to BTN3A1, inducing cytoskeletal changes which in turn stabilize BTN3A1 molecules and allows for subsequent PAgs binding (110).

In contrast to Vγ9Vδ2 T cells the ligands for Vδ2- cells have remained elusive. Several efforts have been made to identify some ligands. Multiple studies have reported some potential ligands (Table 3). However, these ligands are heterogenous with regards to their structure and biology. Their exact mode of recognition remains elusive, adding more complexity to the already confusing landscape.

Ligand name	Cells that recognize	location	reference
BTN3A	Vγ9Vδ2	PB	Salim et al. (114)
BTNL3-BTNL8	Vγ4Vδ1	Intestinal γδ cells	Melandri et al. (115)
EPCR	Vγ4Vδ5	PB	Willcox et al. (116)
Annexin A2	Vγ8Vδ3	--	Marlin et al. (117)
phycoerythrin	Vδ1	PB	Zeng et al. (118)
CD1c	Vδ1	PB	Roy et al. (119)
CD1d	Vδ1	PB	Uldrich et al. (120)
MICA/B	Vδ1	PB	Xu et al. (102)

Table 3. γδ TCR ligands

1.3.4 Adaptive-like γδ T cells

The hallmark of the adaptive immune response is manifested by the ability of cells to display clonal selection and expansion upon recognition of cognate antigen concurrently with the differentiation from a naïve to an effector/memory phenotype. With this concept in mind, several researchers have explored the adaptive immunobiology of γδ T cells (121, 122).

γδ T cells have been widely considered as innate-like. Similar to mucosal associated invariant T (MAIT) cells and invariant natural killer T (iNKT) cells, the predominant γδ cells in the PB display a semi-invariant TCR (Vγ9Vδ2), that features limited diversity and responds to a limited antigen (pAgs) in the context of presenting molecule (BTN3A1). In this paradigm,

TCR can be considered as surrogate pattern recognition receptor. Furthermore, the ability of Vγ9Vδ2 cells to react rapidly in response to stress-induced signals in a non TCR-dependent way supports innate-like paradigm (121).

Davey et al, used NGS and single TCR sequencing to explore the TCR repertoire of Vδ1+ cells. They revealed that Vδ1+ TCR repertoire is initially diverse at birth and become highly focused in adulthood. Furthermore, they explored Vδ1+ TCR repertoire perturbation in response to CMV infection. They revealed significant clonal expansion, suggesting that Vδ1+ cells respond to a driving antigen (CMV) by clonotypic expansion of a few clones. Furthermore, clonality was associated with differentiation from naïve-like (CD27 high) to effector (CD27 low) phenotype (108).

In another study, Davey et al showed that Vδ2+ cells comprise two clonotypically and biologically distinct subsets. An innate-like population that expresses Vγ9+Vδ2+ TCR and respond to PAgs and another small subset that preferentially pairs to non Vγ9 (Vγ9-Vδ2+). Unlike its Vγ9+ counterpart, this subset was unresponsive to PAgs yet responded to TCR stimuli. Furthermore, they displayed clonal expansion in response to CMV infection suggesting adaptive-like biology (122).

1.3.5 Functional diversity of γδ T cells

While murine γδ T cells acquire their functional effector phenotype early during thymic development, human γδ T cells exit the thymus as naïve cells and acquire their functional fate in the periphery. Noteworthy, unlike their mouse counterparts, human γδ T cells are by default programmed to be cytotoxic effector cells that secrete IFNγ and TNFα, thus they have anti-tumorous protective function by nature. However, in presence of highly inflammatory milieu as tumor microenvironment, γδ T cells can undergo reprogramming and acquire IL-17 producing phenotype (IL-17 γδ T cells) (109).

It has been shown that γδ T cells can differentiate into IL-17 producing cells in response to certain cytokines such as IL-1β, IL-23, IL-6 and TGFβ. These cytokines are characteristically heightened in the inflammatory tumor microenvironment as a result of their increased secretion by macrophages and myeloid cells in the tumor microenvironment. Furthermore, it has been shown that microbiota induce IL-17 γδ T cells, which explains the increased proportions of IL-17 γδ T cells in cases of severe meningitis (99).

The pro-tumorous function of IL-17 γδ T cells has been established in mice and human studies, where the presence of IL-17 γδ T cells in higher frequencies was found to be associated with increased tumor growth and metastasis (93).

Mechanistic analysis highlighted several underlying mechanisms that mediate the pathogenic effect of IL-17 γδ T cells (Figure 10); 1- IL-17 produced mainly by IL-17 γδ T cells in tumor microenvironment can act directly on IL-17 receptors expressed by some tumors and induce their growth. 2- IL-17 can induce angiogenesis through upregulating pro-angiogenic factors such as vascular endothelial growth factor (VEGF). 3- A reciprocal cross talk between IL-17

γδ T cells and myeloid cells has been described. IL-17 γδ T cells have been shown to induce G-CSF mediated neutrophil and macrophage mobilization to tumor microenvironment and have been shown to induce neutrophil differentiation into myeloid derived suppressive cells (MDSC) that exert strong suppressive effect on other T cells (123). 4- Through the secretion of the cytokine IL-4 and the expression of programmed cell death protein ligand 1 (PDL-1), IL-17 γδ T cells can exert direct suppressive effect on other T cells. 5- IL-22 produced by IL-17 γδ T cells has been shown to induce tumor cell proliferation (93, 99, 109).

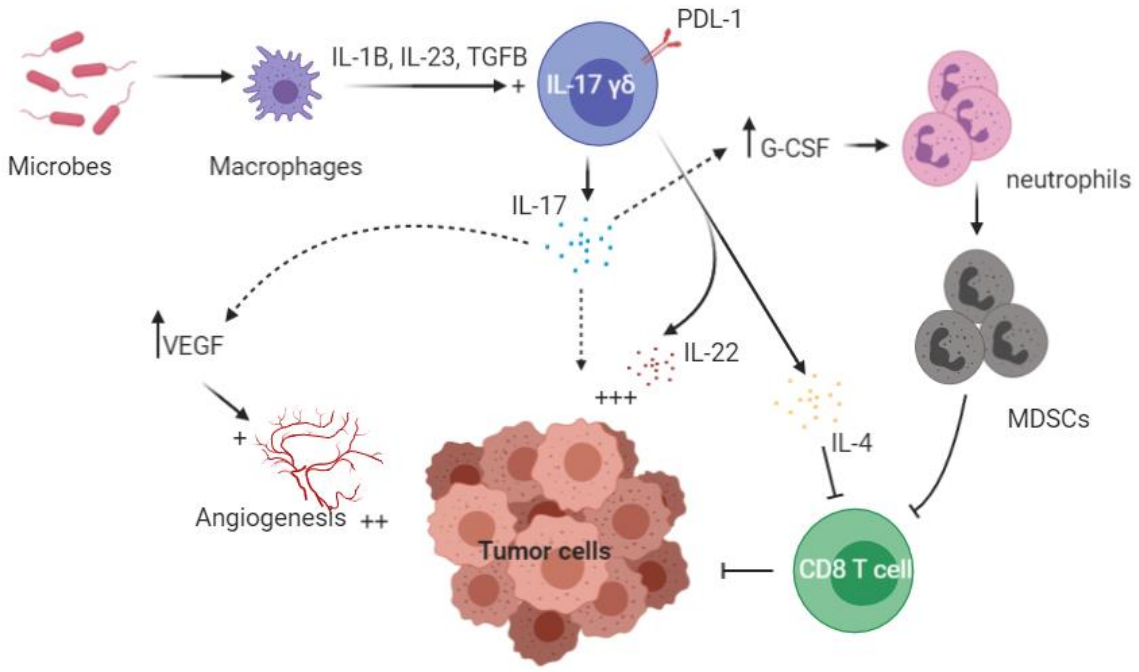

Figure 10. Pro-tumor role of IL-17γδ T cells

1.3.6 γδ T cells in allogeneic HSCT

The antitumor property of γδ T cells has been shown for the first time by Heyday's group in experimental mice model (124). It has soon become well established that γδ T cells can exhibit potent anti-tumor/anti-viral immune responses in the absence of an alloreactive immune response. This feature has placed γδ T cells as a subject of great interest in allogeneic HSCT as they can offer the beneficial GVL effect in the absence of GVHD.

1.3.6.1 Reconstitution following allogeneic HSCT

In contrast to conventional αβ T cells and B cells, γδ T cell recovery occurs shortly after HSCT (125). Hirokawa et al. examined reconstitution of γδ TCR repertoire after allogeneic HSCT. They reported that the γδ TCR repertoire was almost completely recovered a few months after transplantation (126). Although the reconstitution of γδ T cells has been investigated to some degree, the effect of different transplant related factors, such as donor type, conditioning regiment etc. on frequency and TCR diversity remains largely unknown.

The effect of G-CSF mobilization on γδ T cells are controversial. Bian et al. examined phenotypic characteristics of γδ T cells in the peripheral blood of 20 donors before and after G-CSF mobilization. They showed that γδ T cells retained their homeostatic proportions and IFN-γ secreting capabilities following G-CSF (127). Conversely, earlier study showed that G-CSF was associated with TCR repertoire disturbances in the form of alteration of distribution and clonality of some TRG and TRD subfamilies, suggesting a potential immune modulatory effect (128).

Numerus studies have highlighted the importance of conventional αβ T cell recovery after HSCT, but the impact of γδ T cell recovery has not been well described. In this regard, early reconstitution of γδ T cells has been correlated with favorable outcome after allogeneic HSCT. It has been confirmed in multiple clinical studies that patients with elevated γδ T cells numbers have increased leukemia-free survival and overall survival (129, 130). Perko et al. investigated the recovery of γδ T cells in 102 pediatric patients with acute leukemia after allogeneic HSCT. They reported decreased incidence of infection and improved event-free survival in patients with elevated γδ T cells (131).

1.3.6.2 Anti-tumor effect

The role of γδ T cells in tumor surveillance was first demonstrated in a mouse model where mice without γδ T cells were found to be more susceptible to the development of cutaneous malignancy (124). Since then, numerous studies have corroborated and extended these findings in different tumor models (132-135). The anti-tumor effector functions can be mediated by direct cytotoxicity or indirectly through the production of cytokines/chemokines by which they affect other cell types. Additionally, γδ T cells can upregulate surface expression of Fc-receptor FcRγIII (CD16) which can be implicated in tumor cell lysis via antibody-dependent cellular cytotoxicity (ADCC) (92, 105).

In human cancer, the presence of infiltrating γδ T cells has been found in a large range of different tumor types. In a recent large meta-analysis of expression signatures from 18000 human tumor samples covering 39 different tumor, γδ T cell signature was found to be the most favorable significant cancer-wide prognostic immune cell population (136).

1.3.6.3 Anti-infectious properties of γδ T cells

The ability of γδ T cells to protect against infectious pathogens is mediated by several mechanisms. Cytotoxic activities can be mediated by Fas-Fas ligand interaction, perforin/granzyme release, and CD16 mediated ADCC (125). Additionally, γδ T cells can interact with and modulate the activity of other immune cells. For instance, they can promote DC maturation and enhance anti-infectious activities of NK and macrophage. Furthermore, γδ T cells can secrete large amounts of pro-inflammatory cytokines such as IFN-γ, TNFα, IL-17, and IL-22 (90).

In allogeneic HSCT, the anti-bacterial role of γδ T cells is not well displayed. This is due to the administration of prophylactic antibiotics which effectively control bacterial infection. The

anti-viral role is more prominent. CMV reactivation has been shown to be associated with expansion of Vδ2- T cells (137, 138). Furthermore, several studies have highlighted the role of Vδ1 T cells in CMV reactivation after allogeneic HSCT (137, 139). De Paoli et al. showed a possible role of γδ T cells in EBV immune response (140).

1.3.6.4 γδ T cells and GVHD

whether γδ T cells are implicated in immunopathology of GVHD remained an open question. Most evidence indicated that donor γδ T cells are not key effectors in inducing GVHD and their levels in PB following HSCT were not associated to increased severity or incidence of GVHD (92, 125, 135, 141). However, few studies in murine models have shown that γδ T cells contribute to pathogenesis of GVHD (142-144). Maeda et al, investigated the role of host γδ T cells in experimental GVHD model using γδ deficient and wild type mice. They showed mechanistically that host γδ T cells exacerbate GVHD by enhancing the alloreactive capacity of DCs through cell contact dependent manner (144).

Given the early reconstitution of γδ T cells during HSCT, investigating graft composition of γδ T cells can provide insight into their contribution to clinical outcome, particularly aGVHD following HSCT. In this context, Pabst et al. showed increased cumulative incidence of acute GVHD II-IV in patients that received PBSC grafts with higher γδ T cells content (145). However, they examined total γδ T cells and the impact of different subsets were not investigated. Given the heterogeneity of γδ T cells (146, 147), more research is needed in order to elucidate the role of different γδ T cell subsets in GVHD.

1.3.7 γδ T cells as an attractive target for immunotherapy

The ability of γδ T cells to produce large amounts of cytokines, expression of perforin, granzymes, and death receptor ligands, as well as their ability to bypass tumor immune evasion mechanisms based on MHC class I down regulation, has made them an attractive target for cancer immunotherapy (97). However, the potential to use γδ T cells for adoptive cell therapy (ACT) was challenged by their scarcity in PB. Moreover, the standard methods of T cell culture can induce apoptosis in γδ T cells (92, 148). Fortunately, this obstacle was overcome with the discovery that aminobisphosphonate drugs can specifically induce activation and expansion of Vγ9Vδ2 T cells by inhibiting a key enzyme (farnesyl PP synthase) in the mevalonate pathway, leading to accumulation of PAgs (97).

In fact, expansion of γδ T cells from adult PB has been explored with considerable success. For this purpose, two main strategies have been widely used in adoptive cell therapy. *In vivo* Expansion of γδ T cells could be achieved by the administration of bisphosphonates such as zoledronate (Zol). It has been shown that this method not only can expand cells but also increase their lytic function (149, 150). However, it has been reported that γδ T cells rapidly exhaust upon repeated application of Zol (146). *In vitro* techniques involve the stimulation of γδ T cells with natural or synthetic PAgs in presence of exogenous cytokines. More efficient expansion could be achieved following the use of autologous dendritic cells pretreated with Zol (150, 151). Furthermore, the use of anti-apoptotic cytokines, like IL-15, has been reported

to prevent activation-induced cell death of *in vitro* cultured γδ T cells (150, 152). The adoptive transfer of Vδ2 T cells expanded *in vitro* appeared to be well tolerated with no major adverse effects.

Clinical trials using *ex vivo* expanded PB γδ T cells for immunotherapy have been confirmed to be safe and have shown adequate results in patients with malignancies such as renal cell carcinoma (153), melanoma (154), and multiple myeloma (155). Other clinical trials have explored treatment of patients with γδ T cells expanded *in vivo* by bisphosphonates and IL-2 (156, 157). Despite these trials, no large-scale studies with long term follow-up exist to our knowledge.

In contrast to Vγ9Vδ2 T cells, Vδ1 T cells have not received the same attention until recently. several reports have indicated that Vδ1 T cells exhibit more potent cancer killing and express higher levels of CD69, CD107a, granzyme B, TRAIL, and HLA-DR than Vδ2 T cells (158). While Vγ9Vδ2 T cells have been successfully expanded, there are only few attempts for Vδ1 expansion. Lamb et al. used an approach based on αβ T cell depletion from PBMC and using irradiated leukemia feeder cells and low levels of IL-2 (135). Using phytohemagglutinin (PHA) and IL-7, Wu et al. selectively expanded Vδ1 T cells from PB of healthy donors as well as colon cancer patients(158). More recently, Almeida et al. developed a protocol for selective enrichment and expansion of delta one T (DOT) cells (159). In their protocol, Vδ1 cells were stimulated and differentiated by using TCR agonists in presence of IL-4 and IL-15. The expanded DOT cells by this protocol were functionally active and were able to target acute myeloid leukemia via NKp30 (160).

2 AIMS

The hypothesis of my thesis is that γδ T cells specifically attack malignant cells without causing detrimental GVHD in patients after HSCT. We postulate that their development is closely related to whether patients will relapse in their malignant disease.

The results from this work would help to increase patient survival and decrease transplantation-associated complications such as malignant relapse and infectious complications. It will also make it possible for more patients eligible for HSCT

The general aim of my doctoral research project is to investigate the role of the adaptive immune system, with a special focus on γδ T cells, in allogenic HSCT.

More specifically, in paper I we investigated de novo generation of lymphocytes post allogeneic HSCT and addressed whether thymopoiesis and B cell neogenesis are affected by GVHD prophylaxis regimens and their role in predicting clinical outcome.

In papers II-IV, we sought to investigate the graft composition of γδ T cells in detail and addressed whether γδ T cell phenotype/TCR repertoire is correlated to clinical outcome in terms of malignant relapse, viral/bacterial infections and other complications.

Finally, in paper V, we aimed to develop and improve a protocol for γδ T cell expansion from UCB. We then studied the phenotypic and functional properties of expanded cells for future use in adoptive cell immunotherapy.

3 MATERIALS AND METHODS

3.1 SAMPLE CHARACTERISTICS

In my thesis, different sample types were used. In paper I, PB samples were collected from transplanted patients at several time points following allogeneic HSCT. In total 200 patients, with at least one retrievable blood sample were included. Characteristics of patients are shown in table 4

Variable	CsA/Mtx	Tac/Sir
Number of patients	103	97
Median age, years (range)	53 (0.6–71)	51 (2.8–68)
Number of children <18 years	12	13
Sex, number of males/females	57/46	63/34
Median follow-up, years (range)	7.6 (3.9–10.3)	7.2 (3.9–10.3)
Diagnosis, number of patients:		
AML/ALL	27/19	22/22
CLL	7	15
Lymphoma	14	13
MDS	19	14
Other malignancies	7	8
Non-malignant	10	3
Disease stage, early/late	53/50	36/61
Conditioning regimen, number of patients:		
MAC/RIC	28/75	28/69
TBI-based	31	39
ATG	78	69
Donor, number of patients:		
Sibling/MUD	28/75	31/66
Median donor age, years (range)	28 (4–66)	32 (7–66)
Female to male, number of patients	14	14
HSCT graft, number		
BM/PBSCs	21/82	18/79
TNC dose, $\times 10^8$/kg (range)	9.3 (1.8–34.0)	11.1 (1.8–42.8)
CD34+ cell dose, $\times 10^6$/kg (range)	6.6 (1.2–22.8)	6.3 (1.3–19.7)

Table 4. patient characteristics in paper I

In paper II-IV, donor stem cell grafts were collected and used to characterize γδ T cell composition. A total of 105 donor grafts were analyzed in paper II (Table 5) and the results were correlated to clinical outcome in corresponding recipients. In Paper III, the phenotypic and clonotypic characteristics of CD8+γδ T cells were explored in 16 BMSC graft samples from CMV+ (n=7) and CMV- (n=9) donors. In paper IV, CDR3 repertoire of TCR γ chain was analyzed by NGS. In total, 20 PBSC grafts that were transplanted to AML patients were assessed (Table 6).

In paper V, MNCs from UCB (n=8) and healthy donors PB (n=3) were used for *in vitro* expansion and subsequent functional and phenotypic characterization of γδ T cells. UCB units were collected from healthy volunteers giving birth at the maternity ward at Karolinska University Hospital in Huddinge and were unlabeled to prevent tracking of the donor

Characteristics	Recipients	Donors
Total Number	105	105
Gender: Female/Male, n	43/62	40/65
Age, median (IQR), yr.	52 (31.5-61)	30 (23-38.5)
CMV serostatus pre- HCT, - /+, n	21/84	45/58
Donor type, sibling/MUD/others, n		28/66/11
Underlying disease, n		
Acute leukemia	54	
Chronic leukemia	7	
Myelodysplastic syndrome	19	
Others	25	
Disease status: High risk/low risk, n	57/48	
Conditioning regimen, MAC/RIC n	26/79	
Graft source, BM/PB n		16/89
Relapse, n	25	
CMV reactivation, n	40	
ATG: Yes/No, n	70/35	
GvHD prophylaxis, n		
Cyclosporin A + Methothrexate	92	

Table 5. Patient characteristics in paper II

Variable	Recipients	Donors
Total number	20	20
Median age, years (range)	59.5 (38.5-64.5)	30 (23-35.7)
Sex, number of males/females	7/13	11/9
Diagnosis, number of patients	AML (20)	
Conditioning regimen, MAC/RIC	5/15	
Donor type, Sibling/MUD:		3/17
HSCT graft source, number		PB (20)
CMV serostatus (+/-)	15/5	7/13
CMV infection (yes/no)	12/8	
GVHD prophylaxis	CSA+MTX	
aGVHD, (0-I/II-III)	13/7	
cGVHD, no or mild/moderate and severe	17/3	
Relapse, Yes/no	8/12	

Table 6. Patient characteristics in paper IV

3.2 LABORATORY METHODS

Laboratory techniques used in this thesis varied among constituent papers (Figure 11). The main methods used included immunophenotyping, functional assays and molecular PCR-based techniques. The main workflow can be summarized in the following chart.

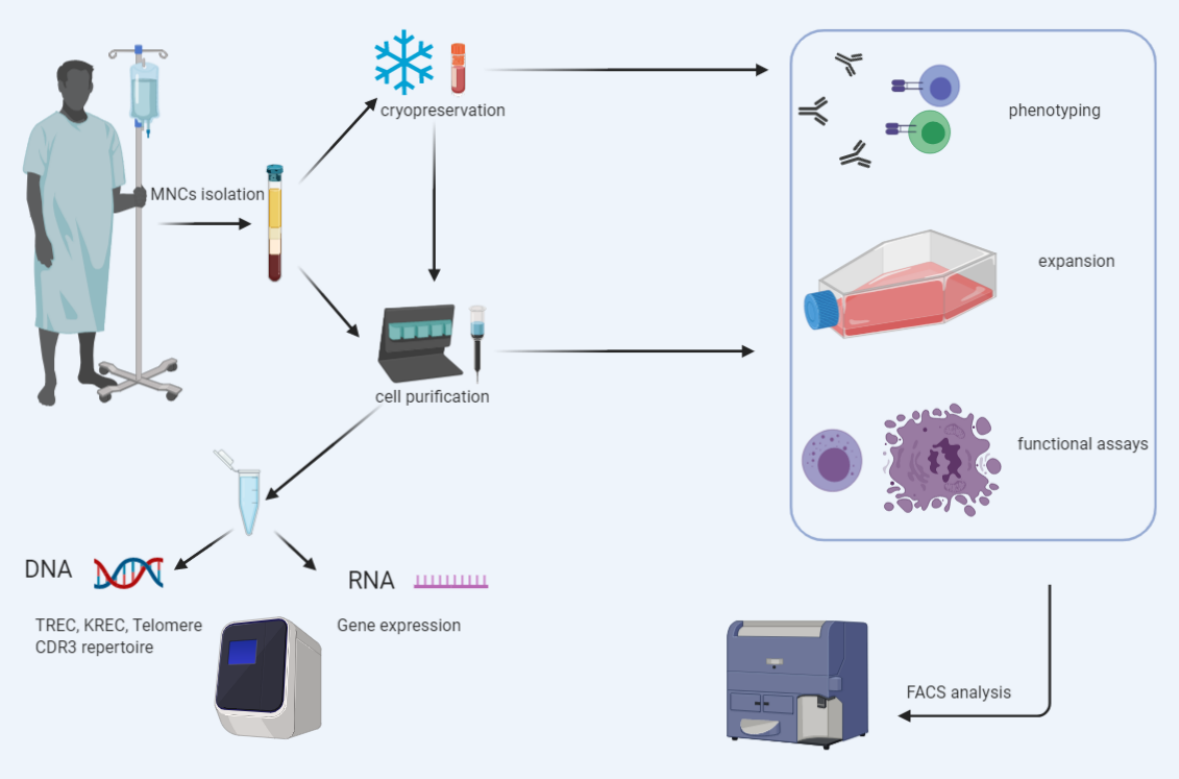

Figure 11. A flowchart showing the workflow and laboratory methods used in the thesis

3.2.1 MNCs isolation and cryopreservation

Samples were collected in vacutainers containing appropriate anticoagulant (Heparin). Mononuclear cells (MNCs) were freshly isolated by density gradient centrifugation using Lymphoprep (Fresenius Kabi, Oslo, Norway). The centrifugation step was set at 800*g* for 20 minutes with no brake. The buffy coat layer containing the MNCs was collected and washed twice with phosphate buffered saline (PBS) prior to cell counting. MNCs were either freshly used in subsequent laboratory methods or cryopreserved (in most cases) at –196°C. Freezing media consisted of 10% DMSO in RPMI-1640 medium (HyClone, Thermo Fisher Scientific Inc., Waltham, MA), containing 10% human AB-serum (Karolinska University Hospital, Huddinge, Sweden) and supplemented with 2 mM L-glutamine (Gibco, Life Technologies, Paisley, UK), 100 IU/mL penicillin G, and 100 mg/mL streptomycin (Gibco).

3.2.2 Immunophenotyping by Flow cytometry

3.2.2.1 Basic principles

Flow cytometry is a powerful high throughput instrument that allows characterization of cells at a single cell level. Flow cytometry consists of two main components: a fluidic system and a detection system (Figure 12) (161).

The role of the fluidic system is to create a laminar flow with hydrodynamic focusing. This hydrodynamic focusing forces the cells in the flow cell chamber to flow in single cell layer, allowing the passage of one single cell through the nozzle at a time.

The detection system is composed of light sources (lasers) that emit lights at different wave lengths, optical filters, and photomultiplier tube (PMT) detectors. As the cells pass through the light beam, light is scattered in different directions and can be collected by different channels. The light detected by the forward scatter channel (FSC) reflects the size of the cell, whereas that detected by side scatter channel (SSC) provides information about cell complexity.

Besides FSC and SSC, more specific markers can be detected by staining cells with fluorochrome-conjugated antibodies. These fluorochromes, once excited by light beam, emit light at specific wavelength that can be collected through appropriate filters and detectors.

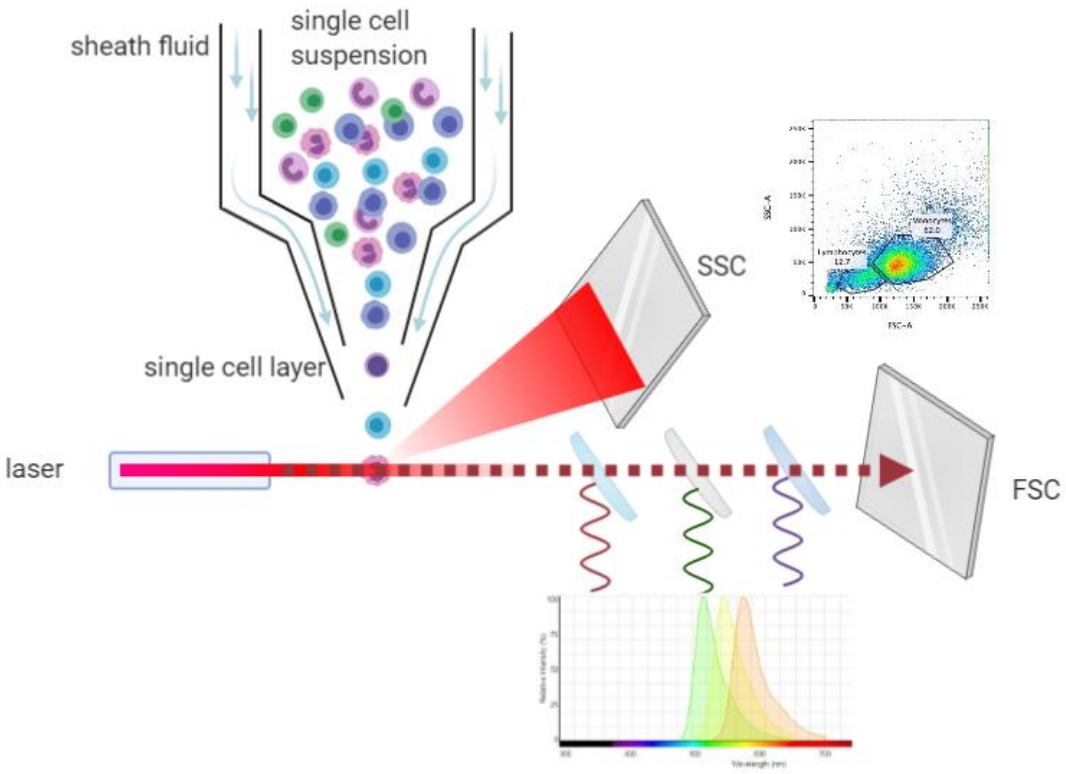

Figure 12. Basic components of flow cytometry

3.2.2.2 Cell surface staining

Cell surface staining was performed as a standard technique in all papers. Briefly, MNCs were washed with PBS and resuspended in PBS in a concentration of 1-2 $\times 10^6$ cells/mL. 200 µl of cell suspension was used for surface staining in plates. Plates were centrifuged and the cell pellets were resuspended in 100 µl of PBS containing specific fluorochrome-conjugated monoclonal antibodies (mAbs). The amount of used mAbs was estimated after initial

titration. Cells were mixed and incubated in fridge (dark) for 30 minutes. After incubation cells were centrifuged and washed twice with PBS and resuspended in 50 µl PBS. Cell viability stain was then added (2 µl of 7-AAD) and plates were incubated for 7 minutes in the dark at room temperature. At the end 200-250 µl of PBS was added and cells were transferred to FACS tubes for analysis.

Samples were acquired by FACS Canto (BD) and results were analyzed by FlowJo software (BD). For each run, appropriate controls were included. Single color stained beads (compensation beads, BD) were used to create the compensation matrix, and fluorochrome minus one (FMO) controls were used for proper gating of cell populations. A list of mAbs used for surface staining are shown in Table 7

Marker	Fluorochrome	Clone	Vendor
7-AAD	-	-	BD Biosciences
ANNEXIN V	FITC		BD Biosciences
CD3	PE	UCHT1	BD Biosciences
CD3	V450	UCHT1	BD Biosciences
CD3	BV510	UCHT1	BD Biosciences
CD4	Alexa Fluor 700	RPA-T4	BD Biosciences
CD8	APC-Cy7	SK1	BD Biosciences
CD8	FITC	SK1	BD Biosciences
CD8	V500	RPA-T8	BD Biosciences
CD19	PE	HIB19	BD Biosciences
CD25	FITC	M-A251	BD Biosciences
CD27	BV421	M-T271	BD Biosciences
CD27	PE	M-T271	BD Biosciences
CD28	FITC	CD28.2	BD Biosciences
CD45RO	APC	UCHL1	BD Biosciences
CD56	BV421	NCAM16.2	BD Biosciences
CD69	FITC	FN50	BD Biosciences
CD94	FITC	HP-3D9	BD Biosciences
CD95	FITC	DX2	BD Biosciences
CD197 (CCR-7)	PE-Cy7	3D12	BD Biosciences
CD314 (NKG2D)	BV421	1D11	BD Biosciences
CD107a	BV510	H4A3	BD Biosciences
TCRαβ	FITC	T10B9.1A-31	BD Biosciences
IFN-γ	PE-Cy7	B27	BD Biosciences
TNF-α	PE-eFluor 610	MAb11	eBioscience
TCR Vδ2	FITC	B6	Biolegend
TCR Vγ9	FITC	B3	Biolegend
CD158	PE-Cy7	DX27	Biolegend
CD152 (CTLA4)	FITC	A3.4H2.H12	Lifespan Biosciences
CD223 (LAG-3)	FITC	17B4	Lifespan Biosciences
CD366 (TIM-3)	APC	F38-2E2	Lifespan Biosciences
CD159a (NKG2A)	APC	REA110	Miltenyi Biotec
TCR pan γδ	PE	REA591	Miltenyi Biotec
CCR-9	APC	Clone # 248621	R&D SYSTEMS
TCR Vδ1	FITC	TS8.2	Thermo Scientific
TCR γδ	FITC	IMMU510	BECKMAN COULTER

Table 7. a list of fluorochrome conjugated mAbs used in the thesis

3.2.2.3 Intracellular staining (ICS)

ICS was performed in paper II to evaluate cytokine and degranulation of γδ T cells in response to mixed lymphocyte reaction (MLR). In such long incubation experiments, brefeldin A (BFA) was added to the media in the last 8 hours of incubation, while CD107a was added at the beginning. After staining for surface markers, cells were fixed and permeabilized using cytofix/cytoperm kit (BD) and were incubated for 15 min in the fridge. Next, cells were washed twice and resuspended in 100 µl of staining buffer solution (Cytofix/cytoperm kit) containing conjugated mAbs for the specific cytokines. After 30 min of incubation in the fridge, cells were washed twice and resuspended in PBS and acquired by FACS.

3.2.3 Mixed lymphocyte reaction (MLR)

MLR was used to study alloreactive responses elicited by T cells (responders) when cocultured with allogeneic irradiated MNCs (stimulators). The irradiation step was crucial to prevent proliferation of stimulators. In paper II, I used a modified MLR protocol to investigate the alloreactivity of γδ T cells. Briefly, MNCs from stem cell grafts or *in vitro* cultured γδ T cells were used as responders. The MLR coculture consisted of allogeneic responder cells that were labeled with 2 µM of CellTrace™ Violet Cell Proliferation Kit (ThermoFisher), and irradiated MNCs (stimulators). A condition without stimulators was used as control. The responder/stimulator ratio was 5:1, and the coculture was incubated for 5 days (37 °C and 5% CO_2). Samples were analyzed by flow cytometry for proliferation and activation markers.

3.2.4 Cell purification and sorting

Cell purification and sorting was performed in all papers. For isolation of untouched T cells pan T cell isolation kit was used. γδ T cell purification was performed using a positive selection kit (human TCR γ/δ+ T Cell Isolation Kit, Miltenyi Biotec), or negative selection kit. For magnetic beads sorting, a selection buffer consisting of PBS containing 0.5% human serum albumin and 2 mM EDTA was used. In paper III, higher purity of CD8+/CD8− γδ fractions could be achieved by FACS sorting (Sony MA900, Sony Biotechnology Inc.).

3.2.5 *Ex vivo* expansion of Vγ9Vδ2 T cells

In vitro expansion of Vγ9Vδ2 T cells from UCB samples was a substantial experiment in paper V. Initially, cryopreserved MNCs from UCB samples were thawed, washed with PBS, and counted. CD56+ cells (NK cells) were depleted prior to culture using CD56-APC conjugated mAbs (BD Bioscience, Franklin Lakes, NJ, USA) and anti-APC microbeads (Miltenyi, Bergisch Gladbach, Germany) according to manufacturers' instructions. CD56 depleted MNCs were counted and then resuspended in RPMI-1640 media (Gibco) containing 10% human AB-serum (Department of Transfusion Medicine at Karolinska University Hospital Huddinge), supplemented with 2mM L-glutamine, 100 IU/ml penicillin G, 100 mg/ml streptomycin, and 0.25 mg/ml amphotericin B. Cells were seeded in appropriate cell

culture flasks in a final concentration of 1×10^6 viable mononuclear cells/ml. At day 0, human recombinant IL-2 and the bisphosphonate zoledronate were added in varying concentrations (0–500 IU/ml and 0–10 µM, respectively). Cultured cells were then incubated at 37°C at 5% CO2. Every other day, viable cells were counted using trypan blue exclusion and IL-2 containing media was added to maintain cell concentration. On day 7, cultured cells were counted, and the media was replaced completely. On day 14, cells were harvested, counted, and cryopreserved as mentioned earlier.

3.2.6 T Cell proliferation assay

T cell proliferation assays were performed as part of MLR or after cytokine or TCR stimulation to assess γδ T cell responses. Briefly, MNCs or purified CD3+ T cells were washed in PBS and centrifuged at 300*g* for 10 minutes. The cell pellet was then resuspended in 1 mL of PBS containing CellTrace violet (Thermofisher) diluted to final concentration of 2.5 µM and incubated in dark for 10 minutes at 37°C. Next, labeled cells were diluted 5 times with complete media, kept for another 5 minutes, and then washed with PBS before use in subsequent experiments. Labeled T cells were cultured in a 96-well plate (1×10^6 cells/mL) in a complete RPMI-1640 medium, either stimulated or unstimulated, and were incubated at 37°C and 5% CO2 for 5 or 7 days. Cells were analyzed by FACS, and proliferating cells were defined as % of Cell-Trace violet (CTV) low cells compared to unstimulated conditions (Figure 13).

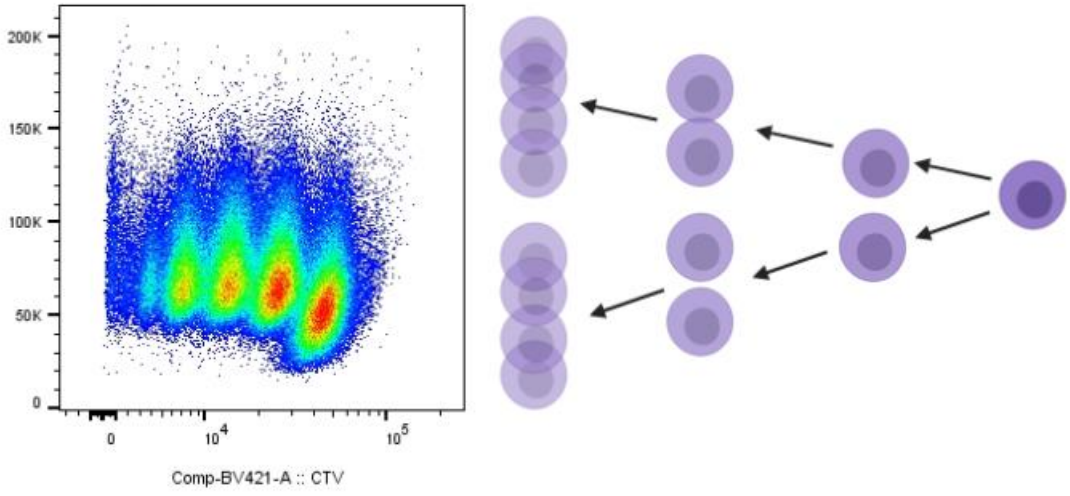

Figure 13. Assessment of cell proliferation using cell trace violet

3.2.7 Cytotoxicity assays

The ability of γδ T cells to kill cholangiocarcinoma cancer cell line (Hucct-1) was assessed in paper V using a cytometry-based assay (162). Briefly, Hucct-1 cells were labeled with the CellTrace™ Violet Cell Proliferation marker (Thermo Fisher). Next, expanded UCB and PB γδ T cells were cocultured with target tumor cells at an effector: target ratio of 10:1 and

incubated at 37°C, with 5% CO_2 for 24 hours. Cell viability was then assessed by flow cytometry in CTV positive cells using annexin V. The % of target cell killing was calculated with the formula

$$\%\ Cytotoxicity = 100 - \frac{\%\ viable\ CTV + cells\ in\ coculture}{\%\ viable\ CTV + cells\ in\ control}\ X100$$

3.2.8 Polymerase chain reaction (PCR)-based methods

3.2.8.1 Basic principles

PCR is a powerful tool that enables amplification and subsequent detection and quantification of DNA and RNA targets (Figure 14). The PCR reaction mix ideally consists of DNA/cDNA template, and a master mix that contains the polymerase enzyme, necessary nucleotides, forward (Fwd) and reverse (Rev) specific primers in final optimized concentrations. Occasionally a fluorescent labeled probe is used. The PCR reaction volume is usually 50, 25, or 10 µl.

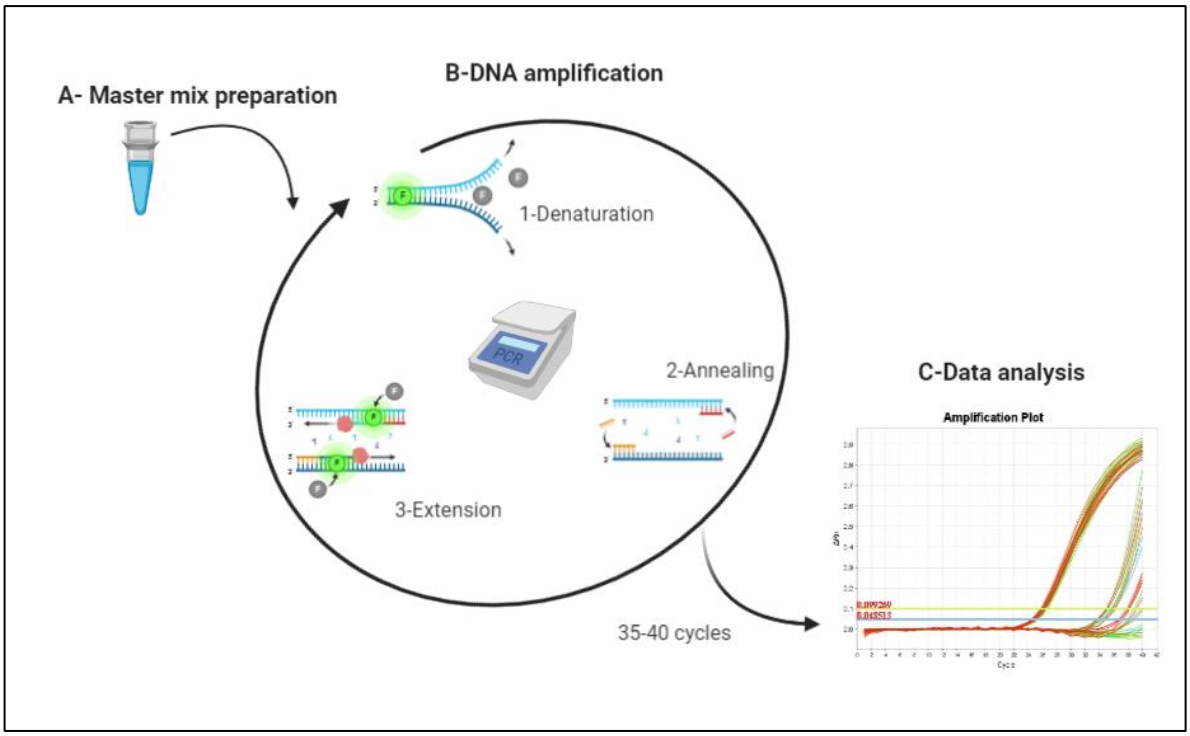

Figure 14. Illustration of main steps of PCR

The amplification of DNA occurs in cycles (35-40). Each PCR cycle consists of several steps that vary in duration and temperature according to the specific application. In general, three main steps are involved: 1-Denaturation step: usually at 95 ºC for 15-30 sec. The aim of denaturation is to separate the double stranded DNA into single strands. 2-Annealing step: during this step, the forward and reverse primers specifically bind (anneal) to their complementary nucleotide sequences in the DNA template. The annealing temperature depends on the melting temperature of each primers. Common commercially available

primers are usually optimized to anneal at 60 ºC for 1 min. 3-Elongation (extension) step: usually at 72 ºC for 1 min. In some cases, the annealing and extension steps are combined in one step.

3.2.8.2 Cytokine gene expression

Cytokine genes were quantified using a two-step reverse transcriptase PCR (RT-PCR) method (163). In the first step, RNA was extracted from sorted γδ T cells (TCR γ/δ T cell isolation kit, Miltenyi) using the PureLink™ RNA Mini Kit (Invitrogen, ThermoFisher) and converted to cDNA using Super- Script™ IV VILO™ Master Mix (ThermoFisher) according to manufacturer's instructions. The cDNA can be used immediately or kept at -20°C.

In the second step, cDNA products resulting from the 1st step were used as template for amplification of specific gene segments using predesigned TaqMan Gene Expression Assays for IL-1B, IL-2, IL-6, IL-7, IL-8, IL-12B, IL-15, and IL-17 genes and human ACTB gene as reference gene (Thermofisher scientific). PCR was performed using a 7500 fast real-time PCR instrument (Applied Biosystems).

3.2.8.3 TREC and KREC assessment

Somatic recombination occurs in all TCR genes. This TCR rearrangement process results in production of circular DNA products known as T cell receptor recombination excision circle (TREC). However, not all TRECs are appropriate for laboratory assessment.

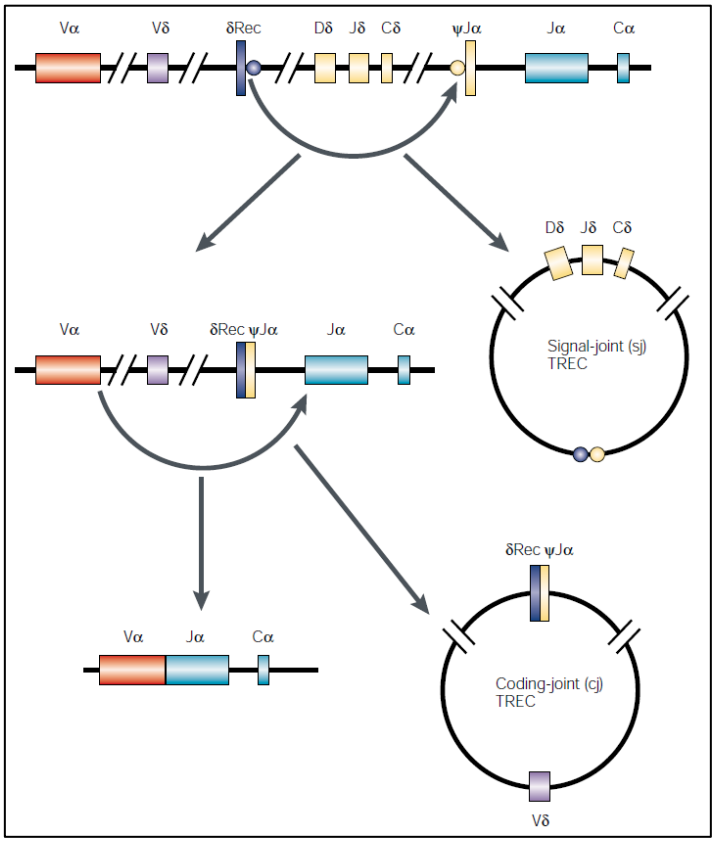

Figure 15. Illustration of signal joint TREC and coding joint TREC

A good candidate TREC must be detectable in peripheral blood and not affected by the different rearrangement possibilities. As rearrangements of *TRB* and *TRG* loci are very early events, their resulting TRECs are extensively diluted before they enter the peripheral blood rendering their levels in the PB below the detection limit (88).

Alternatively, somatic recombination of *TRA* locus demands the deletion of the *TRD* gene locus as it is interspersed with *TRA* along the same chromosomal location. Since this deletion occurs at a relatively late developmental stage, the generated TRECs are less subjected to the dilution effect of thymocyte expansion. Furthermore, this rearrangement results in production of δRec-ΨJα signal joint and coding joint in 70 % of cases (Figure 15) (164).

Likewise, B cells undergo somatic recombination of their immunoglobulin heavy and light chains. The rearrangement process of the kappa gene results in kappa deleting recombination excision circle (KREC) that can be used as surrogate for newly produced naïve B cells (165).

In the first paper, we used a TaqMan real-time PCR based technique to quantify TREC and KREC levels from genomic DNA extracted from CD3+ and CD19+ sorted samples, respectively (Figure 16) (165). Briefly, a multiplex PCR reaction was developed for TREC/GAPDH in a final volume of 25 µl, consisting of 12.5 µl of TaqMan™ 2X Universal PCR Master Mix (Applied Biosystems), sjTREC primers (forward 5'- CAC ATC CCT TTC AAC CAT GCT; reverse 5'-GCC AGC TGC AGG GTT TAGG) and probe (6-FAM-ACA CCT CTG GTT TTT GTA AAG GTG CCC ACT) were added in a final concentration of 300 and 200 nM respectively, and GAPDH primers (forward 5'-GGA CTG AGG CTC CCA CCT TT; reverse 5'-GCA TGG ACT GTG GTC TGC AA) and probe (VIC-CAT CCA AGA CTG GCT CCT CCC TGC) were added in a final concentration of 150 nM and 100 nM respectively. For KREC quantification, we used KREC primers and probes that were described by Sottini et al., (165)in a final concentration of 900 nM and 200 nM respectively and GAPDH as for TREC. PCR amplification was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The PCR cycling conditions were as follows: 1× (95°C for 10 min), 40× (95°C for 15 sec, 60°C for 1 min). TREC and KREC values were calculated relative to GAPDH using the formula $2^{(\Delta Ct)}$, where ΔCt = Ct GAPDH-Ct TREC (KREC)

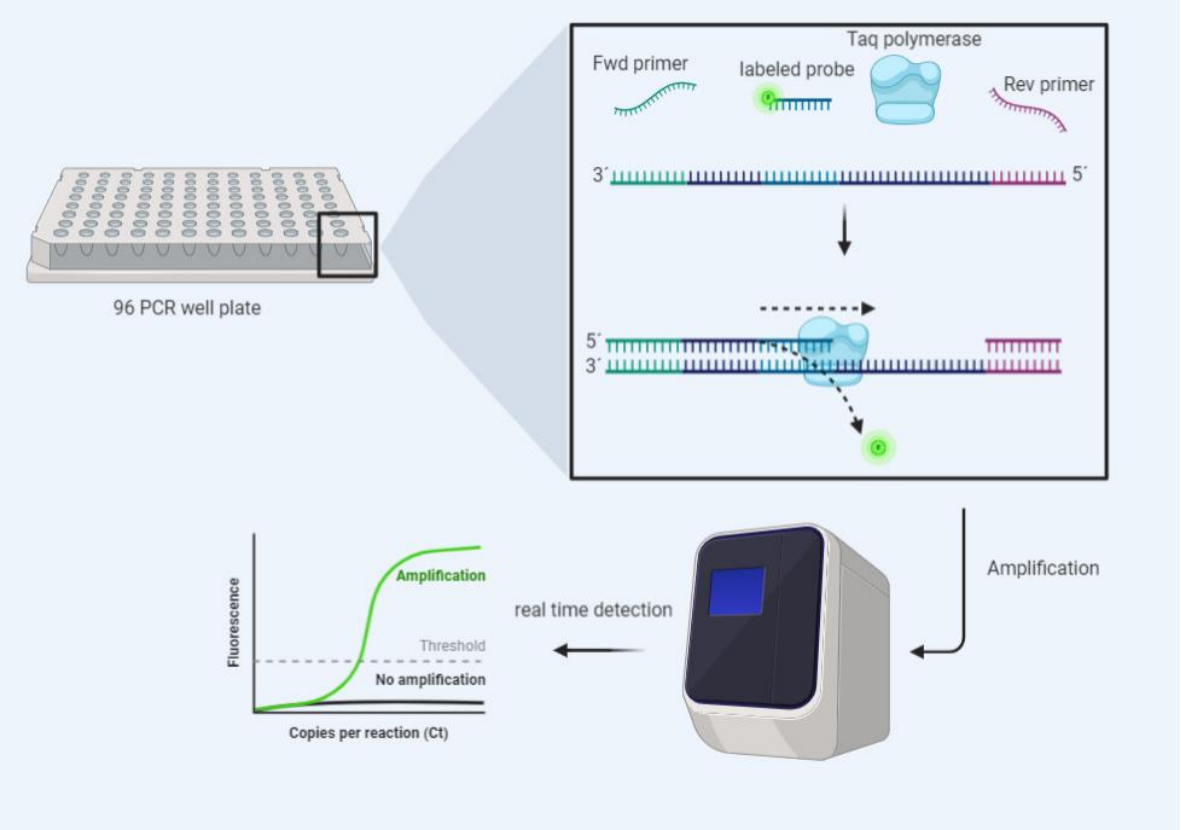

Figure 16. Illustration of the TaqMan PCR method used for TREC and KREC quantification

3.2.8.4 Telomer length (TL) assessment

TL was assessed by quantification of telomere DNA relative to the DNA of a single copy gene (T/S) as described before (166). Briefly, for each sample, telomere and human Beta globulin (HBG) gene DNA were amplified separately as triplicate in a MicroAmp Fast Optical 96 well plates, using SYBR green PCR technique.

A reaction mix was prepared separately for telomere and HBG with a total reaction volume of 20 µl containing Power SYBR green PCR Master Mix (Applied Biosystems) in a final concentration of 1×. For telomere DNA, forward and reverse primers in a final concentration of 100 and 900 nM, respectively, were added. Whereas for HBG DNA, forward and reverse primers in final concentration of 300 and 700 nM, respectively, were added. DNA samples were finally added to each well in a final amount of 25 ng. The PCR was performed on a 7500 fast real-time PCR-system (Applied Biosystems) using the following PCR conditions: 1× (10 min 95°C), 40× (15 sec 95°C, 1 min 60°C). Mean Ct values were determined for both telomere and HBG. And T/S ratio was calculated as follow: $T/S = 2^{\Delta Ct}$ where ΔCt = (mean Ct HBG – mean Ct telomere). The following primers were used: Tel1b-Forward: CGG TTT GTT TGG GTT TGG GTT TGG GTTTGG GTT TGG GTT; Tel2b-Reverse: GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT; Hbg1-Forward: GCT TCT GAC ACA ACT GTG TTC ACT AGC; Hbg2-Reverse: CAC CAA CTT CAT CCA CGT TCA CC

Importantly, SYBR green based method can result in non-specific amplifications. Therefore, results should be carefully analyzed to avoid false results. In order to minimize this limitation, the following measures were implemented: 1- Appropriate controls were used; non template control and positive control sample obtained from acute myeloid leukemia cell line (THP-1). 2-Melting curve analysis was performed after amplification (Figure 17) to discriminate non-specific amplifications. 3-All samples were run as triplicates. Furthermore, samples from different time points for the same recipient were included in the same run simultaneously, to minimize plate to plate variations

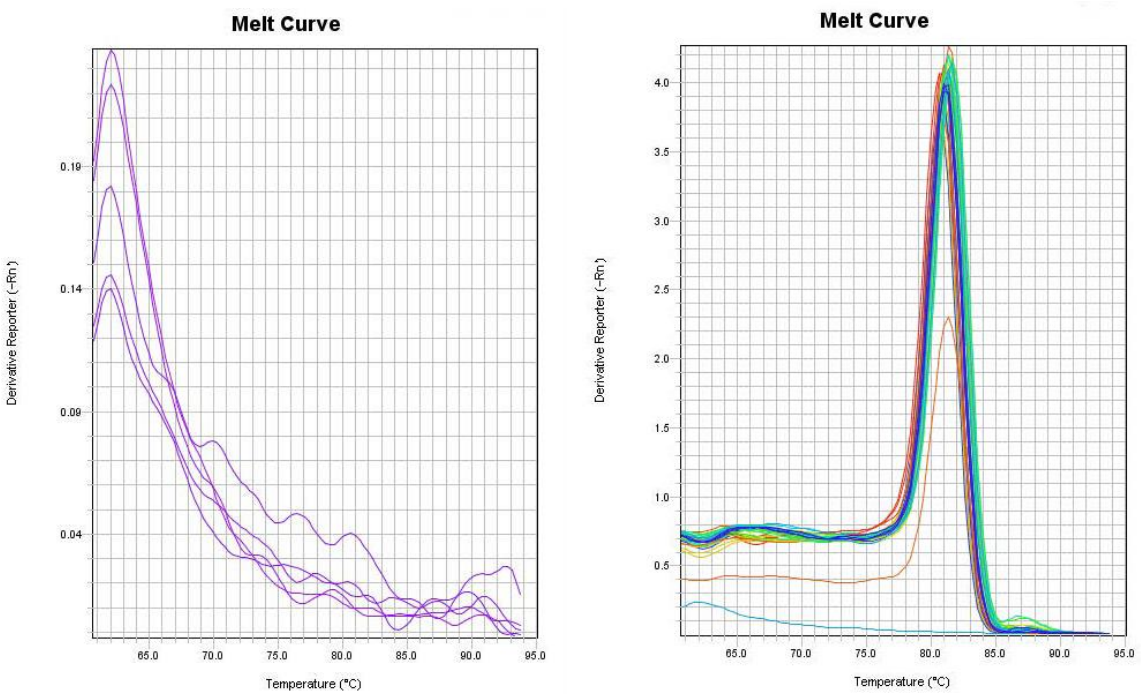

Figure 17. Melting curve performed following SYBR green PCR for TL. To the right a melting curve generated from specific amplification. To the left a melting curve from non-specific amplification

3.2.9 TCR repertoire analysis

3.2.9.1 CDR3 size spectratyping

This technique was used in paper II and V to explore the CDR3 repertoire of γδ T cells (Figure 18). Genomic DNA was used in multiplexed PCR reactions using primers listed in Table 8 to amplify 12 δ and 9 γ chain rearrangements. Briefly, the PCR was performed using AmpliTaq Gold 360 Master Mix (Applied Biosystems), specific primers in a final concentration of 200 or 400 nM as indicated (Table 8 and 9) and 100 ng of DNA, with a thermal cycler PCR machine (PTC-200, MJ Research, Watertown, MA). The process included the following steps: initial denaturation at 95°C for 10 minutes, followed by 35 cycles each of 94°C for 30 sec, 60°C for 45 sec, 72°C for 60 sec, and a final elongation step at 72°C for 10 minutes.

Primer	Sequence
Vδ1	(FAM)-ATGCAAAAAGTGGTCGCTATT
Vδ2	(NED)-ATACCGAGAAAAGGACATCTATG
Vδ3	(VIC)-GTACCGGATAAGGCCAGATTA
Jδ1	GTTCCACAGTCACACGGGTTC
Jδ2	GTTCCACGATGAGTTGTGTTC
Jδ3	CTCACGGGGCTCCACGAAGAG
Jδ4	TTGTACCTCCAGATAGGTTCC
Vγfl	GGAAGGCCCCACAGCRTCTT
Vγ10	AGCATGGGTAAGACAAGCAA
Vγ9	CGGCACTGTCAGAAAGGAATC
Vγ11	CTTCCACTTCCACTTTGAAA
Jγ1.1/2.1	(FAM)-TTACCAGGCGAAGTTACTATGAGC
Jγ1.3/2.3	(NED)-GTGTTGTTCCACTGCCAAAGAG
Jγ1.2	(FAM)-AAGAAAACTTACCTGTAATGATAAGC

Table 8. primer sequences used in γδ TCR spectratyping

A) TCR-δ tubes	V primer/s	J primer/s	Final primer conc.
1	Vδ1 (FAM) Vδ2 (NED) Vδ3 (VIC)	Jδ1	200 nM each
2	Vδ1 (FAM) Vδ2 (NED) Vδ3 (VIC)	Jδ2	200 nM each
3	Vδ1 (FAM) Vδ2 (NED) Vδ3 (VIC)	Jδ3	200 nM each
4	Vδ1 (FAM) Vδ2 (NED) Vδ3 (VIC)	Jδ4	400 nM each
B) TCR-γ tubes			
5	Vγ10 Vγfl	Jγ1.3/2.3 (NED) Jγ1.1/2.1 (FAM)	200 nM each
6	Vγ11	Jγ1.3/2.3 (NED) Jγ1.1/2.1 (FAM)	400 nM each
7	Vγ9	Jγ1.3/2.3 (NED) Jγ1.1/2.1 (FAM)	200 nM each
8	Vγ9	Jγ1.2(FAM)	400 nM each

Table 9. primers used for multiplex PCR

Next, the products of PCR amplification were analyzed to determine their CDR3 length using capillary electrophoresis where each PCR product was mixed with formamid (FA, HiDi Formamide) and a size standard (GeneScan 400HD Rox Size std, Applied Biosystems) in 96-well MicroAmp plates (Applied Biosystems). Samples were analyzed using 3130 × 1 Genetic Analyzer (Applied Biosystems). The results were analyzed using the PeakScanner software (Applied Biosystems).

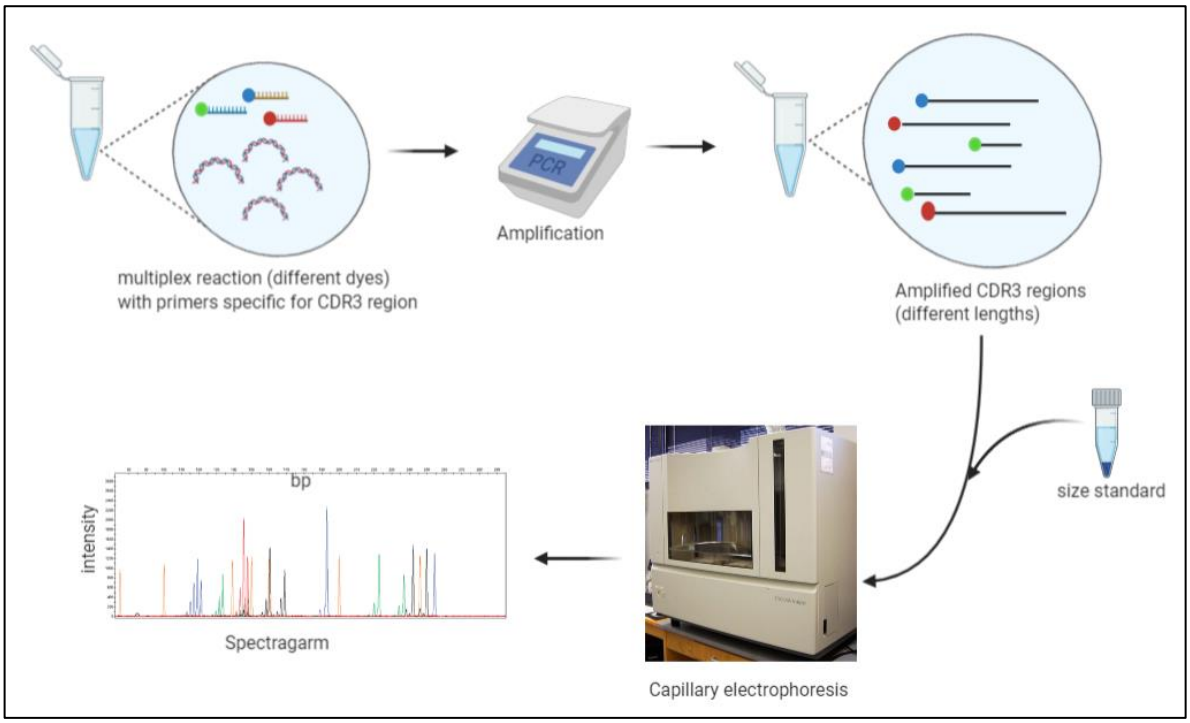

Figure 18. Illustration of the steps of TCR spectratyping

3.2.9.2 Next generation sequencing (NGS)

Basic principles

The advent of NGS has fundamentally revolutionized the field of genomics by providing sequencing solutions that were previously non feasible. NGS technology is rapidly evolving, and different platforms are currently available in the market. However, they use the same basic principle.

NGS and capillary electrophoresis share similar basic concept. NGS however is much rapid and accurate, as it is capable of sequencing millions of fragments in a massively parallel fashion thus reducing the cost of sequencing. In principle, the NGS workflow consists of three basic steps: library preparation, sequencing, and data analysis (Figure 19) (167).

Library preparation: during this step, DNA or RNA are fragmented and ligated to specialized adapters. These fragments can then be amplified and purified. During the sequencing step, libraries are loaded onto a flow cell and placed on the sequencer. The clusters of DNA fragments are amplified, resulting in millions of copies of single-stranded DNA. After sequencing, generated data are analyzed using specialized analysis pipeline.

Figure 19. Basic steps of next generation sequencing

In paper III and paper IV, genomic DNA was extracted from purified γδ T cells using the EZ1 DNA Blood Kit and EZ1 instruments (QIAGEN, Hilden, Germany) and stored at -20°C. The extracted DNA was used for survey-level deep sequencing of the γ-chain using the ImmunoSEQ platform by Adaptive Biotechnologies (Seattle, WA). Briefly, V-J segment rearrangements were amplified in a bias-controlled reaction (168) and sequencing errors were corrected using a clustering algorithm. CDR3 segments were annotated according to the International ImMunoGeneTics collaboration and V and J genes for each rearrangement was identified (169).

Sequencing data were initially analyzed using the ImmunoSEQ Analyzer (adaptivebiotech), followed by post-analysis of TRG repertoire diversity, clonal space homeostasis, segment usage, spectratyping, and repertoire overlap using the tcR package (170), VDJTools (171), and VDJviz browser (172). NGS data are available at the Adaptive Biotechnology ImmunoSEQ site (https://clients.adaptivebiotech.com)

3.3 STATISTICS METHODS

Non-parametric (two-tailed) tests were used throughout the thesis unless specified otherwise. When parametric tests were used the assumptions of normality were confirmed using the Shapiro test and Q-Q plots.

Chi-square test was used to compare categorical variables, while continuous variables were compared using the Mann-Whitney U test. Correlations between given variables were investigated using the nonparametric test of Spearman's rank correlation coefficient.

Analysis of variance (NOVA) followed by post hoc multiple comparisons with Tukey's correction were used when three or more unrelated groups were compared, and repeated measures ANOVA was used for related (paired) groups

γδ Cell subsets were presented as % of total T cells throughout the thesis unless otherwise stated. Survival function was estimated using the Kaplan-Meier method and group differences were compared using the log-rank test. The incidence of TRM was investigated using an estimator of cumulative incidence curves. Patients were censored at the time of death or last follow-up. For multivariate analysis, logistic regression was used to evaluate factors affecting TREC, KREC, and telomeres.

2-sided P value <.05 was considered statistically significant for all tests. Statistical analyses were performed using Statistica software (StatSoft, Tulsa, OK), EZR software, GraphPad Prism version 6.00 for Windows (Graph-Pad Software, La Jolla, California, USA) and SPSS Statistics (IBM, Armonk, NY, USA).

3.4 ETHICAL CONSIDERATIONS

All the research work is conducted in compliance with the declaration of Helsinki, applicable regulations and directions, and is covered by ethical approvals from the Regional ethical committee in Stockholm. All study subjects are covered by the Swedish Patient Insurance (LFF) and the Swedish Pharmaceutical Insurance. It is of upmost importance to be honest, not give false hope and strictly follow ethical and safety guidelines in order to not invoke harm or false expectations. This is also important with regards to research that deals with collection of samples. It is important to be clear that potential results might not directly benefit the study participant but instead future patients.

Additional ethical aspects within the preclinical part of the research include collection of blood samples from healthy individuals/donors and patients. Blood collection of maximum

50 ml/sample is considered safe for the donor and was done at Karolinska University Hospital by qualified personal.

Regarding data management, all data on patients are stored in a quality register located under the supervision of Karolinska University Hospital. The register is continuously updated and controlled. All patients will be informed and give their informed consent prior to being included in the register. Personal identification numbers and all information are kept strictly confidential and patients are coded upon publication or exposure to individuals outside the hospital or project.

4 RESULTS AND DISCUSSION

4.1 EVALUATING TREC, KREC AND TL FOLLOWING ALLOGENEIC HSCT (PAPER I)

Efficient recovery of the adaptive immune system following allogeneic HSCT is pivotal for beneficial outcome. The replenishment of the virtual empty lymphocyte pool occurs through cytokine driven homeostatic expansion of donor mature lymphocytes and through *de novo* generation of naive lymphocytes from HSCs precursors. The latter can be monitored by assessing TREC and KREC levels in the PB (164). These excision circles have been suggested as reliable molecular surrogate markers for recent thymic T cell emigrants and B cell neogenesis, respectively (173).
Additionally, lymphocytes undergo massive proliferation, particularly during the early periods post HSCT. To maintain efficient proliferative capacity, cells require telomere, a short tandem repeat of nucleotide sequences (5´-TTAGGG-3´) found at chromosomal ends. These telomeres undergo continuous shortening with each cell division. Extensive telomere shortening of rapidly proliferating lymphocytes can predispose to immune senescence with resulting impaired immune response or secondary malignancies (174).

We and others have previously assessed TREC, KREC, and TL, to address lymphocyte neogenesis kinetics in relation to different transplant related factors (164, 175). Nevertheless, little is known about the impact of GVHD prophylaxis regimens on newly generated lymphocytes. In this prospective randomized study (paper I), we sought to address whether these markers are influenced by using different GVHD prophylaxis regimens. More specifically, we compared between CsA/Mtx vs Tac/Sir as two GVHD prophylaxis arms.

Our findings indicated no significant differences in TREC, KREC or TL between the two prophylaxis regimens at any assessed time point. Consistently, there was no differences in the absolute counts of the main lymphocyte subsets between the two patient groups. Indeed, in the clinical part of this study, that was published earlier, the incidence and severity of aGVHD was comparable between the two treatment arms, supporting the findings in this paper, as it would have been conceivable to detect differences in these markers if one prophylaxis regimen was associated with increased incidence or severity of GVHD (176).

Tacrolimus and Sirolimus exert their suppressive effects through distinct pathways, by either inhibition of Calcineurin or mTOR respectively. Sirolimus is commonly used to prevent graft rejection in solid organ transplantation (177). In HSCT, the use of Sirolimus was associated with vascular endothelial injury (178). Importantly, in this study Sirolimus was given for relatively shorter duration (<6 months). Thus, the long-term effect of Sirolimus cannot be excluded. Furthermore, Sirolimus was given in combination with Tac (a calcineurin inhibitor), therefore, the sole effect of Sirolimus has not been tested in this study.

In line with previous research, we showed that TREC and KREC levels ascend gradually while TL shortening occurs during follow up. While KREC levels increased significantly by

6 months, TREC levels were significantly increased after 12 months. This indicates that KREC recovers earlier than TREC post allogeneic HSCT. The observed kinetics of TREC recovery fits alongside with the reported thymic rebound and highlights that T cell recovery is much slower process. In this regard multiple studies showed that TREC levels recover slowly and their levels remain low several years after HSCT, suggesting that thymic recovery post HSCT is a slow process or probably never achieved (179).

Consistent with previous studies, we showed a significant association between lower TREC levels and older age, the use of ATG and PB as graft source (Figure 20) (180). Age associated thymic involution is a well know phenomenon (181). The effect of recipient age was prominent in our study as it appeared as independent significant factor in multivariate analysis. This effect should therefore be considered when other factors are interpreted.

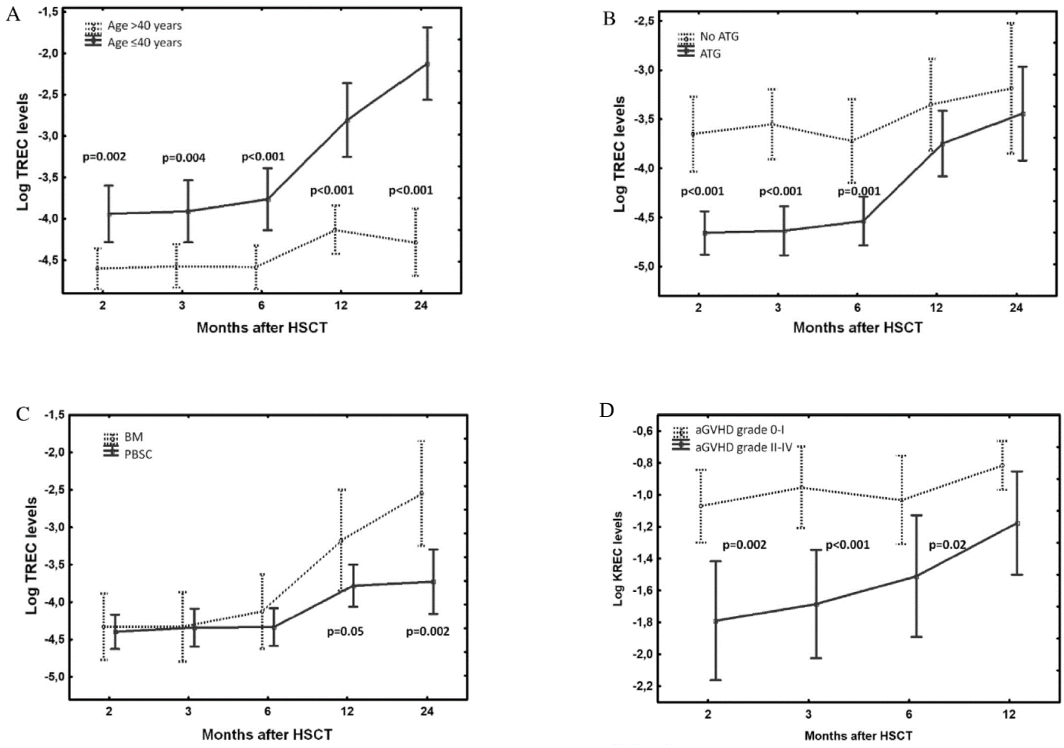

Figure 20. Impacts of patient age (A), ATG treatment (B), graft source (C) on TREC levels after HSCT; Impact of aGVHD (D) on KREC levels after HSCT.

Graft source has been shown to influence the immune reconstitution pattern (75). The comparison between different graft sources is however precluded by the existence of confounders. In this study, BM as graft source was associated with higher TREC levels at 12 and 24 months. This might suggest that BM grafts preferentially contain supporting cells such as mesenchymal stromal cells, that play potential role in thymic regeneration. On the other hand, elevated TREC levels in BM graft recipients could reflect confounding factors. cGVHD is more commonly seen in PB recipients (28) and in the present study, TREC levels were lower in patients with severe cGVHD. Additionally, most BM graft recipients in this study were younger age compared to PB recipients.

In vivo depletion of T cells by ATG is commonly used during conditioning to prevent GVHD and reduce risk of graft rejection (74). Here, we showed that the use of ATG was associated with lower TREC levels. Though ATG has a short half-life (days), the decrease in TREC levels was detected up to 6 months post HSCT. This is of clinical significance since insufficient T cell recovery predisposes to increased risks of opportunistic infections. Patients receiving ATG should therefore be carefully monitored for early signs of infection.

Unlike TREC, aGVHD was the only factor that negatively impacted KREC recovery. Patients with aGVHD grade II-IV had lower KREC levels at 2, 3, and 6 months. Interestingly this difference disappeared by 12 months suggesting that aGVHD has a transient impact on BM.

Our results extend previous work showing that early recovery of TREC and KREC is associated with beneficial outcome. Patients with low TREC levels have higher risk of death due to infection complications. Further studies are required to show the benefit of antimicrobial prophylaxis in those patients.

4.2 EVALUATING THE IMPACT OF GRAFT γδ T CELLS ON ALLOGENEIC HSCT OUTCOME (PAPERS II-IV)

The vast majority of γδ T cells exert potent anti-tumorous, anti-viral immune response independent of MHC recognition. This unique feature of γδ T cells is particularly significant in the field of allogeneic HSCT as it renders γδ T cells able, in theory, to eliminate leukemic blasts without initiating unwanted GVHD.

Multiple studies have investigated the impact of γδ T cells on patient outcome post HSCT. Early reconstitution of γδ T cells was correlated with less incidence of relapse and increased OS. Furthermore, patients with higher γδ T cells have shown less incidence of TRM and severe infection (132, 133, 135). Nevertheless, the role of γδ T cells in GVHD has remained open question. Few studies have suggested a pathogenic role of γδ T cells and potential association with GVHD (144), while large body of evidence indicated that γδ T cells are not key effectors in GVHD initiation.

This contrariety between studies can be attributed to the variation among different studies. Most of the clinical studies have investigated this complex association indirectly by demonstrating the outcome in patients that received manipulated grafts depleted of αβ cells (182, 183), or by monitoring γδ T cell reconstitution post HSCT (126). Importantly, the mechanistic analysis has been conducted in mice models. Given the limitations of using mouse models in the dissection of complex GVHD biology, results from mice studies should be carefully interpreted.

Additionally, few studies have investigated the impact of graft γδ T cells. importantly, most of the available studies have assessed total γδ T cells while the impact of different subsets has not been thoroughly investigated. Given the diversity in function and biology of γδ subsets,

we sought, in papers II-IV, to in-depth characterize intra-graft γδ cells to better alleviate their role in allogeneic HSCT outcome.

In paper II we identified a subset of γδ T cells (CD8+ γδ cells) with a potential alloreactive impact. The graft proportion of this subset was significantly associated with increased incidence of aGVHD grade II-III (Figure 21).

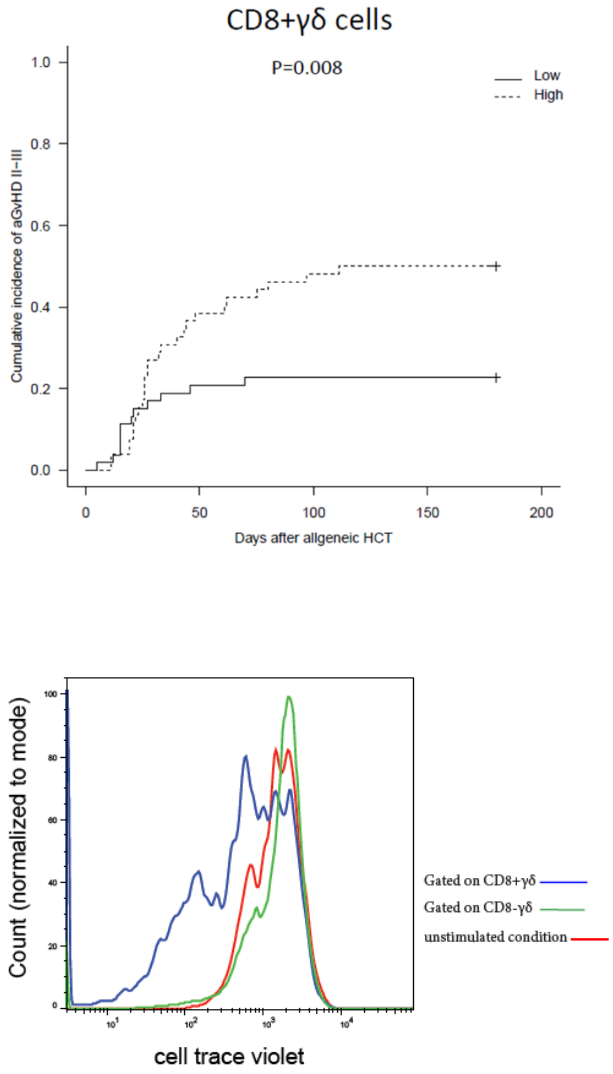

Figure 21. Upper panel, the cumulative incidence of acute GvHD grade II–III for patients who received grafts containing higher (interrupted line) or lower (continuous line) T-cell proportions of CD8+γδ cells. Lower panel, a representative plot of cell proliferation after MLR, using *in-vitro* cultured γδ cells from PB of healthy subjects as responders. Blue histogram represents the MLR condition gated on CD8+γδ cells, green histogram represents MLR condition gated on CD8−γδ cells, and red histogram is the unstimulated condition (responders only).

To account for the impact of ATG in the study we stratified patients in dichotomous groups (ATG and no ATG) and analysed the impact of CD8+ γδ cells. Results were consistent with the main analysis. Furthermore, we demonstrated experimentally that CD8+ γδ cells proliferation was enhanced and displayed activated phenotype when cultured in MLR (Figure 21). Moreover, CD8+ γδ cells showed increased frequencies of chemokine receptor 9 (CCR9) and CD69. Both are gut homing receptor/marker. Collectively, our findings from paper II suggest that CD8+ γδ cells in the stem cell graft could be an unidentified player in GVHD immunopathology.

It is well established now that αβ T cells present in the donor graft are the key players inducing GvHD and their number directly correlates with an increased risk for GvHD (184, 185). To reduce incidence and severity of GVHD, several approaches for graft manipulation have been established using different methods of T cell depletion (185). The selective depletion of αβ T cells while retaining all other beneficial cells including γδ T cells in the graft has the advantage of maintaining antileukemic, antiviral and engraftment-supportive effects (69, 92, 186). Contrastingly, Pabst et al. reported that patients who received PBSC grafts

containing higher doses of γδ T cells developed GVHD more frequently (145). While aGvHD was not correlated with the total graft γδ cell frequency in our study, we showed that a small fraction of γδ cells that co-express CD8 was associated with aGvHD grade II-III both in univariate and multivariable analysis. Therefore, the discrepancies between studies as to the impact of γδ cells may be justified by investigating the proportions of the various distinct subsets, such as CD8+γδ cells.

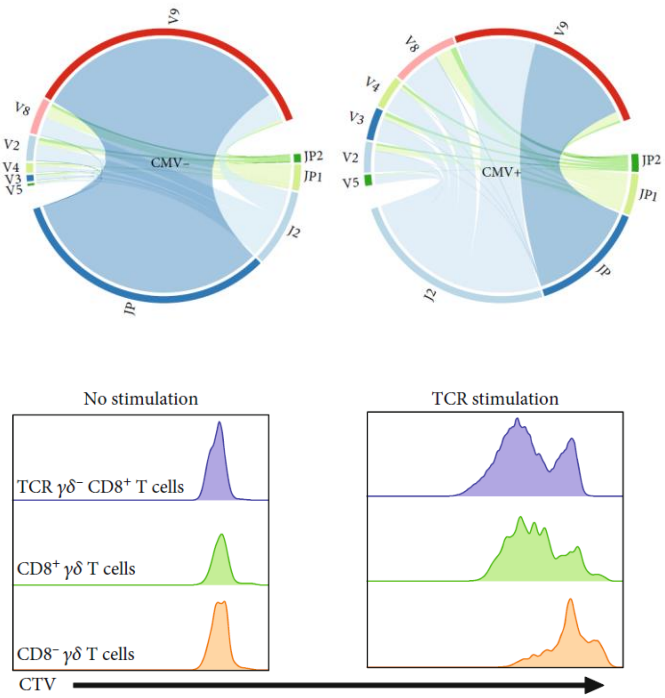

Figure 22. Upper panel, V-J segment pairing abundance in CDR3 junctions of each donor based on CMV status. Chord diagrams are used for visualization. Ribbons connecting segment pairs are scaled by corresponding V-J pair frequency. Lower panel, representative histograms (n = 4) of proliferating T cells from unstimulated or TCR/CD3 stimulated condition, gated on TCR γδ- CD8+ (blue), CD8+γδ (green), and CD8-γδ (orange).

While the vast majority of PB γδ T cells do not express CD4/CD8 co-receptors and are MHC unrestricted, the immunobiology of CD8+ γδ T cells remains poorly understood. Whether the alloreactive response of these cells is MHC restricted or mediated by other undefined ligands is yet to be determined. In a recent study, Kierkels et al. described for the first time a novel allo-HLA–restricted γδ T cell population. Interestingly the identified cells displayed CD8α coreceptor and expressed a TCR comprised of Vγ5Vδ1 (187). This novel subset was shown to recognize a peptide-binding groove of HLA-A*24:02. Therefore, our finding in paper II could reflect analogous population.

Given the poor understanding of CD8+γδ cells immunobiology, in paper III we sought to in-depth characterize this subset to gain more insights about their role. We identified donor CMV status as a significant factor associated with increased graft frequencies of CD8+ γδ cells. Further characterization showed that CD8+ γδ cells predominantly express Vγ9- chain.

We next showed that the increased proportions of CD8+ γδ cells seen in the CMV+ donors were associated with differentiation from naïve (CD27 hi) to effector (CD27 lo) phenotype. Suggesting that CMV driven proliferation of CD8+ γδ cells follows an adaptive-like paradigm. NGS analysis of TRG repertoire highlighted that γδ cells from CMV+ donor graft proliferated in TCR dependent clonal fashion and showed abundant usage of Vγ9- segments. Furthermore, CD8+ γδ cells displayed enhanced response to TCR stimulation and respond differentially to various interleukins (Figure 22). Together, these findings support an adaptive-like yet unconventional phenotype of CD8+ γδ cells.

By combining results from paper II showing potential alloreactive role of intra graft CD8+ γδ cells and paper III showing that CD8+ γδ cells in grafts are CMV responsive, we could try to propose a possible scenario that links between CD8+ γδ cells in grafts and the occurrence of both CMV and GVHD post HSCT.

As CD8+ γδ cells appeared to be CMV responsive, CMV reactivation in patients post HSCT would be expected to preferentially drive the expansion of CD8+ γδ cells particularly in patients that have received grafts from CMV+ donor already enriched with this subset. The expansion of CD8+ γδ cells would then mediate an alloreactive response and GVHD through yet undefined mechanisms. However, considering the complexity of the GVHD underlying immune mechanisms, it is more likely that CD8+ γδ cells are part of a multifaceted model that involves multidirectional cross talks between different immune subsets. Careful dissection of these mechanisms in experimental GVHD mice models can provide more insights.

In paper II, we showed that higher graft content of CD27+ γδ cells were inversely correlated with relapse incidence and associated with less CMV reactivation in patients post HSCT. Unfortunately, we have not conducted functional assays to compare between CD27+ and CD27- γδ cells. Previous research have identified CD27 among other markers that are preferentially expressed by IFNγ producing γδ T cells, whereas IL-17 producing γδ T cells do not express CD27 (188). Given that IL-17 producing γδ T cells are associated with pro-tumorous role, the presence of higher proportions of CD27+ γδ T cells (IFNγ producer) could in part explain the decreased incidence of relapse. However, this functional demarcation based on CD27 applies mostly to murine γδ cells. in humans, the functional division is less prominent. Another explanation could be that CD27 expression by γδ cells reflects a naïve-like phenotype with better proliferative capacity and less exhausted phenotype, in addition to the more diverse TCR repertoire.

In paper II, we used CDR3 size spectratyping method to address the impact of the TCR repertoire on clinical outcome. Though we could observe a potential association between graft γδ TCR repertoire and relapse, interpretation of these results was difficult as a result of sample heterogeneity beside the limitation of the spectratyping method. Based on the encouraging findings in paper II, we extended this work in paper IV by analyzing a homogenous group of 20 PBSC grafts given to AML patients. Analysis of *TRG* repertoire by NGS revealed important findings particularly in grafts given to non-relapsed patients. CDR3 repertoire in grafts given to non-relapsed patients featured a more public repertoire and increased presence of long sequence clonotypes. Further analysis of the amino acid sequences from these CDR3 repertoires showed that 12 public and 4 private sequences were exclusively found in high frequencies in grafts given to non-relapsed patients. Most of these sequences were 42-54 amino acid long. Furthermore, we observed a bias in the V-J pairing in the form of decreased Vγ4-Jγ2/Vγ5-Jγ2/Vγ8-JγP2 and increased Vγ2-JγP1 pairing in grafts given to non-relapsed patients (Figure 23).

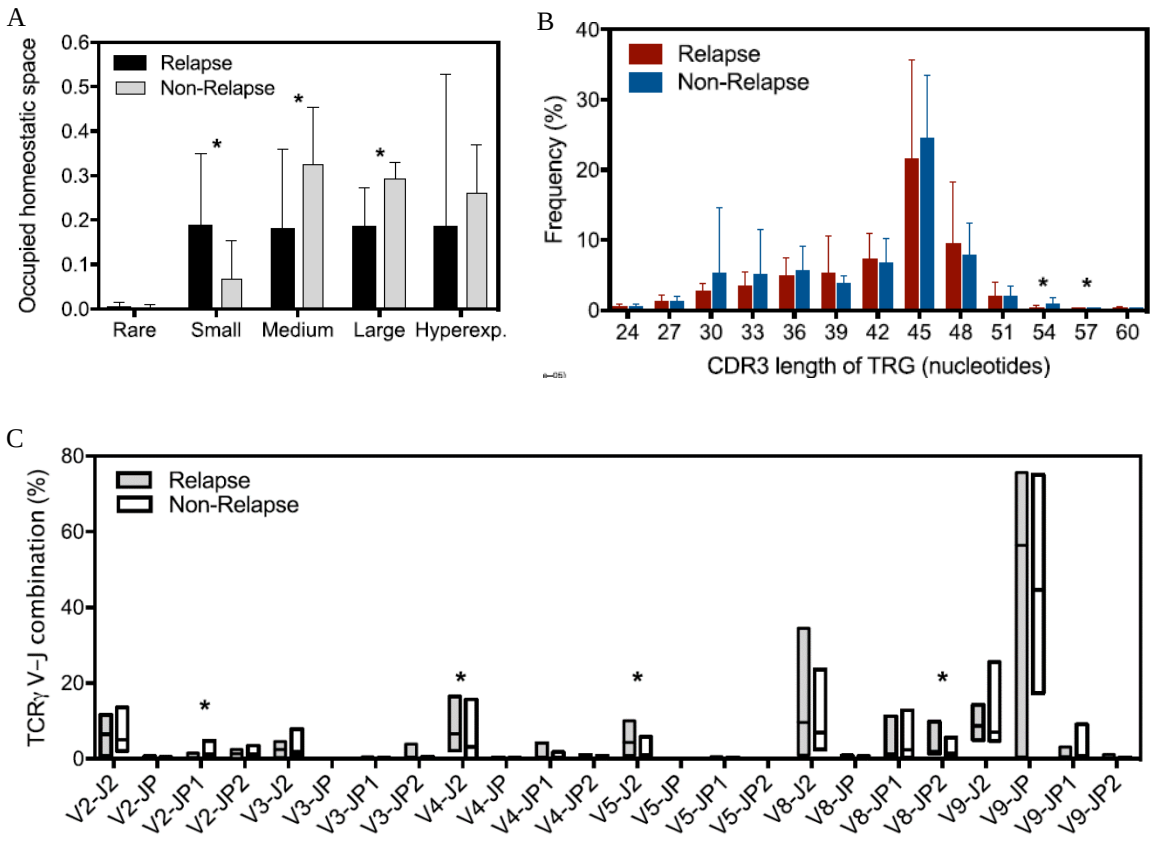

Figure 23. (A) Quantification of homeostatic space taken up by the clones in relapsed and non-relapsed groups. (B) Frequency of unique CDR3 sequences with different nucleotide lengths. (C) Frequency of different TRGV-TRGJ rearrangements in the different groups. Bars represent median and interquartile range. *p < 0.05, two-tailed Mann–Whitney U test

The ability of γδ T cells to lyse leukemia cell lines has been confirmed experimentally. Furthermore, in different allogeneic HSCT settings, increased numbers of γδ T cells were associated with favorable outcome in terms of less relapse and decreased TRM and increased overall survival. Given that γδ T cells reconstitute early after HSCT, graft content of γδ T cells is expected to play protective role during HSCT.

Our results from paper IV suggest that the TCR repertoire composition of infused γδ T cells in terms of occupied homeostatic space, spectratyping, and V and J segment usage, as well as bias in V-J pairing frequencies could be more significant than the quantity of γδ T cells per se in the graft. This again highlights the need of full qualitative characterization of γδ T cells in grafts.

The amino acid sequences that were exclusively enriched in grafts given to non-relapsed patients are most probably of the semi-invariant Vγ9Vδ2 cells. Although we have not analyzed the TRD repertoire in this study, it has been shown previously that Vδ2 exclusively utilize the Vγ9-JγP pairing segment, suggesting a favorable role of certain Vγ9Vδ2 clones. Noteworthy, we showed in paper II that higher graft content of CD27+ γδ T were associated with less relapse. Whether this phenotype was preferentially enriched in these CDR3 sequences is unknown and requires further investigation.

4.3 CHARACTERIZATION OF IN VITRO EXPANDED Vγ9Vδ2 T CELLS FROM UCB (PAPER V)

UCB is an alternative stem cell graft source for allogeneic HSCT and showed outcome comparable to other stem cell sources (189). The advantages of UCB as graft source include their low content of T cells, permitting less stringent HLA matching with relatively lower risk of GVHD. On the other hand, allogeneic HSCT using UCB is associated with prolonged neutrophil engraftment time and increased risk of graft failure. Additionally, the added cost of collection, HLA typing and storage in biobanks renders UCB transplantation more expensive (3).

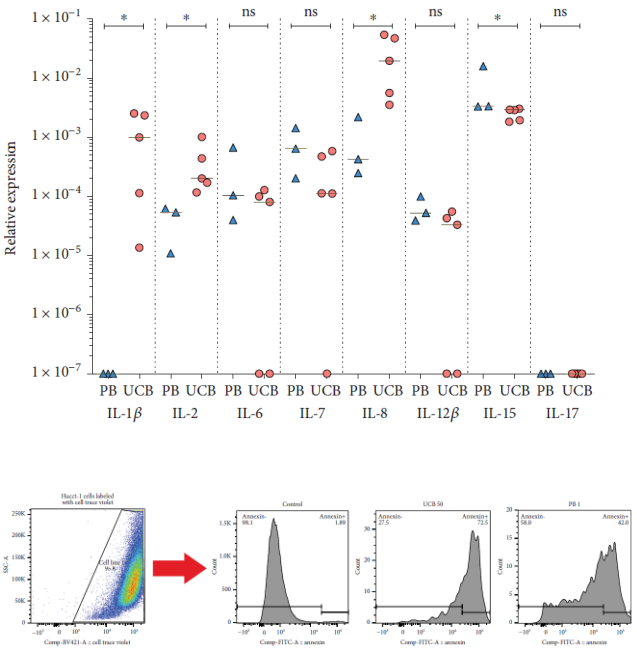

Figure 24. Upper panel, The results of real-time PCR analysis of the gene expression for the cytokines IL-1β, IL-2, IL-6, IL-7, IL-8, IL-12, IL-15, and IL-17 in UCB and PB γδ T cells cultured with 5 μM zoledronate and 200 IU IL-2/ml medium. Lower panel, representative FACS plots of the gating strategy for CTV-labeled tumor cells (left) and histogram plots of annexin V+ gated cells from a control culture (no γδ T cells added), and from cocultures with UCB and PB γδ T cells

In paper V, we used aminobisphosphonate (zoledronate) and IL-2 at different concentrations to optimize a protocol for *in vitro* expansion of UCB Vγ9Vδ2 T cells. We showed that cells cultured in presence of 5 uM zoledronate and (100-200 IU) IL-2/ml showed the highest viability and best fold expansion after 12 days culture. The expanded cells were mainly Vγ9+ and Vδ2+, though other immune cell types were found such as Vδ1 and NK cells.

Immunophenotyping showed that the majority after expansion were differentiated towards central memory with upregulated markers such as TIM-3, PD-1, CTLA-4 and CD95 consistent with activation/exhaustion phenotype.

We then addressed whether the TCR repertoire of expanded cells was altered or skewed. TCR Spectratyping revealed a polyclonal TCR repertoire regardless of the culture condition. This suggests that Vγ9Vδ2 cells respond to PAgs in bulk rather than TCR clonotypic fashion. We also noticed that most of the V-J recombinations were preserved, suggesting that expanded cells retained their baseline TCR repertoire composition and that expansion was not skewed towards certain clones. The polyclonal repertoire of expanded cells could represent potential limitation as the polyclonal expanded cells might contain different functional subsets with different TCR affinities. Therefore, the clinical implication of this issue needs to be addressed carefully in future studies.

To get more insight into the cytokine profile of UCB Vγ9Vδ2 cells, we used real time PCR to quantify several cytokine genes from UCB and PB expanded cells. UCB γδ cells showed higher levels of IL-1B, IL-2, and IL-8. Importantly, both UCB and PB showed very low IL-17 (Figure 24). Finally, we tested their cytotoxic capabilities against cholangiocarcinoma cell line and showed that the ability of expanded UCB γδ cells to kill tumor cell lines was comparable to PB expanded cells (Figure 24).

The delayed immune reconstitution is common following UCB transplantation, increasing the risk of infections. Furthermore, expansion of γδ T cells post HSCT is dependent on graft type and has been shown to be delayed in UCB.

Adoptive cell therapy (ACT) in the form of donor lymphocyte infusion (DLI) can therefore be used to boost immune system. DLI of *in vitro* expanded γδ T cells would have particular significance as their MHC unrestricted feature allows their infusion in large doses with lower risk of GVHD. Additionally, γδ T cells from a third-party donor can be an alternative choice when the original donor cannot be reached. Given the clinical benefit of early reconstitution of γδ T cells, developing a protocol for UCB γδ T expansion is appreciated.

The expansion of Vγ9Vδ2 cells from PB has been intensively practiced. However, extrapolating this method to UCB is challenging. Firstly, γδ T cells account for only smaller fraction of T cells in UCB (<1% of lymphocytes) (190). Furthermore, unlike adult PB, UCB are dominated by Vδ1 whereas Vδ2 cells represent fewer proportions and are mostly functionally immature. Furthermore, It has been reported that UCB Vγ9Vδ2 cells are relatively less responsive to PAgs (191).

Results from paper V highlighted several unique features of *in vitro* expanded Vγ9Vδ2 cells from UCB. Although cells were expanded successfully and the proportions of Vδ2 and Vγ9 were comparable to those from adult PB, the fold expansion in UCB was remarkably lower compared to adult PB expanded cells, reflecting the nature of UCB γδ cells being less responsive to PAgs compared to PB.

Consistent with this finding, it has been shown that neonatal Vδ2 preferentially utilize the Jδ3 segment (192) whereas in adults Jδ1 is mostly used by Vδ2 (193). The Vδ2-Jδ3 regions was found to be enriched in hydrophobic amino acid residues at position 5 that are required for PAgs responsiveness (194).

Another explanation could be that UCB samples were cryopreserved at the time of expansion while fresh PB samples from healthy donors were used. Since monocytes APCs are required for PAgs presentation (195), it is conceivable that their presenting efficiency could be influenced by freezing/thawing process. The use of fresh UCB for expansion of Vγ9Vδ2 cells should be investigated to reveal this.

To compensate for these limitations, additional cytokines such as IL-7, IL-15 and IL-18 need to be investigated either alone or in combination with IL-2. The use of these cytokines has been shown to enhance proliferation and cytotoxic function of γδ T cells (196). Furthermore,

the availability of these cytokines in clinical grade would facilitate their use in ACT. Additionally, the use of substances such as ascorbic acid (vitamin C) has been investigated in PB γδ T cells and showed enhanced proliferation and effector functions of γδ T cells (197). Therefore, investigating these substances in UCB could hold promising results.

Importantly, cytokine data provided in this study should be carefully interpreted as Vγ9Vδ2 cells were not sorted before real time PCR. Given that γδ T cells expanded from UCB contained some Vδ1 and NK cells, the exact source of these cytokines is not confirmed. Therefore, the pro-inflammatory cytokine profile of UCB expanded cells should be confirmed at single cell level. If confirmed, further investigations are required to alleviate their functional implications.

5 CONCLUDING REMARKS

Allogeneic HSCT is potentially curative for several disorders. Nevertheless, it is associated with several complications. Better understanding of the role of different immune cells especially γδ T cells can provide novel therapeutic strategies.

Monitoring TREC, KREC and TL in allogeneic HSCT patients allowed better understanding of the impact of different transplant related factors on adaptive immune reconstitution. Using these markers in the allogeneic HSCT setting could help identifying patients at higher risk for subsequent morbidity and mortality and could enable individualized treatment plans.

Results of TREC and KREC should be interpreted carefully as the level of these excision circles could be altered by other factors such as degradation, apoptosis or longevity of naïve cells.

CMV reactivation, primary malignant relapse and GVHD represent the main causes of transplant related morbidity and mortality following allogeneic HSCT. Though clinically distinct events, a complex relation between these events exists. The underpinning immunological background is still unclear and represents an interesting topic for ongoing research. γδ T cells can be an important player in this context. Reconstitution of γδ T cells occur early after HSCT and the protective role of CMV reactive γδ T cells has been described both in HSCT and solid organ transplantation. However, whether this response is mediated through TCR or other receptors remains elusive.

More efforts are required to identify γδ TCR ligands and to alleviate their mode of recognition.

The immunobiology of CD8+ γδ T cells is poorly known. Their potential role in GVHD must be confirmed in future multi-center studies. Furthermore, the immune mechanisms underpinning their alloreactive response is yet to be identified.

Whether CD8 receptors expressed by few γδ T cells paly a costimulatory role analogous to the conventional CD8 T cells remains unknown and urges for further investigation to alleviate this role and to show whether this function is mediated by CD8αα or CD8αβ receptors.

The role of CD8+γδ T cells in CMV and GVHD should be further investigated and could provide an avenue to better understand the complex relation between CMV and GVHD after HSCT.

The pleotropic function of γδ T cells remains an obstacle against exploiting their full therapeutic benefits. The combination of immunophenotyping, TCR immunosequencing alongside functional assays could help better understanding of the role of different functional subsets.

The TRG sequences identified in this study might be used for future generation of a γδ TCR library that can be used for adoptive immune therapy of AML.

In vitro expansion of Vγ9Vδ2 cells from UCB is a promising approach that can provide a therapeutic potential following UCB HSCT. More studies are warranted to further improve the quantity and quality of expanded cells.

Given the potent anti-tumorous role of Vδ1 cells, more efforts are required to develop and optimize large scale protocols for Vδ1 expansion.

6 ACKNOWLEDGEMENTS

“I am not the best, but I will do my best”. I kept telling myself these words during my PhD period. When I entered the lab for the first time my greatest ambitions were to publish one or two papers. Unexpectedly, I ended with more than I thought. This could have never been accomplished without the help and support I found from everyone. So, this acknowledgement is for those who enabled this thesis to be possible.

First of all, I would like to express my sincere gratitude to my friend and my supervisor at the same time **Michael Uhlin**. Any attempt at any level can't be satisfactorily completed without your support and guidance. You have continuously provided encouragement and was always willing and enthusiastic to assist in any way you could throughout the research project. without you, this work would not have been possible.

I would like to say a special thank you to my co-supervisor, **Jonas Mattsson**, your guidance and overall insights in this field have made this an inspiring experience for me.

I would like to thank my co-supervisors **Moustapha Hassan** and **Sarah Thunberg** for their consistent support and guidance during the last years.

From the bottom of my heart I would like to say big thank you to **Berit** and **Silvia** for their energy, understanding and help throughout my project

I would like to express my gratitude and appreciation for **Isabelle** and **Helen**. whose guidance, support and encouragement has been invaluable throughout my PhD time.

I would like to express my special thanks of gratitude to my opponent **Dieter Kabelitz** for participating and for the valuable discussion

I cannot forget to thank my fellow doctoral and post-doctoral students for their feedback, cooperation and of course friendship: **Arwen**, you have introduced me to the CANTO world and shared your knowledge with unforgotten smile. Very happy to see you back. **Emelie**, whether you are Foord or Rådestad, for me you are the source of happiness and the queen of våfflor. Thanks for providing instant unbiased feedback. **Lucas,** you are a friend, colleague, and an evolutionary mismatched brother. If I would split my PhD period into phases, it would be a *Before Lucas* and an *After Lucas*. There is no need to say that after Lucas was significantly different, in terms of research quality and (diversity). **Lisa-Marie,** you have been a great source of support …and cakes. Your extraordinary planning capabilities saved me a lot of time. **Thomas**, I realized that you are very social person, thanks for the morning talks. **Esther**, for the stimulating questions and sharing my cookies. **Isabella,** for being nice and kind. **Martin,** your extremely outstanding performance during your defense has inspired me (and scared me) a lot. **Laia**, thanks for filling up the blue tips correctly! and for sharing the CANTO with me**. Linda** for the Tabata, **Sofia**, for your help and support. **Rehab**, for your kindness and sense of humor. **Tengyu,** for being a respectful and nice gentleman. **Johan** for the fantastic opportunity to work with you.

I would also like to thank **Mehmet** for providing advice regarding PCR methods and **Bruno**, for making this thesis readable. Also, I would like to thank the **ANA Futura Sony sorter** team for sharing their machine, and the **ANA Futura** service team for their efforts.

I am also grateful to my Egyptian friends **Khaled**, **Osama, Ibrahim, Mohamed, Kareem and Hatem** for all the unconditional support during these years.

I am grateful to my dear wife **Yasmin** and my sons **Omar** and **Zeyad**, who have provided me with moral and emotional support in my life.

I would like to express my gratitude for my colleagues and advisors at the National liver Institute, especially Dr. **Ashraf El-Fert** and **Dr. Hala El-Said** who were so generous and have helped me in many ways.

And finally, last but by no means least, many thanks to all patients who are the real heroes behind this work. Also, I would like to thank the Stockholm county Council, Swedish research Council, Children Cancer Foundation, Radiumhemmets Forskningsfonder, and the Egyptian government for their funding and financial support.

7 REFERENCES

1. Gratwohl A, Baldomero H, Passweg J. Hematopoietic stem cell transplantation activity in Europe. Curr Opin Hematol. 2013;20(6):485-93.

2. Jimenez M, Martinez C, Ercilla G, Carreras E, Urbano-Ispizua A, Aymerich M, et al. Reduced-intensity conditioning regimen preserves thymic function in the early period after hematopoietic stem cell transplantation. Experimental hematology. 2005;33(10):1240-8.

3. Kekre N, Antin JH. Hematopoietic stem cell transplantation donor sources in the 21st century: choosing the ideal donor when a perfect match does not exist. Blood. 2014;124(3):334-43.

4. Gratwohl A, Pasquini MC, Aljurf M, Atsuta Y, Baldomero H, Foeken L, et al. One million haemopoietic stem-cell transplants: a retrospective observational study. Lancet Haematol. 2015;2(3):e91-100.

5. Arnaout K, Patel N, Jain M, El-Amm J, Amro F, Tabbara IA. Complications of allogeneic hematopoietic stem cell transplantation. Cancer Invest. 2014;32(7):349-62.

6. Morris ES, Hill GR. Advances in the understanding of acute graft-versus-host disease. British journal of haematology. 2007;137(1):3-19.

7. Shlomchik WD. Graft-versus-host disease. Nat Rev Immunol. 2007;7(5):340-52.

8. Tugues S, Amorim A, Spath S, Martin-Blondel G, Schreiner B, De Feo D, et al. Graft-versus-host disease, but not graft-versus-leukemia immunity, is mediated by GM-CSF-licensed myeloid cells. Science translational medicine. 2018;10(469).

9. Holtan SG, Pasquini M, Weisdorf DJ. Acute graft-versus-host disease: a bench-to-bedside update. Blood. 2014;124(3):363-73.

10. Blazar BR, Murphy WJ, Abedi M. Advances in graft-versus-host disease biology and therapy. Nat Rev Immunol. 2012;12(6):443-58.

11. Zeiser R, Blazar BR. Acute Graft-versus-Host Disease - Biologic Process, Prevention, and Therapy. The New England journal of medicine. 2017;377(22):2167-79.

12. Pidala J, Wang T, Haagenson M, Spellman SR, Askar M, Battiwalla M, et al. Amino acid substitution at peptide-binding pockets of HLA class I molecules increases risk of severe acute GVHD and mortality. Blood. 2013;122(22):3651-8.

13. Petersdorf EW. The major histocompatibility complex: a model for understanding graft-versus-host disease. Blood. 2013;122(11):1863-72.

14. Stern M, Brand R, de Witte T, Sureda A, Rocha V, Passweg J, et al. Female-versus-male alloreactivity as a model for minor histocompatibility antigens in hematopoietic stem cell transplantation. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2008;8(10):2149-57.

15. Jaglowski SM, Devine SM. Graft-versus-host disease: why have we not made more progress? Curr Opin Hematol. 2014;21(2):141-7.

16. Zeiser R, Penack O, Holler E, Idzko M. Danger signals activating innate immunity in graft-versus-host disease. Journal of molecular medicine. 2011;89(9):833-45.

17. Duffner UA, Maeda Y, Cooke KR, Reddy P, Ordemann R, Liu C, et al. Host dendritic cells alone are sufficient to initiate acute graft-versus-host disease. J Immunol. 2004;172(12):7393-8.

18. Yi T, Chen Y, Wang L, Du G, Huang D, Zhao D, et al. Reciprocal differentiation and tissue-specific pathogenesis of Th1, Th2, and Th17 cells in graft-versus-host disease. Blood. 2009;114(14):3101-12.

19. Jankovic D, Ganesan J, Bscheider M, Stickel N, Weber FC, Guarda G, et al. The Nlrp3 inflammasome regulates acute graft-versus-host disease. The Journal of experimental medicine. 2013;210(10):1899-910.

20. Heimesaat MM, Nogai A, Bereswill S, Plickert R, Fischer A, Loddenkemper C, et al. MyD88/TLR9 mediated immunopathology and gut microbiota dynamics in a novel murine model of intestinal graft-versus-host disease. Gut. 2010;59(8):1079-87.

21. Staffas A, Burgos da Silva M, van den Brink MR. The intestinal microbiota in allogeneic hematopoietic cell transplant and graft-versus-host disease. Blood. 2017;129(8):927-33.

22. Mathewson ND, Jenq R, Mathew AV, Koenigsknecht M, Hanash A, Toubai T, et al. Gut microbiome-derived metabolites modulate intestinal epithelial cell damage and mitigate graft-versus-host disease. Nat Immunol. 2016;17(5):505-13.

23. Kruger WH, Bohlius J, Cornely OA, Einsele H, Hebart H, Massenkeil G, et al. Antimicrobial prophylaxis in allogeneic bone marrow transplantation. Guidelines of the infectious diseases working party (AGIHO) of the german society of haematology and oncology. Annals of oncology : official journal of the European Society for Medical Oncology. 2005;16(8):1381-90.

24. Jenq RR, Ubeda C, Taur Y, Menezes CC, Khanin R, Dudakov JA, et al. Regulation of intestinal inflammation by microbiota following allogeneic bone marrow transplantation. The Journal of experimental medicine. 2012;209(5):903-11.

25. Chakraverty R, Cote D, Buchli J, Cotter P, Hsu R, Zhao G, et al. An inflammatory checkpoint regulates recruitment of graft-versus-host reactive T cells to peripheral tissues. The Journal of experimental medicine. 2006;203(8):2021-31.

26. Sprent J, Schaefer M, Gao EK, Korngold R. Role of T cell subsets in lethal graft-versus-host disease (GVHD) directed to class I versus class II H-2 differences. I. L3T4+ cells can either augment or retard GVHD elicited by Lyt-2+ cells in class I different hosts. The Journal of experimental medicine. 1988;167(2):556-69.

27. Jones SC, Murphy GF, Friedman TM, Korngold R. Importance of minor histocompatibility antigen expression by nonhematopoietic tissues in a CD4+ T cell-mediated graft-versus-host disease model. J Clin Invest. 2003;112(12):1880-6.

28. Zeiser R, Blazar BR. Pathophysiology of Chronic Graft-versus-Host Disease and Therapeutic Targets. The New England journal of medicine. 2017;377(26):2565-79.

29. Boyiadzis M, Arora M, Klein JP, Hassebroek A, Hemmer M, Urbano-Ispizua A, et al. Impact of Chronic Graft-versus-Host Disease on Late Relapse and Survival on 7,489 Patients after Myeloablative Allogeneic Hematopoietic Cell Transplantation for Leukemia. Clinical cancer research : an official journal of the American Association for Cancer Research. 2015;21(9):2020-8.

30. Jagasia MH, Greinix HT, Arora M, Williams KM, Wolff D, Cowen EW, et al. National Institutes of Health Consensus Development Project on Criteria for Clinical Trials in

Chronic Graft-versus-Host Disease: I. The 2014 Diagnosis and Staging Working Group report. Biol Blood Marrow Transplant. 2015;21(3):389-401 e1.

31. Flowers ME, Inamoto Y, Carpenter PA, Lee SJ, Kiem HP, Petersdorf EW, et al. Comparative analysis of risk factors for acute graft-versus-host disease and for chronic graft-versus-host disease according to National Institutes of Health consensus criteria. Blood. 2011;117(11):3214-9.

32. Burkett PR, Meyer zu Horste G, Kuchroo VK. Pouring fuel on the fire: Th17 cells, the environment, and autoimmunity. J Clin Invest. 2015;125(6):2211-9.

33. Fujiwara H, Maeda Y, Kobayashi K, Nishimori H, Matsuoka K, Fujii N, et al. Programmed death-1 pathway in host tissues ameliorates Th17/Th1-mediated experimental chronic graft-versus-host disease. J Immunol. 2014;193(5):2565-73.

34. Ringden O, Labopin M, Gorin NC, Le Blanc K, Rocha V, Gluckman E, et al. Treatment with granulocyte colony-stimulating factor after allogeneic bone marrow transplantation for acute leukemia increases the risk of graft-versus-host disease and death: a study from the Acute Leukemia Working Party of the European Group for Blood and Marrow Transplantation. J Clin Oncol. 2004;22(3):416-23.

35. She K, Gilman AL, Aslanian S, Shimizu H, Krailo M, Chen Z, et al. Altered Toll-like receptor 9 responses in circulating B cells at the onset of extensive chronic graft-versus-host disease. Biol Blood Marrow Transplant. 2007;13(4):386-97.

36. Zhao D, Young JS, Chen YH, Shen E, Yi T, Todorov I, et al. Alloimmune response results in expansion of autoreactive donor CD4+ T cells in transplants that can mediate chronic graft-versus-host disease. J Immunol. 2011;186(2):856-68.

37. Le Huu D, Matsushita T, Jin G, Hamaguchi Y, Hasegawa M, Takehara K, et al. Donor-derived regulatory B cells are important for suppression of murine sclerodermatous chronic graft-versus-host disease. Blood. 2013;121(16):3274-83.

38. MacDonald KP, Hill GR, Blazar BR. Chronic graft-versus-host disease: biological insights from preclinical and clinical studies. Blood. 2017;129(1):13-21.

39. Ferrara JL, Harris AC, Greenson JK, Braun TM, Holler E, Teshima T, et al. Regenerating islet-derived 3-alpha is a biomarker of gastrointestinal graft-versus-host disease. Blood. 2011;118(25):6702-8.

40. Hartwell MJ, Ozbek U, Holler E, Renteria AS, Major-Monfried H, Reddy P, et al. An early-biomarker algorithm predicts lethal graft-versus-host disease and survival. JCI insight. 2017;2(3):e89798.

41. Paczesny S. Discovery and validation of graft-versus-host disease biomarkers. Blood. 2013;121(4):585-94.

42. Zinocker S, Sviland L, Dressel R, Rolstad B. Kinetics of lymphocyte reconstitution after allogeneic bone marrow transplantation: markers of graft-versus-host disease. J Leukoc Biol. 2011;90(1):177-87.

43. Koreth J, Matsuoka K, Kim HT, McDonough SM, Bindra B, Alyea EP, 3rd, et al. Interleukin-2 and regulatory T cells in graft-versus-host disease. The New England journal of medicine. 2011;365(22):2055-66.

44. Paczesny S, Braun TM, Levine JE, Hogan J, Crawford J, Coffing B, et al. Elafin is a biomarker of graft-versus-host disease of the skin. Science translational medicine. 2010;2(13):13ra2.

45. Wang H, Clouthier SG, Galchev V, Misek DE, Duffner U, Min CK, et al. Intact-protein-based high-resolution three-dimensional quantitative analysis system for proteome profiling of biological fluids. Molecular & cellular proteomics : MCP. 2005;4(5):618-25.

46. Ranganathan P, Heaphy CE, Costinean S, Stauffer N, Na C, Hamadani M, et al. Regulation of acute graft-versus-host disease by microRNA-155. Blood. 2012;119(20):4786-97.

47. Kim DH, Jung HD, Lee NY, Sohn SK. Single nucleotide polymorphism of CC chemokine ligand 5 promoter gene in recipients may predict the risk of chronic graft-versus-host disease and its severity after allogeneic transplantation. Transplantation. 2007;84(7):917-25.

48. Martin PJ, Fan W, Storer BE, Levine DM, Zhao LP, Warren EH, et al. Replication of associations between genetic polymorphisms and chronic graft-versus-host disease. Blood. 2016;128(20):2450-6.

49. Nash RA, Storb R. Graft-versus-host effect after allogeneic hematopoietic stem cell transplantation: GVHD and GVL. Current Opinion in Immunology. 1996;8(5):674-80.

50. Kolb HJ, Mittermuller J, Clemm C, Holler E, Ledderose G, Brehm G, et al. Donor leukocyte transfusions for treatment of recurrent chronic myelogenous leukemia in marrow transplant patients. Blood. 1990;76(12):2462-5.

51. Weiden PL, Flournoy N, Thomas ED, Prentice R, Fefer A, Buckner CD, et al. Antileukemic effect of graft-versus-host disease in human recipients of allogeneic-marrow grafts. The New England journal of medicine. 1979;300(19):1068-73.

52. Weiden PL, Sullivan KM, Flournoy N, Storb R, Thomas ED. Antileukemic effect of chronic graft-versus-host disease: contribution to improved survival after allogeneic marrow transplantation. The New England journal of medicine. 1981;304(25):1529-33.

53. Sullivan KM, Weiden PL, Storb R, Witherspoon RP, Fefer A, Fisher L, et al. Influence of acute and chronic graft-versus-host disease on relapse and survival after bone marrow transplantation from HLA-identical siblings as treatment of acute and chronic leukemia. Blood. 1989;73(6):1720-8.

54. Collins RH, Jr., Rogers ZR, Bennett M, Kumar V, Nikein A, Fay JW. Hematologic relapse of chronic myelogenous leukemia following allogeneic bone marrow transplantation: apparent graft-versus-leukemia effect following abrupt discontinuation of immunosuppression. Bone marrow transplantation. 1992;10(4):391-5.

55. Higano CS, Brixey M, Bryant EM, Durnam DM, Doney K, Sullivan KM, et al. Durable complete remission of acute nonlymphocytic leukemia associated with discontinuation of immunosuppression following relapse after allogeneic bone marrow transplantation. A case report of a probable graft-versus-leukemia effect. Transplantation. 1990;50(1):175-7.

56. Odom LF, August CS, Githens JH, Humbert JR, Morse H, Peakman D, et al. Remission of relapsed leukaemia during a graft-versus-host reaction. A "graft-versus-leukaemia reaction" in man? Lancet. 1978;2(8089):537-40.

57. Horowitz MM, Gale RP, Sondel PM, Goldman JM, Kersey J, Kolb HJ, et al. Graft-versus-leukemia reactions after bone marrow transplantation. Blood. 1990;75(3):555-62.

58. Chen BJ, Deoliveira D, Cui X, Le NT, Son J, Whitesides JF, et al. Inability of memory T cells to induce graft-versus-host disease is a result of an abortive alloresponse. Blood. 2007;109(7):3115-23.

59. Xystrakis E, Bernard I, Dejean AS, Alsaati T, Druet P, Saoudi A. Alloreactive CD4 T lymphocytes responsible for acute and chronic graft-versus-host disease are contained within the CD45RChigh but not the CD45RClow subset. European journal of immunology. 2004;34(2):408-17.

60. Dutt S, Tseng D, Ermann J, George TI, Liu YP, Davis CR, et al. Naive and memory T cells induce different types of graft-versus-host disease. J Immunol. 2007;179(10):6547-54.

61. Young JS, Wu T, Chen Y, Zhao D, Liu H, Yi T, et al. Donor B cells in transplants augment clonal expansion and survival of pathogenic CD4+ T cells that mediate autoimmune-like chronic graft-versus-host disease. J Immunol. 2012;189(1):222-33.

62. Ratanatharathorn V, Logan B, Wang D, Horowitz M, Uberti JP, Ringden O, et al. Prior rituximab correlates with less acute graft-versus-host disease and better survival in B-cell lymphoma patients who received allogeneic peripheral blood stem cell transplantation. British journal of haematology. 2009;145(6):816-24.

63. Khoder A, Sarvaria A, Alsuliman A, Chew C, Sekine T, Cooper N, et al. Regulatory B cells are enriched within the IgM memory and transitional subsets in healthy donors but are deficient in chronic GVHD. Blood. 2014;124(13):2034-45.

64. Ghayur T, Seemayer TA, Kongshavn PA, Gartner JG, Lapp WS. Graft-versus-host reactions in the beige mouse. An investigation of the role of host and donor natural killer cells in the pathogenesis of graft-versus-host disease. Transplantation. 1987;44(2):261-7.

65. Tanaka M, Kobayashi S, Numata A, Tachibana T, Takasaki H, Maruta A, et al. The impact of the dose of natural killer cells in the graft on severe acute graft-versus-host disease after unrelated bone marrow transplantation. Leuk Res. 2012;36(6):699-703.

66. Ram R, Gafter-Gvili A, Yeshurun M, Paul M, Raanani P, Shpilberg O. Prophylaxis regimens for GVHD: systematic review and meta-analysis. Bone Marrow Transplant. 2009;43(8):643-53.

67. Kanakry CG, Ganguly S, Zahurak M, Bolanos-Meade J, Thoburn C, Perkins B, et al. Aldehyde dehydrogenase expression drives human regulatory T cell resistance to posttransplantation cyclophosphamide. Science translational medicine. 2013;5(211):211ra157.

68. Munchel AT, Kasamon YL, Fuchs EJ. Treatment of hematological malignancies with nonmyeloablative, HLA-haploidentical bone marrow transplantation and high dose, post-transplantation cyclophosphamide. Best practice & research Clinical haematology. 2011;24(3):359-68.

69. Handgretinger R. Negative depletion of CD3(+) and TcRalphabeta(+) T cells. Curr Opin Hematol. 2012;19(6):434-9.

70. Bray RA, Hurley CK, Kamani NR, Woolfrey A, Muller C, Spellman S, et al. National marrow donor program HLA matching guidelines for unrelated adult donor hematopoietic cell transplants. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 2008;14(9 Suppl):45-53.

71. Morishima Y, Sasazuki T, Inoko H, Juji T, Akaza T, Yamamoto K, et al. The clinical significance of human leukocyte antigen (HLA) allele compatibility in patients

receiving a marrow transplant from serologically HLA-A, HLA-B, and HLA-DR matched unrelated donors. Blood. 2002;99(11):4200-6.

72. Petersdorf EW, Hansen JA, Martin PJ, Woolfrey A, Malkki M, Gooley T, et al. Major-histocompatibility-complex class I alleles and antigens in hematopoietic-cell transplantation. The New England journal of medicine. 2001;345(25):1794-800.

73. Ho CM, McCarthy PL, Wallace PK, Zhang Y, Fora A, Mellors P, et al. Immune signatures associated with improved progression-free and overall survival for myeloma patients treated with AHSCT. Blood Adv. 2017;1(15):1056-66.

74. Cavazzana-Calvo M, Andre-Schmutz I, Dal Cortivo L, Neven B, Hacein-Bey-Abina S, Fischer A. Immune reconstitution after haematopoietic stem cell transplantation: obstacles and anticipated progress. Current opinion in immunology. 2009;21(5):544-8.

75. Ogonek J, Kralj Juric M, Ghimire S, Varanasi PR, Holler E, Greinix H, et al. Immune Reconstitution after Allogeneic Hematopoietic Stem Cell Transplantation. Frontiers in immunology. 2016;7:507.

76. Gordon J, Manley NR. Mechanisms of thymus organogenesis and morphogenesis. Development. 2011;138(18):3865-78.

77. Nishino M, Ashiku SK, Kocher ON, Thurer RL, Boiselle PM, Hatabu H. The thymus: a comprehensive review. Radiographics. 2006;26(2):335-48.

78. Miller JF. Immunological function of the thymus. Lancet. 1961;2(7205):748-9.

79. Komanduri KV. Thymic function and allogeneic T-cell responses in stem-cell transplantation. Cytotherapy. 2002;4(4):333-42.

80. Zuniga-Pflucker JC. T-cell development made simple. Nat Rev Immunol. 2004;4(1):67-72.

81. Krenger W, Blazar BR, Hollander GA. Thymic T-cell development in allogeneic stem cell transplantation. Blood. 2011;117(25):6768-76.

82. Gaballa A, Sundin M, Stikvoort A, Abumaree M, Uzunel M, Sairafi D, et al. T Cell Receptor Excision Circle (TREC) Monitoring after Allogeneic Stem Cell Transplantation; a Predictive Marker for Complications and Clinical Outcome. Int J Mol Sci. 2016;17(10).

83. Toubert A, Glauzy S, Douay C, Clave E. Thymus and immune reconstitution after allogeneic hematopoietic stem cell transplantation in humans: never say never again. Tissue antigens. 2012;79(2):83-9.

84. Mackall CL, Bare CV, Granger LA, Sharrow SO, Titus JA, Gress RE. Thymic-independent T cell regeneration occurs via antigen-driven expansion of peripheral T cells resulting in a repertoire that is limited in diversity and prone to skewing. J Immunol. 1996;156(12):4609-16.

85. Mackall CL, Hakim FT, Gress RE. T-cell regeneration: all repertoires are not created equal. Immunol Today. 1997;18(5):245-51.

86. Jimenez M, Ercilla G, Martinez C. Immune reconstitution after allogeneic stem cell transplantation with reduced-intensity conditioning regimens. Leukemia. 2007;21(8):1628-37.

87. Przybylski GK, Kreuzer KA, Siegert W, Schmidt CA. No recovery of T-cell receptor excision circles (TRECs) after non-myeloablative allogeneic hematopoietic stem cell transplantation is correlated with the onset of GvHD. J Appl Genet. 2007;48(4):397-404.

88. Gaballa A, Clave E, Uhlin M, Toubert A, Arruda LCM. Evaluating Thymic Function After Human Hematopoietic Stem Cell Transplantation in the Personalized Medicine Era. Frontiers in Immunology. 2020;11(1341).

89. Kabelitz D, Serrano R, Kouakanou L, Peters C, Kalyan S. Cancer immunotherapy with γδ T cells: many paths ahead of us. Cellular & Molecular Immunology. 2020.

90. Zheng J, Liu Y, Lau YL, Tu W. gammadelta-T cells: an unpolished sword in human anti-infection immunity. Cell Mol Immunol. 2013;10(1):50-7.

91. Lafont V, Sanchez F, Laprevotte E, Michaud HA, Gros L, Eliaou JF, et al. Plasticity of gammadelta T Cells: Impact on the Anti-Tumor Response. Front Immunol. 2014;5:622.

92. Lamb LS, Jr., Lopez RD. gammadelta T cells: a new frontier for immunotherapy? Biol Blood Marrow Transplant. 2005;11(3):161-8.

93. Silva-Santos B, Serre K, Norell H. gammadelta T cells in cancer. Nat Rev Immunol. 2015;15(11):683-91.

94. Lawand M, Dechanet-Merville J, Dieu-Nosjean MC. Key Features of Gamma-Delta T-Cell Subsets in Human Diseases and Their Immunotherapeutic Implications. Frontiers in immunology. 2017;8:761.

95. O'Brien RL, Born WK. gammadelta T cell subsets: a link between TCR and function? Seminars in immunology. 2010;22(4):193-8.

96. Handgretinger R, Schilbach K. The potential role of gammadelta T cells after allogeneic HCT for leukemia. Blood. 2018;131(10):1063-72.

97. Peters C, Kouakanou L, Oberg HH, Wesch D, Kabelitz D. In vitro expansion of Vgamma9Vdelta2 T cells for immunotherapy. Methods in enzymology. 2020;631:223-37.

98. Serrano R, Wesch D, Kabelitz D. Activation of Human gammadelta T Cells: Modulation by Toll-Like Receptor 8 Ligands and Role of Monocytes. Cells. 2020;9(3).

99. Silva-Santos B, Mensurado S, Coffelt SB. gammadelta T cells: pleiotropic immune effectors with therapeutic potential in cancer. Nature reviews Cancer. 2019;19(7):392-404.

100. Hoeres T, Smetak M, Pretscher D, Wilhelm M. Improving the Efficiency of Vgamma9Vdelta2 T-Cell Immunotherapy in Cancer. Front Immunol. 2018;9:800.

101. Adams EJ, Gu S, Luoma AM. Human gamma delta T cells: Evolution and ligand recognition. Cell Immunol. 2015;296(1):31-40.

102. Xu B, Pizarro JC, Holmes MA, McBeth C, Groh V, Spies T, et al. Crystal structure of a gammadelta T-cell receptor specific for the human MHC class I homolog MICA. Proc Natl Acad Sci U S A. 2011;108(6):2414-9.

103. Prinz I, Silva-Santos B, Pennington DJ. Functional development of gammadelta T cells. European journal of immunology. 2013;43(8):1988-94.

104. Rock EP, Sibbald PR, Davis MM, Chien YH. CDR3 length in antigen-specific immune receptors. The Journal of experimental medicine. 1994;179(1):323-8.

105. Minculescu L, Sengelov H. The Role of Gamma Delta T Cells in Haematopoietic Stem Cell Transplantation. Scandinavian Journal of Immunology. 2015;81(6):459-68.

106. Davey MS, Willcox CR, Hunter S, Kasatskaya SA, Remmerswaal EBM, Salim M, et al. The human Vdelta2(+) T-cell compartment comprises distinct innate-like Vgamma9(+) and adaptive Vgamma9(-) subsets. Nature communications. 2018;9(1):1760.

107. Ravens S, Schultze-Florey C, Raha S, Sandrock I, Drenker M, Oberdorfer L, et al. Human gamma delta T cells are quickly reconstituted after stem-cell transplantation and show adaptive clonal expansion in response to viral infection. Nature Immunology. 2017;18(4):393-+.

108. Davey MS, Willcox CR, Joyce SP, Ladell K, Kasatskaya SA, McLaren JE, et al. Clonal selection in the human Vdelta1 T cell repertoire indicates gammadelta TCR-dependent adaptive immune surveillance. Nature communications. 2017;8:14760.

109. Sebestyen Z, Prinz I, Dechanet-Merville J, Silva-Santos B, Kuball J. Translating gammadelta (gammadelta) T cells and their receptors into cancer cell therapies. Nature reviews Drug discovery. 2020;19(3):169-84.

110. Sebestyen Z, Scheper W, Vyborova A, Gu S, Rychnavska Z, Schiffler M, et al. RhoB Mediates Phosphoantigen Recognition by Vgamma9Vdelta2 T Cell Receptor. Cell Rep. 2016;15(9):1973-85.

111. Vavassori S, Kumar A, Wan GS, Ramanjaneyulu GS, Cavallari M, El Daker S, et al. Butyrophilin 3A1 binds phosphorylated antigens and stimulates human gammadelta T cells. Nat Immunol. 2013;14(9):908-16.

112. Wang H, Henry O, Distefano MD, Wang YC, Raikkonen J, Monkkonen J, et al. Butyrophilin 3A1 plays an essential role in prenyl pyrophosphate stimulation of human Vgamma2Vdelta2 T cells. J Immunol. 2013;191(3):1029-42.

113. Sandstrom A, Peigne CM, Leger A, Crooks JE, Konczak F, Gesnel MC, et al. The intracellular B30.2 domain of butyrophilin 3A1 binds phosphoantigens to mediate activation of human Vgamma9Vdelta2 T cells. Immunity. 2014;40(4):490-500.

114. Salim M, Knowles TJ, Baker AT, Davey MS, Jeeves M, Sridhar P, et al. BTN3A1 Discriminates gammadelta T Cell Phosphoantigens from Nonantigenic Small Molecules via a Conformational Sensor in Its B30.2 Domain. ACS chemical biology. 2017;12(10):2631-43.

115. Melandri D, Zlatareva I, Chaleil RAG, Dart RJ, Chancellor A, Nussbaumer O, et al. The gammadeltaTCR combines innate immunity with adaptive immunity by utilizing spatially distinct regions for agonist selection and antigen responsiveness. Nat Immunol. 2018;19(12):1352-65.

116. Willcox CR, Pitard V, Netzer S, Couzi L, Salim M, Silberzahn T, et al. Cytomegalovirus and tumor stress surveillance by binding of a human gammadelta T cell antigen receptor to endothelial protein C receptor. Nat Immunol. 2012;13(9):872-9.

117. Marlin R, Pappalardo A, Kaminski H, Willcox CR, Pitard V, Netzer S, et al. Sensing of cell stress by human gammadelta TCR-dependent recognition of annexin A2. Proc Natl Acad Sci U S A. 2017;114(12):3163-8.

118. Zeng X, Wei YL, Huang J, Newell EW, Yu H, Kidd BA, et al. gammadelta T cells recognize a microbial encoded B cell antigen to initiate a rapid antigen-specific interleukin-17 response. Immunity. 2012;37(3):524-34.

119. Roy S, Ly D, Castro CD, Li NS, Hawk AJ, Altman JD, et al. Molecular Analysis of Lipid-Reactive Vdelta1 gammadelta T Cells Identified by CD1c Tetramers. J Immunol. 2016;196(4):1933-42.

120. Uldrich AP, Le Nours J, Pellicci DG, Gherardin NA, McPherson KG, Lim RT, et al. CD1d-lipid antigen recognition by the gammadelta TCR. Nat Immunol. 2013;14(11):1137-45.

121. Davey MS, Willcox CR, Baker AT, Hunter S, Willcox BE. Recasting Human Vdelta1 Lymphocytes in an Adaptive Role. Trends Immunol. 2018;39(6):446-59.

122. Davey MS, Willcox CR, Hunter S, Oo YH, Willcox BE. Vdelta2(+) T Cells-Two Subsets for the Price of One. Front Immunol. 2018;9:2106.

123. Coffelt SB, Kersten K, Doornebal CW, Weiden J, Vrijland K, Hau CS, et al. IL-17-producing gammadelta T cells and neutrophils conspire to promote breast cancer metastasis. Nature. 2015;522(7556):345-8.

124. Girardi M, Oppenheim DE, Steele CR, Lewis JM, Glusac E, Filler R, et al. Regulation of cutaneous malignancy by gammadelta T cells. Science (New York, NY). 2001;294(5542):605-9.

125. Hu Y, Cui Q, Luo C, Luo Y, Shi J, Huang H. A promising sword of tomorrow: Human gammadelta T cell strategies reconcile allo-HSCT complications. Blood Rev. 2016;30(3):179-88.

126. Hirokawa M, Horiuchi T, Kawabata Y, Kitabayashi A, Miura AB. Reconstitution of gammadelta T cell repertoire diversity after human allogeneic hematopoietic cell transplantation and the role of peripheral expansion of mature T cell population in the graft. Bone Marrow Transplant. 2000;26(2):177-85.

127. Bian Z, Xu LP, Fu Q, Huo M, Liu L, Zhao X, et al. Homeostatic gammadelta T Cell Contents Are Preserved by Granulocyte Colony-Stimulating Factor Priming and Correlate with the Early Recovery of gammadelta T Cell Subsets after Haploidentical Hematopoietic Stem Cell Transplantation. Biol Blood Marrow Transplant. 2018;24(2):252-9.

128. Xuan L, Wu X, Zhang Y, Fan Z, Ling Y, Huang F, et al. Granulocyte colony-stimulating factor affects the distribution and clonality of TRGV and TRDV repertoire of T cells and graft-versus-host disease. J Transl Med. 2011;9:215.

129. Lamb LS, Jr., Henslee-Downey PJ, Parrish RS, Godder K, Thompson J, Lee C, et al. Increased frequency of TCR gamma delta + T cells in disease-free survivors following T cell-depleted, partially mismatched, related donor bone marrow transplantation for leukemia. J Hematother. 1996;5(5):503-9.

130. Godder KT, Henslee-Downey PJ, Mehta J, Park BS, Chiang KY, Abhyankar S, et al. Long term disease-free survival in acute leukemia patients recovering with increased gammadelta T cells after partially mismatched related donor bone marrow transplantation. Bone Marrow Transplant. 2007;39(12):751-7.

131. Perko R, Kang G, Sunkara A, Leung W, Thomas PG, Dallas MH. Gamma delta T cell reconstitution is associated with fewer infections and improved event-free survival after hematopoietic stem cell transplantation for pediatric leukemia. Biol Blood Marrow Transplant. 2015;21(1):130-6.

132. Airoldi I, Bertaina A, Prigione I, Zorzoli A, Pagliara D, Cocco C, et al. gammadelta T-cell reconstitution after HLA-haploidentical hematopoietic transplantation depleted of TCR-alphabeta+/CD19+ lymphocytes. Blood. 2015;125(15):2349-58.

133. Arruda LCM, Gaballa A, Uhlin M. Impact of gammadelta T cells on clinical outcome of hematopoietic stem cell transplantation: systematic review and meta-analysis. Blood Adv. 2019;3(21):3436-48.

134. Kondo M, Sakuta K, Noguchi A, Ariyoshi N, Sato K, Sato S, et al. Zoledronate facilitates large-scale ex vivo expansion of functional gammadelta T cells from cancer patients for use in adoptive immunotherapy. Cytotherapy. 2008;10(8):842-56.

135. Lamb LS, Jr., Musk P, Ye Z, van Rhee F, Geier SS, Tong JJ, et al. Human gammadelta(+) T lymphocytes have in vitro graft vs leukemia activity in the absence of an allogeneic response. Bone Marrow Transplant. 2001;27(6):601-6.

136. Gentles AJ, Newman AM, Liu CL, Bratman SV, Feng W, Kim D, et al. The prognostic landscape of genes and infiltrating immune cells across human cancers. Nature medicine. 2015;21(8):938-45.

137. Knight A, Madrigal AJ, Grace S, Sivakumaran J, Kottaridis P, Mackinnon S, et al. The role of Vdelta2-negative gammadelta T cells during cytomegalovirus reactivation in recipients of allogeneic stem cell transplantation. Blood. 2010;116(12):2164-72.

138. Prinz I, Thamm K, Port M, Weissinger EM, Stadler M, Gabaev I, et al. Donor Vdelta1+ gammadelta T cells expand after allogeneic hematopoietic stem cell transplantation and show reactivity against CMV-infected cells but not against progressing B-CLL. Experimental hematology & oncology. 2013;2(1):14.

139. Scheper W, van Dorp S, Kersting S, Pietersma F, Lindemans C, Hol S, et al. gammadeltaT cells elicited by CMV reactivation after allo-SCT cross-recognize CMV and leukemia. Leukemia. 2013;27(6):1328-38.

140. De Paoli P, Gennari D, Martelli P, Cavarzerani V, Comoretto R, Santini G. Gamma delta T cell receptor-bearing lymphocytes during Epstein-Barr virus infection. J Infect Dis. 1990;161(5):1013-6.

141. Drobyski WR, Majewski D, Hanson G. Graft-facilitating doses of ex vivo activated gammadelta T cells do not cause lethal murine graft-vs.-host disease. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 1999;5(4):222-30.

142. Blazar BR, Taylor PA, Panoskaltsis-Mortari A, Barrett TA, Bluestone JA, Vallera DA. Lethal murine graft-versus-host disease induced by donor gamma/delta expressing T cells with specificity for host nonclassical major histocompatibility complex class Ib antigens. Blood. 1996;87(2):827-37.

143. Huang Y, Cramer DE, Ray MB, Chilton PM, Que X, Ildstad ST. The role of alphabeta- and gammadelta-T cells in allogenic donor marrow on engraftment, chimerism, and graft-versus-host disease. Transplantation. 2001;72(12):1907-14.

144. Maeda Y, Reddy P, Lowler KP, Liu C, Bishop DK, Ferrara JL. Critical role of host gammadelta T cells in experimental acute graft-versus-host disease. Blood. 2005;106(2):749-55.

145. Pabst C, Schirutschke H, Ehninger G, Bornhauser M, Platzbecker U. The graft content of donor T cells expressing gamma delta TCR+ and CD4+foxp3+ predicts the risk of acute graft versus host disease after transplantation of allogeneic peripheral blood stem cells

from unrelated donors. Clinical cancer research : an official journal of the American Association for Cancer Research. 2007;13(10):2916-22.

146. Kabelitz D, Kalyan S, Oberg HH, Wesch D. Human Vdelta2 versus non-Vdelta2 gammadelta T cells in antitumor immunity. Oncoimmunology. 2013;2(3):e23304.

147. Xiang Z, Tu W. Dual Face of Vgamma9Vdelta2-T Cells in Tumor Immunology: Anti- versus Pro-Tumoral Activities. Frontiers in immunology. 2017;8:1041.

148. Daniele N, Scerpa MC, Caniglia M, Bernardo ME, Rossi C, Ciammetti C, et al. Transplantation in the onco-hematology field: focus on the manipulation of alphabeta and gammadelta T cells. Pathology, research and practice. 2012;208(2):67-73.

149. Chaleff S, Otto M, Barfield RC, Leimig T, Iyengar R, Martin J, et al. A large-scale method for the selective depletion of alphabeta T lymphocytes from PBSC for allogeneic transplantation. Cytotherapy. 2007;9(8):746-54.

150. Braza MS, Klein B. Anti-tumour immunotherapy with Vgamma9Vdelta2 T lymphocytes: from the bench to the bedside. British journal of haematology. 2013;160(2):123-32.

151. Cabillic F, Toutirais O, Lavoue V, de La Pintiere CT, Daniel P, Rioux-Leclerc N, et al. Aminobisphosphonate-pretreated dendritic cells trigger successful Vgamma9Vdelta2 T cell amplification for immunotherapy in advanced cancer patients. Cancer immunology, immunotherapy : CII. 2010;59(11):1611-9.

152. Izumi T, Kondo M, Takahashi T, Fujieda N, Kondo A, Tamura N, et al. Ex vivo characterization of gammadelta T-cell repertoire in patients after adoptive transfer of Vgamma9Vdelta2 T cells expressing the interleukin-2 receptor beta-chain and the common gamma-chain. Cytotherapy. 2013;15(4):481-91.

153. Kobayashi H, Tanaka Y, Yagi J, Osaka Y, Nakazawa H, Uchiyama T, et al. Safety profile and anti-tumor effects of adoptive immunotherapy using gamma-delta T cells against advanced renal cell carcinoma: a pilot study. Cancer Immunol Immunother. 2007;56(4):469-76.

154. Cordova A, Toia F, La Mendola C, Orlando V, Meraviglia S, Rinaldi G, et al. Characterization of human gammadelta T lymphocytes infiltrating primary malignant melanomas. PLoS One. 2012;7(11):e49878.

155. Abe Y, Muto M, Nieda M, Nakagawa Y, Nicol A, Kaneko T, et al. Clinical and immunological evaluation of zoledronate-activated Vgamma9gammadelta T-cell-based immunotherapy for patients with multiple myeloma. Exp Hematol. 2009;37(8):956-68.

156. Dieli F, Vermijlen D, Fulfaro F, Caccamo N, Meraviglia S, Cicero G, et al. Targeting human {gamma}delta} T cells with zoledronate and interleukin-2 for immunotherapy of hormone-refractory prostate cancer. Cancer Res. 2007;67(15):7450-7.

157. Wilhelm M, Smetak M, Schaefer-Eckart K, Kimmel B, Birkmann J, Einsele H, et al. Successful adoptive transfer and in vivo expansion of haploidentical gammadelta T cells. J Transl Med. 2014;12:45.

158. Wu D, Wu P, Wu X, Ye J, Wang Z, Zhao S, et al. Ex vivo expanded human circulating Vdelta1 gammadeltaT cells exhibit favorable therapeutic potential for colon cancer. Oncoimmunology. 2015;4(3):e992749.

159. Almeida AR, Correia DV, Fernandes-Platzgummer A, da Silva CL, da Silva MG, Anjos DR, et al. Delta One T Cells for Immunotherapy of Chronic Lymphocytic

Leukemia: Clinical-Grade Expansion/Differentiation and Preclinical Proof of Concept. Clinical cancer research : an official journal of the American Association for Cancer Research. 2016;22(23):5795-804.

160. Di Lorenzo B, Simoes AE, Caiado F, Tieppo P, Correia DV, Carvalho T, et al. Broad Cytotoxic Targeting of Acute Myeloid Leukemia by Polyclonal Delta One T Cells. Cancer immunology research. 2019;7(4):552-8.

161. Adan A, Alizada G, Kiraz Y, Baran Y, Nalbant A. Flow cytometry: basic principles and applications. Critical reviews in biotechnology. 2017;37(2):163-76.

162. Van Acker HH, Anguille S, Willemen Y, Van den Bergh JM, Berneman ZN, Lion E, et al. Interleukin-15 enhances the proliferation, stimulatory phenotype, and antitumor effector functions of human gamma delta T cells. J Hematol Oncol. 2016;9(1):101.

163. Wacker MJ, Godard MP. Analysis of one-step and two-step real-time RT-PCR using SuperScript III. Journal of biomolecular techniques : JBT. 2005;16(3):266-71.

164. Douek DC, Vescio RA, Betts MR, Brenchley JM, Hill BJ, Zhang L, et al. Assessment of thymic output in adults after haematopoietic stem-cell transplantation and prediction of T-cell reconstitution. Lancet. 2000;355(9218):1875-81.

165. Sottini A, Serana F, Bertoli D, Chiarini M, Valotti M, Vaglio Tessitore M, et al. Simultaneous quantification of T-cell receptor excision circles (TRECs) and K-deleting recombination excision circles (KRECs) by real-time PCR. J Vis Exp. 2014(94).

166. Cawthon RM. Telomere measurement by quantitative PCR. Nucleic acids research. 2002;30(10):e47.

167. Ambardar S, Gupta R, Trakroo D, Lal R, Vakhlu J. High Throughput Sequencing: An Overview of Sequencing Chemistry. Indian journal of microbiology. 2016;56(4):394-404.

168. Carlson CS, Emerson RO, Sherwood AM, Desmarais C, Chung MW, Parsons JM, et al. Using synthetic templates to design an unbiased multiplex PCR assay. Nature communications. 2013;4:2680.

169. Sherwood AM, Desmarais C, Livingston RJ, Andriesen J, Haussler M, Carlson CS, et al. Deep sequencing of the human TCRgamma and TCRbeta repertoires suggests that TCRbeta rearranges after alphabeta and gammadelta T cell commitment. Science translational medicine. 2011;3(90):90ra61.

170. Nazarov VI, Pogorelyy MV, Komech EA, Zvyagin IV, Bolotin DA, Shugay M, et al. tcR: an R package for T cell receptor repertoire advanced data analysis. BMC bioinformatics. 2015;16:175.

171. Shugay M, Bagaev DV, Turchaninova MA, Bolotin DA, Britanova OV, Putintseva EV, et al. VDJtools: Unifying Post-analysis of T Cell Receptor Repertoires. PLoS computational biology. 2015;11(11):e1004503.

172. Bagaev DV, Zvyagin IV, Putintseva EV, Izraelson M, Britanova OV, Chudakov DM, et al. VDJviz: a versatile browser for immunogenomics data. BMC genomics. 2016;17:453.

173. Gaballa A, Norberg A, Stikvoort A, Mattsson J, Sundberg B, Uzunel M, et al. Assessment of TREC, KREC and telomere length in long-term survivors after allogeneic HSCT: the role of GvHD and graft source and evidence for telomere homeostasis in young recipients. Bone marrow transplantation. 2018;53(1):69-77.

174. Baerlocher GM, Rovo A, Muller A, Matthey S, Stern M, Halter J, et al. Cellular senescence of white blood cells in very long-term survivors after allogeneic hematopoietic stem cell transplantation: the role of chronic graft-versus-host disease and female donor sex. Blood. 2009;114(1):219-22.

175. Saglio F, Cena S, Berger M, Quarello P, Boccasavia V, Ferrando F, et al. Association between thymic function and allogeneic hematopoietic stem cell transplantation outcome: results of a pediatric study. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 2015;21(6):1099-105.

176. Torlen J, Ringden O, Garming-Legert K, Ljungman P, Winiarski J, Remes K, et al. A prospective randomized trial comparing cyclosporine/methotrexate and tacrolimus/sirolimus as graft-versus-host disease prophylaxis after allogeneic hematopoietic stem cell transplantation. Haematologica. 2016;101(11):1417-25.

177. MacDonald AS. Rapamycin in combination with cyclosporine or tacrolimus in liver, pancreas, and kidney transplantation. Transplantation proceedings. 2003;35(3 Suppl):201S-8S.

178. Cutler C, Stevenson K, Kim HT, Richardson P, Ho VT, Linden E, et al. Sirolimus is associated with veno-occlusive disease of the liver after myeloablative allogeneic stem cell transplantation. Blood. 2008;112(12):4425-31.

179. Storek J, Joseph A, Espino G, Dawson MA, Douek DC, Sullivan KM, et al. Immunity of patients surviving 20 to 30 years after allogeneic or syngeneic bone marrow transplantation. Blood. 2001;98(13):3505-12.

180. Jimenez M, Martinez C, Ercilla G, Carreras E, Urbano-Ispizua A, Aymerich M, et al. Clinical factors influencing T-cell receptor excision circle (TRECs) counts following allogeneic stem cell transplantation in adults. Transplant immunology. 2006;16(1):52-9.

181. Hakim FT, Memon SA, Cepeda R, Jones EC, Chow CK, Kasten-Sportes C, et al. Age-dependent incidence, time course, and consequences of thymic renewal in adults. J Clin Invest. 2005;115(4):930-9.

182. Airoldi I, Prigione I, Bertaina A, Cocco C, Pagliara D, Zorzoli A, et al. Recovery Of Gamma/Delta plus T Cells After Transplantation With Alpha-Beta+/CD19+Lymphocyte Depleted Hematopoietic Stem Cells From HLA-Haploidentical Donors. Blood. 2013;122(21).

183. Park M, Im HJ, Lee YJ, Park N, Jang S, Kwon SW, et al. Reconstitution of T and NK cells after haploidentical hematopoietic cell transplantation using alpha beta T cell-depleted grafts and the clinical implication of gamma delta T cells. Clinical Transplantation. 2018;32(1).

184. Daniele N, Scerpa MC, Caniglia M, Ciammetti C, Rossi C, Bernardo ME, et al. Overview of T-cell depletion in haploidentical stem cell transplantation. Blood transfusion = Trasfusione del sangue. 2012;10(3):264-72.

185. Minculescu L, Sengelov H. The role of gamma delta T cells in haematopoietic stem cell transplantation. Scandinavian journal of immunology. 2015;81(6):459-68.

186. Lang P, Teltschik HM, Feuchtinger T, Muller I, Pfeiffer M, Schumm M, et al. Transplantation of CD3/CD19 depleted allografts from haploidentical family donors in paediatric leukaemia. British journal of haematology. 2014;165(5):688-98.

187. Kierkels GJJ, Scheper W, Meringa AD, Johanna I, Beringer DX, Janssen A, et al. Identification of a tumor-specific allo-HLA-restricted gammadeltaTCR. Blood Adv. 2019;3(19):2870-82.

188. Ribot JC, deBarros A, Pang DJ, Neves JF, Peperzak V, Roberts SJ, et al. CD27 is a thymic determinant of the balance between interferon-gamma- and interleukin 17-producing gammadelta T cell subsets. Nat Immunol. 2009;10(4):427-36.

189. Berglund S, Magalhaes I, Gaballa A, Vanherberghen B, Uhlin M. Advances in umbilical cord blood cell therapy: the present and the future. Expert Opin Biol Ther. 2017;17(6):691-9.

190. Placido R, Auricchio G, Gabriele I, Galli E, Brunetti E, Colizzi V, et al. Characterization of the immune response of human cord-blood derived gamma/delta T cells to stimulation with aminobisphosphonate compounds. International journal of immunopathology and pharmacology. 2011;24(1):101-10.

191. Dimova T, Brouwer M, Gosselin F, Tassignon J, Leo O, Donner C, et al. Effector Vgamma9Vdelta2 T cells dominate the human fetal gammadelta T-cell repertoire. Proc Natl Acad Sci U S A. 2015;112(6):E556-65.

192. McVay LD, Carding SR, Bottomly K, Hayday AC. Regulated expression and structure of T cell receptor gamma/delta transcripts in human thymic ontogeny. The EMBO journal. 1991;10(1):83-91.

193. Tamura N, Holroyd KJ, Banks T, Kirby M, Okayama H, Crystal RG. Diversity in junctional sequences associated with the common human V gamma 9 and V delta 2 gene segments in normal blood and lung compared with the limited diversity in a granulomatous disease. The Journal of experimental medicine. 1990;172(1):169-81.

194. Wang H, Fang Z, Morita CT. Vgamma2Vdelta2 T Cell Receptor recognition of prenyl pyrophosphates is dependent on all CDRs. J Immunol. 2010;184(11):6209-22.

195. Miyagawa F, Tanaka Y, Yamashita S, Minato N. Essential requirement of antigen presentation by monocyte lineage cells for the activation of primary human gamma delta T cells by aminobisphosphonate antigen. J Immunol. 2001;166(9):5508-14.

196. Nishio N, Fujita M, Tanaka Y, Maki H, Zhang R, Hirosawa T, et al. Zoledronate sensitizes neuroblastoma-derived tumor-initiating cells to cytolysis mediated by human gammadelta T cells. Journal of immunotherapy. 2012;35(8):598-606.

197. Peters C, Kouakanou L, Kabelitz D. A comparative view on vitamin C effects on alphabeta- versus gammadelta T-cell activation and differentiation. J Leukoc Biol. 2020;107(6):1009-22.