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The Role of γδ T Cells in Haematopoietic Stem Cell Transplantation and Malignancies

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The Role of γδ T Cells in Haematopoietic Stem Cell Transplantation and Malignancies

Thesis for Doctoral Degree (Ph.D.)

By

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Dedicated with love to my beloved parents, my father (Sultan) and my mother (Moodi), my wife and soulmate (Mazaya), my brothers (Fahad and Abdullah) and my sisters (Lana, Lama, and Shatha), my niece and nephew, and my entire family, both near and far.

Popular science summary of the thesis

The immune system is a defense arm of our bodies that protects us from infections (e.g. viruses and bacteria) and diseases like cancers. This defense arm is classified into the innate and adaptive immune systems. Innate immunity is the initial line of defense that offers a fast-acting response within minutes or hours upon exposure to foreign dangers. If foreign dangers overcome the initial defense, adaptive immunity combats the foe to ensure our safety. Within the defense arm, γδ T cells are a special force that act as quick responders to attack cancer or infectious foes like innate immunity, but they are part of the adaptive immunity, so they can also recall past invaders, providing a more robust response upon their subsequent appearance. During allogeneic haemopoietic stem cell transplantation (aHCT)-a medical procedure frequently used for blood disorders such as leukemia-a healthy immune system issue from donor is given to patients to replace the defective one. However, this procedure is not straightforward: rapid immune system's recovery is needed to restore the defense to combat infections and any remaining cancer cells. Unlike most T cells, γδ T cells recover quickly and are considered one of the first lines of defense after aHCT. γδ T cells have also been used in medical applications in the war against blood cancers (leukemia).

In this thesis, we focused on different areas of γδ T cell biology. For example, is the recovery of γδ T cells to the normal level in patients (recipients) after aHCT comparable to those found in healthy donors? What is the relationship between γδ T cell recovery and previous clinical outcomes of patients, i.e., which γδ T cell subtypes are good or bad? Can we boost the anti-cancer functionality of γδ T cells with a **NEW TYPE** of targeted immunotherapy called a bispecific T-cell engager (BTE)? This engager is specially designed to drive γδ T cells toward leukemia cells, allowing the γδ T cells to directly kill the cancer. How can a natural drug, i.e. thymoquinone (TQ), make leukemia more vulnerable to attack by γδ T cells?

Study I: We found that γδ T cells in transplant recipients recovered to normal levels and closely mirrored those of their healthy donors at cellular and molecular levels. This recovery was linked to clinical outcomes, with some γδ T cell subtypes providing long-lived protection against previous virus infection, while some others were associated with adverse event severity, such as graft-versus-host disease (GVHD). GVHD occurs when donor-origin cells attack healthy tissues inside the recipient's body. This subtype of γδ T cells could become a potential target for improving post-aHCT outcomes in the future.

Study II: This part of our research focused on finding a new treatment option for leukemia patients who cannot receive high doses of conventional chemotherapy due to their advanced age or morbidities (e.g. heart diseases). One interesting approach we tested involves a modern immunotherapy (BTE) form. The BTE acts like a molecular bridge: one end attaches to cancer cells (target cells) while the other attaches to and induces the activation of γδ T cells (effector cells). We showed that γδ T cells significantly boosted their ability to kill different types of leukemia cells in combination with BTE. Moreover, γδ T cells produced a high level of cytokines that help fight leukemia cells. In essence, this kind of synergistic therapy, which combines γδ T cells with BTE, offers a promising treatment alternative with potentially fewer side effects than conventional treatments.

Study III: We introduced an additional strategy to boost the cancer-fighting effect of γδ T cells against leukemia. This strategy adds another layer by using TQ, a natural compound derived from the medicinal plant Nigella sativa. TQ can enhance leukemia cells' recognition or "sensing" by γδ T cells. We used low doses of TQ to reduce the risk of potential toxicity. The treatment of leukemia cells with TQ induced a noticeable increase in the levels of specific surface receptors on the leukemia cells. These receptors are essential for the attraction of γδ T cells. Accordingly, pre-treatment of leukemia cells with TQ significantly increased the anticancer function of γδ T cells.

Overall, the findings from these studies offer valuable knowledge into the role of γδ T cells in the context of aHCT and blood cancers. The promise of γδ T cell therapy presents a new strategy and hope for safer and more effective leukemia treatments in combination with BTE antibody and TQ.

الملخص العلمي لرسالة الدكتوراة

الدراسة الأولى

خلال العقود الماضية بدئ في استخدام الخلايا الجذعية المكونة للدم Allogeneic haematopoietic stem cell transplantation كعلاج للعديد من أمراض سرطان الدم (اللوكيميا) او الامراض الوراثية مثل الانيميا. حيث أن هذا العلاج يستهدف إلى استبدال الجهاز المناعي المصاب داخل المريض بجهاز مناعي سليم من شخص متبرع. لكن نجاح هذا العلاج يعتمد بدرجة كبيرة على عودة الجهاز المناعي للعمل بكفاءة بعد الزراعة الخلوية وخاصة الخلايا التائية التي تحمي الجسم من العدوى الميكروبية والسرطان. الخلايا التائية ليست جميعا متشابهة من ناحية وظائفها المناعية، هناك نوع خاص يعرف باسم الخلايا التائية $\gamma\delta$ (جاما دلتا)، قد أظهرت الدراسات العلمية السابقة من خلال النتائج السريرية أنها تلعب دورا مهما في حياة المرضى خصوصا في الأشهر الأولى بعد الزراعة الخلوية من تقليل خطرالإصابة بالميكروبات وكذلك من عودة الإصابة باللوكيميا. لكن لم يكن معروفا ما إذا كانت هذه الخلايا تحافظ على وظائفها وقدراتها الدفاعية بعد مرور سنوات طويلة بعد الزراعة الخلوية.

في هذه الدراسة لقد تم تحليل خصائص الخلايا التائية جاما دلتا من ٢٠ مريضا عاشوا لفترة طويلة حتى ١٨ سنة بعد الزراعة الخلوية، كما تمت مقارنة خصائص الخلايا التائية من المرضى أيضا مع المتبرعين الأصحاء باستخدام تقنيات متقدمة مثل التدفق الخلوي (Flow cytometry) وكذلك تقنية تحليل التسلسل الجيني من الجيل الثاني (Next generation sequencing). وقد توصلت الدراسة بأن الخلايا التائية جاما دلتا قد استعادت وظائفها وخصائصها المناعية على المستوى الخلوي والجزيئي الجيني والتي كانت مشابهة للمتبرعين الأصحاء. حيث أن التركيب الجيني لمستقبل الخلايا التائية جاما دلتا عاد بشكل كبير إلى حالته الطبيعية، مما يعني ان التنوع المناعي قد تعافى وأصبح لديه القدرة للتصدي للعديد من الميكروبات والأمراض. كما أظهرت الخلايا التائية جاما دلتا قدرتها على السيطرة على فيروس CMV ، وهو أحد الفيروسات الشائعة التي قد تؤدي إلى مضاعفات بعد الزراعة الخلوية. لكن لوحظ وجود بعض المرضى عانوا من مضاعفات مناعية مزمنة تعرف باسم (GVHD)، حيث أن كان لديهم ارتفاع معين من أحد أنواع خلايا جاما دلتا، وقد يكون هذا النوع مرتبط بزيادة شدة GVHD. حيث أن هذه النتائج توصلت بان الخلايا التائية جاما دلتا يمكنها ان تتجدد وتحتفظ بقدراتها الدفاعية لعقود بعد زراعة النخاع، مع ملاحظة وجود نوع معين من الخلايا التائية جاما دلتا يبدو مرتبطا بزيادة شدة GVHDالمزمن، مما قد يفتح الباب امام تطوير علاجات تستهدف هذا النوع المعين من الخلايا التائية جاما دلتا لتقليل المضاعفات.

الدراسة الثانية

على الرغم من التقدم الكبير في علاج امراض الدم من ضمنها سرطان اللوكيميا، ما يزال خطر الانتكاس بعد زراعة الخلايا الجذعية المكونة للدم مرتفعا. ويعزى ذلك إلى عدم القضاء التام على الخلايا الجذعية السرطانية المكونة الى سرطان اللوكيميا قبل الزرع. في هذا السياق هذه الخلايا الجذعية السرطانية تحتوي على مستقبل +CD34، ولذلك في هذه الدراسة لقد طورنا جسم مضاد ثنائي النوعية (BTE) يستهدف مستقبل +CD34 على هذه الخلايا. حيث أن أحد مميزات هذا الجسم المضاد الثنائي يحتوي على طرفين للارتباط الخلوي، الطرف الأول يرتبط مع الخلية السرطانية من خلال مستقبل +CD34 والطرف الاخر يرتبط مع الخلايا التائية جاما دلتا من خلال مستقبل +CD3. في هذه الدراسة لأول مرة تم اختبار فعالية الجسم المضاد مع الخلايا التائية جاما دلتا كخلايا قاتلة على أكثر من نوع على الخلايا اللوكيميا مخبريا. وقد أظهرت النتائج ان الخلايا التائية أظهرت قدرة قتل أعلى مع الجسم المضاد الثنائي على كافة الخلايا اللوكيميا المستهدفة، وذلك من خلال زيادة انتاج السيتوكينات السامة التي تفرزها الخلايا التائية جاما دلتا من اجل قتل هذه الخلايا اللوكيميا. هذا الدمج ما بين الجسم المضاد الثنائي مع الخلايا التائية جاما دلتا قد يفتح افاق علاجية واعدة ضد انتكاس سرطان اللوكيميا.

الدراسة الثالثة

في هذه الدراسة استكمال للدراسة الثانية والتي تهدف الى تطوير علاجي مدمج يتكون من العلاج المناعي وكذلك العلاج الكيميائي ضد الخلايا اللوكيميا. لقد بحثنا في دمج العلاج الكيميائي لكن بجرعات منخفضة مع العلاج المناعي لتقليل الاثار الجانبية. في هذا الإطار برز مركب الثيموكينون (Thymoquinone) المستخلص من نبات الحبة السوداء Nigella sativa ، أحد المركبات النباتية التي لديه خصائص مضادة للسرطان وقادر على تعديل الاستجابات المناعة. هدفت هذه الدراسة إلى اختبار قدرة الثيموكينون على جعل خلايا اللوكيميا أكثر حساسية لقتلها بواسطة الخلايا التائية جاما دلتا في المختبر. أظهرت النتائج أن معالجة خلايا اللوكيميا بجرعة منخفضة من الثيموكينون (٥ ميكرومول) قبل مزجها مع الخلايا التائية جاما دلتا أدت الى زيادة ملحوظة في قتل الخلايا اللوكيميا. كما ان استخدام الجسم المضاد عزز فعالية الخلايا التائية جاما دلتا ضد خلايا اللوكيميا بعد معالجة الثيموكينون. تشير هذه النتائج الي ان مركب الثيموكينون قد يكون وسيلة فعالة لجعل الخلايا اللويكيميا أكثر عرضة للهجوم المناعي، مما قد يساعد في تقديم استراتيجيات علاجية جديدة وامنة لعلاج سرطان اللوكيميا في المستقبل.

Abstract

Allogeneic haematopoietic stem cell transplantation (aHCT) is a potentially curative immunotherapeutic approach for several lethal hematological disorders. However, morbidity and mortality rates remain high after aHCT due to complications such as relapse, graft-versus-host disease (GVHD), and infections. Successful aHCT requires rapid and effective immune reconstitution, particularly within the T-cell compartment, to protect against opportunistic infections and to eliminate residual tumor cells without aggravating GVHD during the immunocompromised period following transplantation. Early reconstitution of γδ T cells plays a substantial role in immune surveillance post-aHCT via mediating anti-infection and anticancer immunity, correlating with favorable clinical outcomes with less GVHD incidence. These unique features have attracted increasing attention towards harnessing γδ T cells as effector cells for cancer immunotherapy. This thesis presents three papers to bring further knowledge on the role of γδ T cells in the dimension of aHCT and their potential use in hematological malignancy therapy.

Study I aimed to investigate long-term homeostatic steady-state γδ T cell reconstitution after aHCT, focusing on its associations with previous clinical outcomes. We performed an in-depth analysis of γδ T cell phenotypes, TCR-γ repertoire, and functional responses upon stimulation in 20 recipient/donor pairs using multiparametric flow cytometry and next-generation sequencing of the TCR-γ chain. Results showed a comparable phenotypic profile between recipients and donors. Upon PMA/Ionomycin stimulation, recipient γδ T cells secreted high levels of cytokines. Furthermore, the TRG repertoire in recipients was almost completely restored, with no significant differences in diversity, clonality, or gene segment usage compared to donors. However, we found an association between overrepresented donor-derived clonotypes and elevated HLA-DR expression in Vδ1 T cells with increased severity of chronic GVHD in some recipients.

Study II focused on augmenting the antileukemic activity of expanded γδ T cells by CD34/CD3 bispecific T-cell engager (BTE) in vitro. We demonstrated that the CD34/CD3 BTE effectively activates and redirects γδ T cells (effector cells) against CD34-expressing leukemia cell lines (target cells), as evidenced by their specific cytotoxicity in a dose-dependent manner and high cytokine release. Furthermore, CD34/CD3 BTE induced γδ T cell-mediated killing of primary CD34+ AML blasts. In the presence of CD34/CD3, γδ T cells showed superior antileukemic activity compared to conventional αβ T cells, while demonstrating no cytotoxic effects against CD34+ normal cells.

In Study III, we aimed to further enhance the antileukemic activity of expanded γδ T cells by sensitizing leukemia cells with thymoquinone (TQ) treatment. TQ is a phytochemical compound featuring epigenetic activity and immunomodulatory properties with growing evidence as a potent anticancer candidate. We observed that γδ T cells exhibited rapid and increased cytotoxicity when co-cultured with pre-treated leukemia cell lines with TQ compared to vehicle control and untreated conditions. This enhanced cytotoxicity could be attributed to the upregulated expression of NKG2D and DNAM-1 ligands on leukemia cells after TQ treatment. We also showed that TQ pretreatment in leukemia cell lines supports the condition of the γδ T cells-based CD34/CD3 approach.

Altogether, this thesis investigated that long-term γδ T cell reconstitution reaches a homeostatic state with a normalized repertoire. Elevated HLA-DR expression on Vδ1 T cells in cGVHD recipients could be a potential therapeutic target, warranting further investigation. It also highlights the potential use of expanded γδ T cells in hematological malignancy therapy by targeting CD34 and sensitizing leukemia cell lines through thymoquinone for CD34/CD3 BTE treatment.

List of scientific papers

I. **Alagrafi F**, Stikvoort A, Gaballa A, Solders M, Ringden O, Poiret T, *Arruda LC, *Uhlin M. γδ T cell characterisation in the long term after haematopoietic stem cell transplantation and its impact on CMV control and cGVHD severity. Clin Transl Immunology. 2025 Mar 7;14(3):e70027. doi: 10.1002/cti2.70027. PMID: 40059884; PMCID: PMC11886888.

II. **Al Agrafi F**, Gaballa A, Hahn P, Arruda LCM, Jaramillo AC, Witsen M, Lehmann S, Önfelt B, Uhlin M, Stikvoort A. Selective lysis of acute myeloid leukemia cells by CD34/CD3 bispecific antibody through the activation of γδ T-cells. Oncoimmunology. 2024 Jul 27;13(1):2379063. doi: 10.1080/2162402X.2024.2379063. PMID: 39076247; PMCID: PMC11285226.

III. **Alagrafi F,** Stikvoort A, Gaballa A, Saher O, Bazaz S, Sundin M, *Poiret T, *Uhlin M. Enhancing γδ T cell cytotoxicity against leukemia cells through thymoquinone sensitization and CD34/CD3 bispecific antibody activation. **Manuscript**.

*Shared last authorship

Scientific papers not included in the thesis

I. Gaballa A, **Alagrafi F**, Uhlin M, Stikvoort A. Revisiting the Role of γδ T Cells in Anti-CMV Immune Response after Transplantation. Viruses. 2021 May 29;13(6):1031. doi: 10.3390/v13061031. PMID: 34072610; PMCID: PMC8228273.

Contents

List of abbreviations

αβ	Alpha-beta
γδ	Gamma-delta
a(c)GVHD	Acute (chronic) graft versus host disease
ADCC	Antibody-dependent cellular cytotoxicity
aHCT	Allogeneic haematopoietic stem cell transplantation
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APC	Antigen-Presenting Cells
BM	Bone Marrow
BSA	Bovine serum albumin
BsAbs	Bispecific antibodies
BTE	Bispecific T-cell engager
BTN	Butyrophilin
CCR	Chemokine receptor
CD	Cluster of differentiation
CDR3	Complementary determining region 3
CMV	Cytomegalovirus
CR	Complete remission
CTV	CellTrace™ Violet
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
CXCR	C-X-C motif chemokine receptor
CLL-1	C-type lectin-like molecule-1
DMSO	Dimethyl sulfoxide
DNAM-1	DNAX accessory molecule 1
DN	Double-negative
DP	Double-positive
DNMT	DNA methyltransferases
EBV	Epstein-Barr virus

ELISA	Enzyme-linked immunosorbent assay
EPCR	Endothelia protein C receptor
EphA2	Ephrin type-A receptor 2
ETPs	Early T cell precursors
FACS	Fluorescence Actived Cell Sorting
FAK	Focal adhesion kinase
Fc	Fragment domain
FMO	Fluorochrome-minus-one
FSC	Forward scatter
G-CSF	Granulocyte colony-stimulating factor
HDAC	Histone deacetylase
HLA	Human leukocyte antigen
HMBPP	(E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate
HSCs	Hematopoietic stem cells
HSPCs	Hematopoietic stem and progenitor cells
HSV	Herpes simplex virus
IPP	Isopentenyl pyrophosphate
IFN-γ	Interferon γ
IL	Interleukin
LFS	Leukaemia-free survival
LSCs	Leukemic stem cells
mAbs	Monoclonal antibodies
MAC	Myeloablative conditioning
MACS	Magnetic Activated Cell Sorting
MAIT	Mucosal-Associated Invariant T cells
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
MICA/B	MHC class I chain-related molecules A/B
MIP-1β	Macrophage inflammatory protein 1-β

MMPs	Metalloproteinases
MNCs	Mononuclear cells
NCRs	Natural cytotoxicity receptors
NGS	Next-generation sequencing
NK	Natural killer
NKRs	NK receptors
NKT	Natural Killer T cells
NKG2D	Natural killer group 2 member D
NRM	Non-relapse mortality
PAgs	Phosphoantigens
PB	Peripheral blood
PBS	Phosphate-buffered saline
PBSCs	Peripheral blood stem cells
PLGA	Poly-lactide-co-glycolide
PMT	Photomultiplier tube
PTX	Paclitaxel
PVR	Polyoma virus receptor
PD-1	Programmed cell death protein 1
PDL1	Programmed death-ligand 1
RIC	Reduced-intensity conditioning
ROX	Reactive oxygen species
scFv	Single-chain variable fragment
SSC	Side scatter
SP	Single positive
TCRs	T cell receptors
TdT	Terminal deoxynucleotigen transferase
TLR	Toll-like receptor
TQ	Thymoquinone
TREC	T-cell receptor excision circle

TRG	T cell receptor γ-chain
TRD	T cell receptor δ-chain
Tregs	Regulatory T cells
TSPs	Thymus-seeding progenitor cells
TNF	Tumor necrosis factor
UCB	Umbilical cord blood
ULBP	UL16-binding proteins
VH	Variable heavy
VL	Variable light
VZV	Varicella-zoster virus
V(D)J genes	Variable, diversity, joining
ZOL	Zoledronate

1 Background

1.1 Human γδ T cell biology

1.1.1 αβ and γδ T cells

T cells are one of the most multifunctional immune cells, performing various roles, including B cells activation, destroying infected/tumor cells and regulating immune responses (1). Two major groups of T cell "siblings" can be classified based on the expression of the heterodimers TCR αβ or γδ (2). Although they arise from the same common progenitor cells, they are different in their development, activation, response mode, and proportion (2).

Conventional T cells usually refer to the αβ linage and react to specific processed peptides via the interaction of their TCR (1). αβ T cells are the most frequent compared to other non-conventional T cells (account for 70-90 %) (3). For their activation, αβ T cells require an appropriate antigen stimulation in the form of a peptide presented by the MHC (also called human leukocyte antigen in humans; HLA complex) molecule and a co/stimulatory signal (e.g. CD80/CD86 or CD70), both provided by an APC (1, 4, 5). The activation process occurs via the TCR when it binds to a HLA molecule and co-stimulatory molecules, e.g. CD28 and 4-1BB amongst others on T cells (5). Classically, αβ T cells can be classified into CD4+ T cells that recognize antigen presented on HLA class II and CD8+ T cells recognize antigen on HLA class I (1).

Unconventional T cells can be classified into two main categories: unconventional αβ T cells such as MAIT and invariant NKT cells and unconventional γδ T cells (6). Both types recognize non-peptidic antigens presented by non-classical MHC molecules (6). Discovered in the 1980s, γδ T cells have a distinct T cell receptors (TCRs) consisting of γ and δ chains (7-10). Unlike αβ T cells, γδ T cells are a small fraction commonly detected at a frequency 1-10% of all circulating CD3+ T cells in the blood; however, they comprise the majority of resident T cells in mucosal and epithelial tissues (2, 3). Most γδ T cells are CD4 and CD8 negative, but a small fraction can express CD8 (2). Antigen recognition by γδ T cells is not restricted to HLA molecules which can result in a rapid immune response to foreign antigens compared to αβ T cells (11, 12). The MHC-independent nature of γδ T cells implies that they may good candidate for allogeneic therapies as they can overcome antigen escape mechanisms, by their innate recognition pattern which offer persistence in eradicating residual tumor cells. They are also less likely to cause alloreactivity such as graft-versus-host disease (GVHD) in the context of allogeneic haematopoietic stem cell transplantation (aHCT) (13, 14) or when used

as allogeneic cells in adoptive cell therapy (15, 16). Moreover, this unique recognition capability allows γδ T cells to respond to a diverse array of structurally different ligands, including unprocessed peptides, soluble proteins, and phospholipids (17). These characteristics enable γδ T cells to get activated and kill pathogens and tumor cells directly also in the first line of defense (12, 15, 18). In addition, γδ T cells have several other innate-like properties and express TLRs (e.g. TLR2, 3, 4, and 5) (19) and a variety of NK receptors (NKRs), which augment their antitumor activity (15, 17). Furthermore, these cells can play an adjuvant role by acting as professional APC to prime CD4+ and CD8+ T cells (17, 19). Recently, advances in molecular analyses have improved our understanding of γδ T cell biology and their pleiotropic function both in innate and adaptive immunity, offering opportunities for therapeutic application (2).

1.1.2 γδ T cell subsets: distribution and antigen recognition

γδ T cells are a heterogenous population of lymphocytes, distributed in peripheral blood, lymphoid tissue and various epithelial tissues (2). In both humans and mice, the γδ TCR is composed of γ and δ chains; however, there are species-specific differences in the chain rearrangement, subsets, antigen recognition and functions (20). In mice, γδ T cells are primarily classified according to their γ chain usage into principal subsets (Vγ1-Vγ7) (20). Human γδ T cells are defined by their δ chain usage into four subsets (Vδ1, Vδ2, Vδ3, and Vδ5) with, the Vδ1 and Vδ2 subsets being the most prevalent with distinct tissue localization and antigen recognition (**Figure 1**) (2, 20).

1.1.2.1 Vδ2 subset

Within all Vδ2 subsets, Vγ9Vδ2 T cells dominate the γδ T cell population in adult peripheral blood, accounting for 50-90 % of total circulating γδ T cells (21). However, a small fraction of Vδ2 can co-expressed with non-Vγ9 chain (21). The non-Vγ9Vδ2 T cells exhibit clonal expansion and differentiation in response to CMV infection, similar to Vδ1 T cells (21, 22).

Vγ9Vδ2 T cells are characterized by their different mechanisms of antigen recognition: TCR-independent or TCR-dependent (23). Through their TCR-dependent recognition, Vγ9Vδ2 T cells specifically react toward natural metabolites known as phosphoantigens (PAgs). These PAgs are non-peptidic phosphorylated antigens (23). For instance, (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP), a metabolite produced exogenously by many bacteria through a non-mevalonate pathway, is considered a potent activator for Vγ9Vδ2 T cells (24). While in the eukaryotic cells, Vγ9Vδ2 T cells can also detect

isopentenyl pyrophosphate (IPP) as a tumor-associated antigen (25). IPP is endogenously produced by tumor cells via the mevalonate pathway to contribute to cholesterol synthesis to satisfy their energy needs (23, 25). The enhanced metabolism of tumor cells lead to overproduced IPP, above normal physiological range (25). Notably, these PAgs, originating from infected or malignant cells, are sensed by γδ T cells through antigen-presenting molecules of the butyrophilin family (BTN), specifically BTN3A1 and BTN2A1 (26).

Besides TCR-dependent recognition, like NK cells, Vγ9Vδ2 T cells also express NKG2D and DNAM-1 receptors (17). These receptors enable the targeting of stressed ligands expressed on transformed and infected cells. Among these ligands, MHC class I chain-related molecules (MICA, MICB) and UL16-binding proteins (ULBP) function as NKG2D ligands on human tumor cells. Moreover, DNAM ligands nectin-2 and polyoma virus receptor (PVR), abundantly expressed on tumor cells, engage the Vγ9Vδ2 T cells to target tumor cells (17). Another antitumor activity, Vγ9Vδ2 T cells, can also mediate cancer lysis through cell engager antibodies or antibody-dependent cellular cytotoxicity (ADCC) by expressing Fc receptor FcRγIII (CD16) (17).

1.1.2.2 Vδ1 subset

Vδ1 T cells are more abundant in epithelial tissues including the gut, skin (dermis), liver, and other mucosal tissues (2, 17), where they contribute to local immune surveillance and tissue homeostasis. In peripheral blood, Vδ1 T cells represent the second most common γδ T cell subset, accounting for up to 30 % of circulating γδ T cells in adult (17, 21), and comprise nearly 40 % of intraepithelial lymphocytes (2, 17). They play a crucial role in antiviral immunity, particularly against cytomegalovirus (CMV) infection (18).

Like Vγ9Vδ2 T cells, Vδ1 T cells rely on both TCR-mediated and innate NKRs-mediated recognition (17). In addition to expressing NKG2D and DNAM-1, Vδ1 T cells also can upregulate natural cytotoxicity receptors (NCRs) such as NKp30, NKp44, and NKp46 under a specific condition. NKp46 is frequently expressed on Vδ1 T cells in the intestinal epithelium, characterizing a subset with induced capacity to eliminate colorectal cancer cells (27). NKp30 and NKp44 are typically expressed in Vδ1 T cells following IL-2 or IL-15 stimulation in vitro (28, 29).

Vδ1 TCRs have a wide variety of recognition capabilities but are not responsive to PAgs (17). They can recognize lipid antigens presented by CD1c, CD1d, and CD1b molecules (30-32). Additionally, Vδ1 T cells exhibit selective responsiveness to BTNL3/BTNL8 expressed on gut epithelial cells and may play a role in tissue homeostasis. (33). Vδ1 T cells are found to recognize stress-induced ligands such

as Annexin A2 upon co-culture with cancer cells (34). Annexin A2 is an intracellular protein upregulated during oxidative stress or cellular transformation (34). Upon cellular stress, annexin A2 can translocate to the cell surface by binding anionic phospholipids on the plasma membrane, thereby facilitating immune recognition (34). Interestingly, these cells can also detect metabolic dysregulation in tumor cells through ephrin type-A receptor 2 (EphA2) (35). EphA2 blockade reduced Vδ1 T cell–mediated tumor lysis, indicating its role in tumor recognition by these cells (36). Moreover, Vδ1 T have been shown to bind MR1, a non-polymorphic MHC-related molecule in a manner independent of antigen presentation (37).

1.1.2.3 Vδ1- Vδ2- subsets

Vδ1- Vδ2- T cells are mainly Vδ3 and Vδ5 γδ T cells (17). These subsets remain less studied compared to Vδ1 and Vδ2 subsets. Vδ3 T cells, although rare in the blood, are enriched in the liver and gut (17). They share functional properties such as recognition of glycolipid antigens with Vδ1 T cells. However, expanded Vδ3 T cells recognize only CD1d but cannot recognize CD1a and CD1b, as demonstrated by the exclusive killing of CD1d+ target cells (38). They can also exert antitumor functions through recognizing annexin A2 molecule. Additionally, Vδ3 T cells have exhibited independent reactivity to MR1 irrespective of antigen presentation (39).

On the other hand, Vδ5 T cells are detectable in peripheral blood and exhibit distinct ligand recognition (17). They can directly recognize the endothelia protein C receptor (EPCR) on stressed cells in lipid-independent manner, resembling the binding mode of an antibody (40).

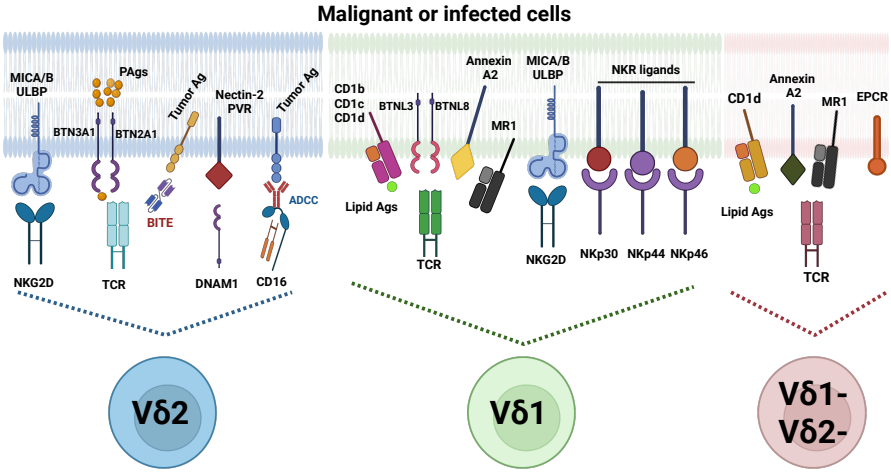


Figure 1*. Antigen recognition by γδ T subsets. (created by BioRender).*

1.1.3 T (γδ) cell development in thymus: human view

The thymic gland is the primary site for the development of αβ and γδ T cell lineages, providing a stromal environment that can support their maturation (41). T cell development entails several phases, including early thymocyte differentiation, TCR gene rearrangement, and lineage divergence into αβ and γδ T cells. The divergence between αβ and γδ T cell lineages depends on the strength of TCR signaling and Notch signaling during thymocyte differentiation (2, 41, 42). As illustrated in **Figure 2**, initially, the differentiation of T cells begins in the bone marrow from haematopoietic stem and progenitor cells (HSPCs) (2, 41). Some of these progenitors, known as thymus-seeding progenitor cells (TSPs), migrate to and seed the thymus, characterized by a CD34+ CD1- phenotype (2, 41). TCR gene rearrangements are initiated early at the CD34+ CD1- stage before full T-cell commitment, which is associated with the commencement of RAG expression (41, 43).

Upon entering the thymus, these progenitors further develop into early T cell precursors (ETPs). The ETPs lose CD44dim expression and acquire CD1 expression (CD34+CD1+) in response to Notch signaling (41, 44). During this commitment process, these cells are also accompanied by upregulation of IL-7 receptor (45) and transcription factors BCL11B (46) and GATA3, which are more suppressive of alternative fates (47). As a result, committed thymocytes experience TCR gene rearrangements, leading to the development and formation of either a pre-TCRβ complex (αβ lineage) or a γδ TCR (γδ lineage) (41).

Beyond γδ TCR formation, human γδ T cell differentiation proceeds via two distinct pathways, influenced by the strength of both the TCR and Notch signaling (2, 41, 42). In the Notch-independent pathway, γδ T cells diverge from αβ T cells most commonly at the double-negative (DN) stages, with further maturation marked by downregulation of CD1 expression (42). This would suggest that Notch signaling is no longer essential in this route (2, 42). A study has shown that the divergence of γδ T cells from precursors of β-selected cells is associated with chromatin accessibility driven by AP-1 activity (48). This indicates that AP-1 works as a pioneer factor by facilitating the opening of chromatin regions, which in turn enhances TCR signaling. These chromatin changes enable γδ T cells to respond more effectively to TCR signals, whereas immature β-selected cells exhibit elevated expression of repressive genes that temporary prevent premature gene activation in response to TCR signaling (48). Furthermore, CD8αα SP TCR γδ+ cells have been suggested to arise directly from this DN stage (42).

Trans-differentiation from the double-positive (DP) stage has also been proposed in human γδ T cell differentiation (42), particularly via JAG2/Notch3 signaling (2, 49). During the Notch-dependent pathway, immature CD4 single-positive (SP) γδ T cells are first generated before developing into immature DP γδ T cells that ultimately, lead to the differentiation of mature CD8αβ TCR γδ cells (42). These findings suggest that γδ T cell development can arise independently from a transient DP stage of pre-TCR-mediated β-selection (42). However, unlike αβ T cells (50), the process of positive and negative selection during human γδ T cell development is still not fully understood and remains to be elucidated.

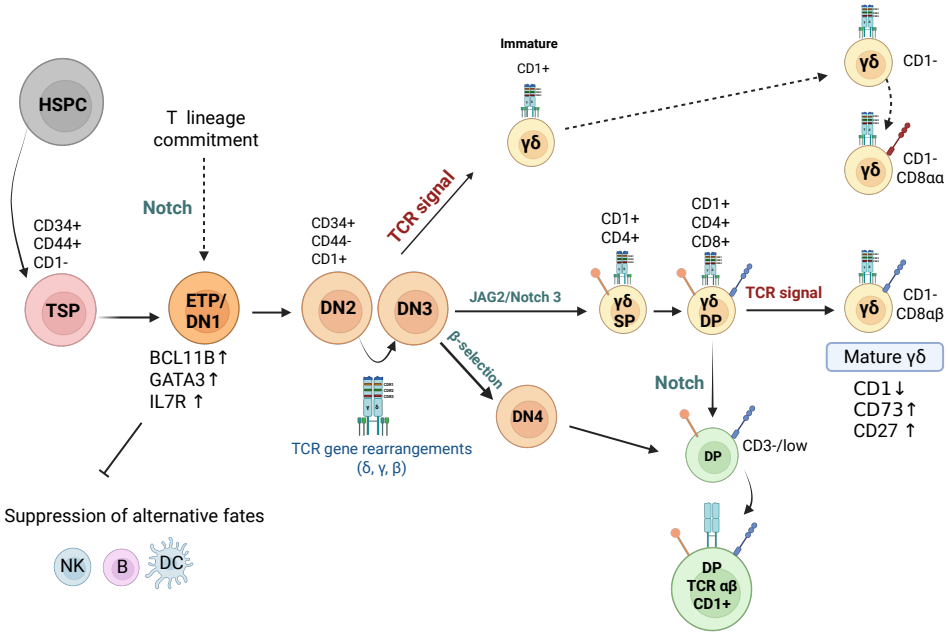


***Figure 2**. The potential mechanisms of γδ T cell development in the human thymus. (created by BioRender).*

1.1.4 Lineage and effector commitment

In mice, γδ T cells are pre-programmed during thymic development into specific effector lineages, such as IL-17-producing γδ T cells (γδT17) or IFN-γ-producing γδ T cells (γδT1) (51). The acquisition of the effector function by human γδ T cells during development is a matter of debate. There is a consensus that human γδ T cells almost leave the thymus in a naïve state and acquire their effector functions in the periphery (41). Supporting this, human γδ thymocytes isolated from pediatric tissue presented more naïve cells compared to γδ peripheral blood cells and lack cytolytic activity against leukemic cells. These thymic γδ cells were able to acquire a cytolytic function once stimulated with IL-2 and IL-15, suggesting that γδ cells achieve their functional maturation outside the thymus (52).

However, despite mounting evidence, the effector commitment of γδ T cells occurs in the periphery. Several studies suggested that, under certain circumstances, γδ T cells are pre-programmed for cytokine production during fetal thymic development in response to infectious conditions (53, 54). Tieppo et al. described that fetal γδ T thymocytes exhibit a distinct effector profile, characterized by the expression of IFN-γ and granzyme molecules in comparison to postnatal γδ T thymocytes, and even to fetal αβ T thymocytes and postnatal αβ T thymocytes (54). The overexpression of the RNA-binding protein Lin28b in HSPCs has been proposed to generate the effector commitment of fetal γδ T cells (54). Indeed, this overexpression by Lin28b appears to reduce the suppression imposed by let-7 microRNAs, subsequently leading to increased PLZF (ZBTB16) expression in both ex vivo fetal γδ thymocytes and γδ T cells derived from fetal HSPCs (54). Given that the innate lymphocyte transcription factor PLZF (ZBTB16) is essential for developing multiple innate-like lymphocytes (55), Lin28b may likely support this developmental pathway (54). Additionally, it has been reported that a small subset of effector γδ T cell clusters, γδT17, γδT1, and Th1-like cells, can arise during fetal thymic development and persist into adult life, where they are identified based on PLZF expression and other functional markers (e.g. RORC & IFNG) (56). Based on scRNA analysis and the fetal thymus dataset, γδT17 and γδT1 cells showed overlapping transcriptional profiles between cord blood and adult peripheral blood, which aligned with gene signatures from fetal thymic development (56). Another study found that fetal γδ T cells exhibit an expanded and differentiated phenotype due to CMV infection in utero during early gestation in contrast to non-CMV infection (53). Overall, human γδ T cells remain predominantly naïve in the thymus, with a possible pre-programming of effector cells only in the fetal thymus under certain conditions.

1.1.5 γδ TCR repertoire composition

The gene rearrangement in γδ TCR γ-chain (TRG) and δ-chain (TRD) creates a diverse repertoire for an efficient immunological response (20, 41). The γδ TCR consists of three complementarity-determining regions (CDR1, CDR2, and CDR3) both in the TRG and TRD chains. The CDR3 region represents the most variable region generated during junctional diversity, providing individual γδ T cell clonotypes, whereas CDR1 and CDR2 are encoded by germline variable genes (57). In humans, the TRG genes located on chromosome 7, comprise six functional variable genes (Vγ2-5, Vγ8, and Vγ9), five joining genes (JP1, JP, J1, JP2, and J2), linked with two content regions (C1 and C2). The TRD genes are located on chromosome 14 within the TCR-α (TRA) locus, including eight functional V genes (Vδ1, Vδ2, Vδ3, Vα14/Vδ4, Vα29/Vδ5, Vα23/Vδ6, Vα36/Vδ7, and Vα38/Vδ8), of which Vδ1 and Vδ2 are the most frequently used, and to a lesser extent Vδ3. These

V genes can rearrange with three diversity genes (D1–D3), four J genes (J1–J4), connected with a single consent region (2, 20).

The diversification process entails the somatic recombination of gene fragments called V, D (applicable only for TRD), and J, which are subsequently joined together with random insertion or removal of junctional nucleotides (**Figure 3**). After recombination, the transcribed V(D)J fragments in combination with the C region, leads to the formation of a unique γδ TCR. Additionally, this recombination process occurs independently for the h TRG and TRD chains, providing further combinatorial diversity from chain pairing (2, 20, 58). Theoretically, γδ T cells are estimated to be able to generate a vast TCR diversity of up to 10^{17}–10^{18} distinct clonotypes through heterodimeric pairing, which is comparable to/higher than the diversity in TCR αβ (2). However, in reality, γδ T cells exhibit less diversity than αβ T cells in individuals (57, 59). This lower diversity is coming not only from biased gene recombination processes that favor the pairing of Vγ9 with Vδ2 (the Vγ9Vδ2 T cells) (60, 61) , but also from functional selection toward specific antigens., leading to oligoclonal expansion (21, 62). For example, the Vδ1 repertoire shows low clonal focusing on cord blood but becomes highly focused in blood from adults (21). This shift is driven by antigenic selection (e.g., CMV-dominant clones), which expand postnatally, leading to a more private and individualized repertoire in adults (62).

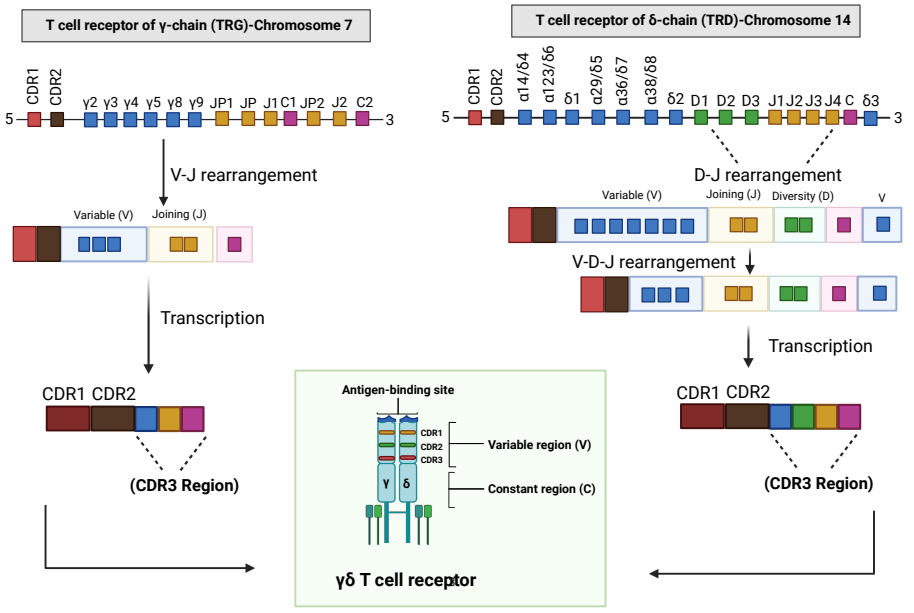


***Figure 3**. Illustration of the human γδ TCR repertoire composition and V(D)J recombination for diversity generation. (created by BioRender).*

1.1.6 Overview of γδ TCR repertoire dynamics

The repertoire of γδ T cells can be described by different nomenclatures based on its diversity, shared clonality, and clonal dominance, as shown in **Figure 4** (58). The Vδ1 chain exhibits more flexibility to pair with different γ9 chains compared to the semi-invariant pairing of the Vδ2 chain which almost exclusively pairs with the γ9 chain (63, 64). The pairing of the Vδ1 chain, most commonly involves the Vγ2, Vγ3, Vγ4, and Vγ5 chains, and rarely with the γ9 chain (63, 64). In early life, the Vδ1 T cell repertoire is polyclonal and highly diverse, displaying an unfocused repertoire in the cord blood (21, 62). With age, the Vδ1 T cell repertoire becomes a more private repertoire and is more clonally focused. This privacy is driven by a few dominant clones, unique to everyone (62). Additionally, these expanded clones showed more differentiated phenotypes, characterized by downregulation of CD27 expression (62). Despite this effector differentiation, the expanded Vδ1 clones were able to proliferate in response to IL-15 and TCR stimulation. Importantly, these clonotypes remain prevalent at high frequencies for 12-18 months later, where they made up more than 50% of the total repertoire in the same donors, indicating their long-term stability over time (62). Accordingly, Vδ1 T cells can display adaptative immunity, marked by a memory phenotype and clonal expansion, similar to αβ T cells (65, 66). Additionally, the repertoire privacy observed is primarily linked to the TRD chain, while the TRG chain is generally shared across individuals (21). It has also been found that the TRD exhibits a more diverse repertoire compared to the TRD due to the presence of the D gene segments that generates two junctions (V–D and D–J) for rearrangement, leading to increased potential diversity when compared to the single V–J junction found in the TRG chain (57)

In contrast to Vδ1, the Vδ2 T cell repertoire is mainly semi-invariant (Vγ9Vδ2 T cells) in peripheral blood, which is enriched by public Vγ9 clonotypes (60, 61). The publicity of Vγ9Vδ2 T cells primarily results from the evolutionary conservation of Vγ9JP clones, caused by convergent recombination in adults or is generated by a germline-encoded process in fetal/neonatal with low terminal deoxynucleotidyl transferase (TdT) (60). These Vγ9JP clones are critical for the phosphoantigen reactivity of the Vγ9Vδ2 T cells, featuring a shared repertoire across individuals (12). After birth, the Vγ9Vδ2 T cell repertoire undergoes a polyclonal expansion in response to microbial-derived PAgs (12). Consistent with this, a study found overlaps between adult and cord blood repertoires in canonical PAg–recognizing Vγ9 sequences. This overlap reflects a pre-selected repertoire with PAgs specificity that undergoes polyclonal expansion after birth (21). After birth, the Vγ9Vδ2 T cell repertoire undergoes a polyclonal expansion in response to microbial-derived PAgs (12). Moreover, in one study, TCR repertoire analysis

showed that adult Vγ9Vδ2 T cells exhibit a polyclonal reaction to PAgs stimulation in vitro, without affecting their repertoire diversity. Even proliferation, the cells retained their (67).

Despite the extensive polyclonal expansion of Vγ9Vδ2 T cells occurs after birth, their frequencies decline with aging, whereas Vδ1 T cells are either maintained or may even expand (68). with ageing, a shift from Vγ9Vδ2 T cells toward Vγ2Vδ1 T cells is observed, particularly in memory populations (66). This might be driven by antigenic selection over time due to environmental/chronic infection (66). Overall, the unique innate and adaptive roles of γδ T cells arise from specific cell subsets with distinct gene rearrangement patterns.

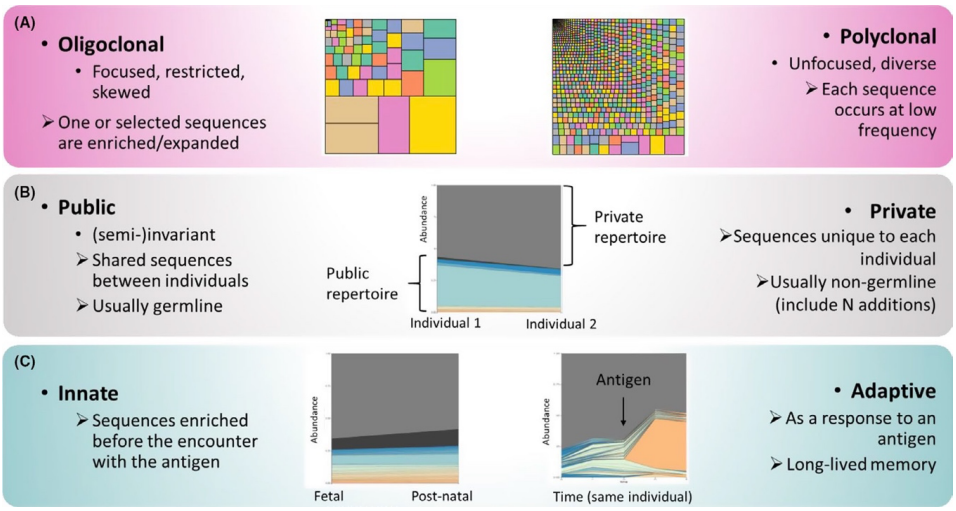


***Figure 4**. Overview of the human γδ TCR repertoire dynamics. Reproduced with permission from Papadopoulou (58).*

1.1.7 Developmental waves of γδ T cell subsets

Human γδ T cells, specifically the Vγ9Vδ2 subset, represent the earliest T cell lineage generated in the fetal liver, detectable between 5-6 weeks of gestation, and in the fetal thymus after 7-9 weeks of gestation (69, 70). Following their development, these cells exit the thymus and significantly increase in the fetal blood until mid-gestation and constitute the majority of the γδ T cells, reflecting this early developmental wave (60, 71). Later, the frequency of Vγ9Vδ2 T cells progressively declines until birth (71). This phenomenon can be attributed to the migration of Vγ9Vδ2 T cells into the fetal intestine around 20 weeks of gestation, as indicated by shared TCR sequences between the fetal intestine, liver, and thymus (58, 70). Over time, this early enrichment of Vγ9Vδ2 T cells in the fetal intestine is gradually replaced with a more diverse population dominated mainly

by Vδ1 T cells (58, 72). Vδ1 T cells, along with a minor population of Vδ3 T cells, emerge during mid-gestation (71). From mid-gestation onwards, Vδ1 T cells continue to increase and become the dominant cells at birth, subsequently populating the fetal blood, pediatric thymus, and epithelial tissues (41, 71, 73, 74). Therefore, the proportion of Vδ2 is outnumbered by Vδ1 at birth (41, 71) (**Figure 5**).

The ratio of Vδ1 to Vδ2 T cells is crucial during the progression of gestation (75). Research indicates that pregnant women with a history of recurrent miscarriages, along with those in mid to late gestation, are at risk for preterm birth, show a lower percentage of circulating Vδ1 T cells and a higher percentage of Vδ2 T cells when compared to healthy pregnancies (75, 76).

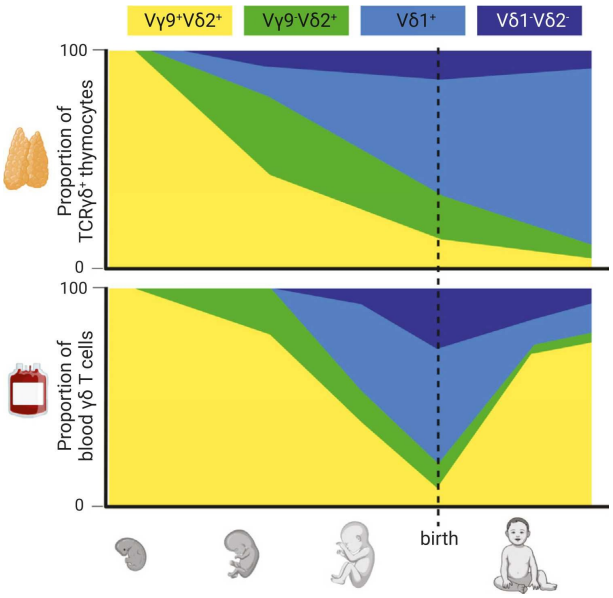


***Figure 5**. Illustration of the developmental waves of human γδ T cell subsets during gestation and after birth in the blood and thymus. Reproduced from Boehme et al (41), licensed under CC BY-NC-ND 4.0.*

The neonatal period represents a phase of high vulnerability, as the newborn transitions from a sterile environment and must efficiently develop their own immunity against a wide range of pathogens (77). Similarly, this is also a main concern in immunocompromised patients following aHCT, where the adaptive immune system requires years to fully reconstitute (78). In both cases, the early development of γδ T cells during fetal life or the early reconstitution following aHCT play an important role in providing immediate immune protection upon environmental exposure (71, 78).

In preterm neonates, there is a rapid polyclonal expansion of Vγ9Vδ2 T cells, including public clones (12). After tracking these cells from matched samples of preterm neonates across weeks 1-2 and 3-5, there was a significant increase in Vγ9Vδ2 T cell frequencies. After 4 weeks of age, their frequencies reached 90 % of the total γδ T cell lineage, indicating a rapid expansion immediately after birth (12). It has been suggested that bacterial growth from gut microbiota or food products after birth supplies PAgs, thereby promoting the expansion of Vγ9Vδ2 T cells, which ultimately emerge as the predominant γδ T cell lineage in adult circulating blood (12, 60, 61). Another study showed that Vγ9Vδ2 T cells from 10-week-old infants exhibit a differentiated, activated, and cytotoxic profile, as demonstrated with rapid IFN-γ production, which were more comparable to that observed in adult rather than cord blood (79). Therefore, Vγ9Vδ2 T cells experience polyclonal expansion with robust functional maturation in neonates. This extensive expansion endows early antimicrobial protection with innate immunity after birth (12, 79). It can also compensate for the late maturation of αβ T cells (80).

The Vδ1 T cell repertoire is more diverse and polyclonal than the PAgs-directed Vγ9Vδ2 in cord blood (62). A study showed that the frequencies of Vδ1 and Vδ3 T cells are significantly elevated in CMV-infected infants compared to non-infected ones. These subsets also display an activated and more differentiated phenotype, distinct from Vγ9Vδ2 T cells, suggesting an adaptive-like response to CMV (81). In CMV-infected infants, the frequency of Vδ1 and Vδ3 gradually increased over different months during their first year of life. However, their frequencies subsequently decreased to levels comparable to CMVsero+ in both children and adults, suggesting CMV shapes the γδ T cell repertoire in infants (81).

1.2 Allogeneic haematopoietic stem cell transplantation (aHCT)

Allogeneic haematopoietic stem cell transplantation (aHCT) refers to the replacement of a defective hematopoietic system in a patient with a healthy immune system from a donor. aHCT represents a curative approach to treat children and adults with life-threatening hematological malignancies (e.g. acute or chronic leukemia, multiple myeloma, and lymphoma) and certain non-malignant disease (e.g. aplastic anemia). The purpose with the aHCT is to obtain a graft versus leukemia (GVL) effect of donor cells in patients against malignant cells and/or producing a new functional immunity in immunodeficiency cases (82).

As shown in **Figure 6**, when performing a aHCT, suitable donor selection, stem cell source, and conditioning regimen are all essential parameters to optimize the GVL effect and avoid transplant rejection (82). The aim of the donor selection is

to find human leukocyte antigen (HLA) matching between recipients and donors for a better outcome. HLA genes are highly polymorphic and vary across people. HL-A, -B, -C, -DP, -DQ, and DR genes are considered when matching between patients and donors (83). Other important factors include donor gender, age, and CMV serostatus (82, 83). For instance, a female donor to a male recipient is not preferred as it is associated with an increased rate of graft-versus-host disease (GVHD) due to generated antibodies against the Y-chromosome during pregnancy (84). In addition, an older age of the donor is also associated with an increased risk of non-relapse mortality (NRM) and a high relapse rate post-transplantation (85, 86). Pediatric patients receiving grafts from parental donors had increased frequency of senescent CD4+ CD28- T cells post aHCT. These cells exhibited reduced cytokine production and impaired functionality when stimulated by PMA and TCR compared to their parental donors (87).

There is a consecutive order of donor selection with siblings and matched related donors being the first choice (82). If not available, unrelated matched donors is the second option. However, in certain geographical areas, it remains a challenge to find a fully matched donor due to the diversity of ethnicities and low donor availability in existing registries (82, 88). Alternatively, if the unrelated matched donor is unavailable, an umbilical cord or a half-matched-related donor, known as HLA-haploidentical, is also possible. (82). The immunological immaturity of umbilical cord cells enables greater tolerance for HLA mismatches during donor selection.

Before the graft infusion, a conditioning regimen treatment is administrated to the patient. It consists of chemo and radiotherapy or only chemotherapy to suppress the host immune system of the recipient, destroy remaining malignant cells, and facilitate engraftment of the new stem cells (82, 83). The conditioning regimen can be categorized as high-intensity myeloablative conditioning (MAC) or reduced-intensity conditioning (RIC). MAC regimen is associated with a lower relapse incidence at the expense of increased toxicity while RIC is less toxic, allowing aHCT in patients who are elderly or have comorbidities, and are currently widely applied in malignant and non-malignant diseases (82, 83).

Haematopoietic stem cells (HSCs) can be harvested from three sources: bone marrow aspirates (BM), mobilized peripheral blood stem cells (PBSCs), and umbilical cord blood (UCB) (83). The procedure of BM collection requires an operation under general anesthesia accompanied by a putative risk for the donor while PBSC are obtained from healthy donors following mobilization with granulocyte colony-stimulating factor (G-CSF), allowing migration of HSCs into circulating peripheral blood and then collection by leukapheresis. This is now the most commonly used procedure to collect HSCs for aHCT (83). Procedure aside,

the immune system reconstitution of the PBSC graft is faster than BM grafts due to a higher number of HSC (CD34+ cells) and T cells (CD3) present (89). Conversely, the BM graft presents less incidence of GVHD because of the lower numbers of T cells as compared to PBSC graft (90). UCB grafts are more commonly used in children since its main limitation is insufficient number of HSCs which delays immune reconstitution compared to BM and PBSC graft cells (83). Therefore, a double UCB transplantation has been introduced to enhance graft cell dosage for adult patients (91, 92).

Despite significant advances in aHCT, it is still impeded by complications that lead to considerable morbidity and mortality. Main complications include disease relapse, GVHD and infections (86). GVHD is caused by alloreactivity and cytotoxic effects of activated donor T cells. It's classified as acute or chronic based on the time and affected organ (86). Acute GVHD typically arises between 30-100 days post-aHCT, most often involving the skin, liver, and gastrointestinal tract. Its grading is assessed the severity of each affected organ on a scale from stage 0 to 4, which are then combined into an overall grade (I–IV). For instance, in life-threatening patients, multi-organ involvement is classified as grade IV. In contrast, chronic GVHD is a long-term complication after aHCT that can affect more organs. Its severity is assessed based on the clinical manifestations in the affected organs and categorized as mild, moderate, or severe (93, 94). Bacterial and viral infections are other complications that can lead to morbidity and mortality; for example, CMV reactivation is the most prevalent as will be outlined later (86).

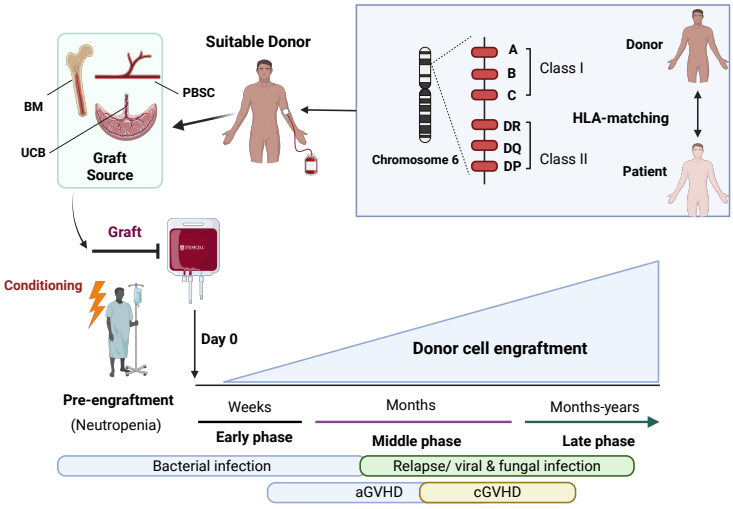


***Figure 6**. Illustration of aHCT procedure. (created by BioRender).*

1.2.1 Mechanisms of T cell reconstitution following aHCT

aHCT entails a conditioning regimen (chemotherapy and/or radiotherapy) as a part of the grafting procedure to eradicate the tumors and suppress recipient alloreactivity toward the donor before graft infusion (82, 83). Consequently, the severe immunodeficiency induced by the depletion of the innate and adaptive immune systems is one of the primary concerns with treated patient following aHCT (86). Therefore, early reconstitution and restoration of the function of the donor-derived immune system essential for the recovery and long-term survival of patients after aHCT. This results in successful aHCT and better clinical outcomes (13, 95).

After aHCT, immune reconstitution is a complex and gradual process. The innate immune system recovers first and normally within one month. In contrast, the kinetics of the adaptive immune system reconstitution is frequently delayed and it takes months to years to reach complete competence (96). This delayed reconstitution is attributed to inadequate thymic recovery affected by the conditioning regimen and immunosuppressive treatment after transplantation (86) . As a part of the adaptive system, T cell recovery post-aHCT is dependent on two primary mechanisms: thymic function and the peripheral niche as shown in **Figure 7** (86, 97). The early T cell reconstitution is provided by peripheral clonal expansion of the donor derived mature naïve and memory T cells that were transferred from the graft within the recipient. This process is so called the thymic-independent mechanism and relies on the peripheral niche, which offer a T cell survival resource, but it can also be affected by immunosuppressive treatment (98). This thymic independent mechanism offers transient protection but insufficient immune response due to a limited repertoire. (86, 97). The thymic-dependent mechanism corresponds to the *de novo* generation of naïve T cells from the thymus occurs later post-aHCT as it is dependent of successful engraftment (86, 97). This process forms the long-term reconstitution with an increasing TCR diversity which is associated with a more favourable long-term clinical outcome (86, 98, 99).

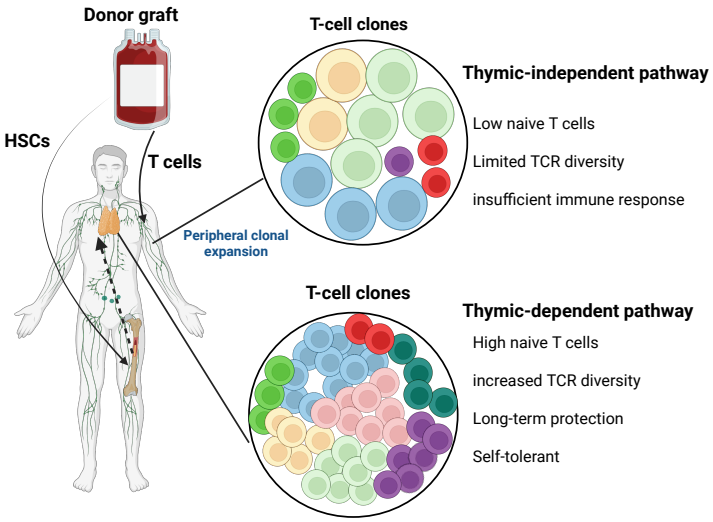


***Figure 7**. Illustration of T cell reconstitution mechanisms after aHCT. (created by BioRender).*

Successful aHCT is strongly linked to the thymic function because of its central role in T cell regeneration and homeostasis. However, the quality of T cell reconstitution can vary between individual patients depending on several parameters such as age, conditioning regimen, graft source, GVHD, and infection (100).

1.2.2 Significant impact of γδ T cell reconstitution post aHCT

The role of γδ T cells and their subsets in aHCT has been the subject of multiple studies in the past decade (101). Early post-aHCT, functional γδ T cells provide an effective graft versus leukemia (GVL), increased patient survival, and protection against infections (94, 102, 103). As γδ T cells are non-alloreactive with an HLA-independent recognition ability (2), their elevated frequency is associated with fewer relapse and less GVHD incidence in transplanted patients (14, 104). Due to their unique characteristics, γδ T cells facilitate allo-engraftment by mediating anti-infective and anti-cancer activities (94, 103). making them ideal effector cells in the aHCT procedure (105) or cancer immunotherapy platform (15). Indeed, several clinical strategies have enhanced γδ T cell functionality in vivo using certain drugs (e.g. Zoldernate) or antibodies or ex vivo expansion for adoptive transfer post aHCT (105, 106).

Several studies have demonstrated that γδ T cells reconstitute faster, within a few weeks post-transplantation, than other T cell subsets after aHCT as shown in **Figure 8** (97, 107, 108). This is followed by CD8+ then CD4+ αβ T cells. The αβ subsets recover within around 100 days or later and can take up years for reconstitution (97). Bian et al. found that the early recovery of γδ T cells in haplo-aHCT recipients were significantly correlated with homeostatic donor γδ T cell contents (109). In another study of γδ T cell reconstitution were found to be the predominant T cell population in paediatric haplo-aHCT during the first week's post transplantation (107). Although it is believed that homeostatic peripheral expansion of γδ T cell is the primary source of this cell population during the first year after aHCT (110). The role of thymic and/or extrathymic differentiation from T cell progenitors post-transplant cannot be ruled out, as some γδ T cell clones in the recipient's blood were found only after aHCT with no presence detected before the transplant or in the donor graft (110). Another study also demonstrated a rapid reconstitution of naïve γδ T cells arising from the thymus within the first three months after aHCT and the recipients' γδ T cell repertoires exhibiting distinct clonotypes, with few shared clones between donor–recipient pairs (108). These findings suggest that γδ T cell recovery relies on both peripheral expansion and thymopoiesis (97, 108, 110).

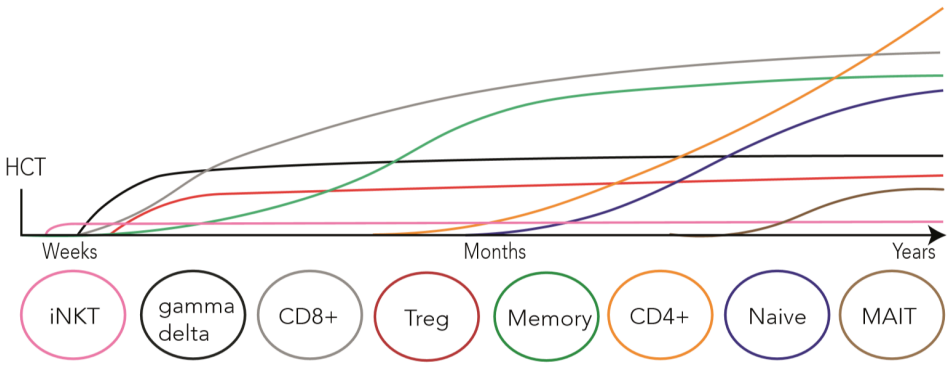


***Figure 8**. Illustration of speed of the reconstitution of different T cell population after aHCT. Reproduced from Dekker et al (97).*

1.2.3 γδ T cell and clinical outcome: GVHD

Despite, there are some studies that have reported an association of γδ T cells with GVHD: In the 1990s, Viale et al. observed that a higher proportion of total γδ T cells was found in patients who developed acute GVHD up to 3 months post-aHCT compared to patients who did not develop GVHD (111). Similarly, evidence from mouse studies reported that grafts enriched with γδ T cells led to an increased risk of acute GVHD (112, 113). In 2005, research demonstrated that host-

derived γδ T cells aggravate GVHD in a mouse model by augmenting the activation of host APCs (DCs) through cell-to-cell contact, thus inducing their allo-stimulatory capability (114). Another study found a significant correlation between a high dosage of γδ T cells in PBSC graft and aGVHD grade II-IV (115). However, this study was only based on total γδ T as single-cell population without addressing the impact of the different γδ T cell subsets (115).

Reversely, many studies have demonstrated that γδ T cells contribute to better clinical outcomes post-aHCT: In the late 1990s, several studies demonstrated that higher frequencies of γδ T cell in peripheral blood facilitates engraftment and improves the disease-free survival of patients after aHCT. Their proportion markedly decreased in GVHD patients (116, 117). In lethally irradiated mice, the transplantation of large doses of ex vivo activated γδ T cells into irradiated hosts has shown equivalent survival to control mice (T cell-depleted BM alone), without any increase in incidence of GVHD. Moreover, the activated γδ T cells promoted the engraftment of irradiate host and augmented hematopoietic reconstitution in these mice compared to T cell-depleted BM only. The limited number of transplanted αβ T cells has caused fatal GVHD, whereas a large dose of activated γδ T cells did not result in significant GVHD in transplanted mice (118). Consistently with these findings, in 2001, it was shown that allogeneic donor-derived γδ T cells could be specifically expanded when co-cultured with primary acute lymphoblastic leukemia (ALL) blasts from the patient. The blasts were also shown to be efficiently lysed by the γδ T cells. In contrast the cells did not proliferate in a mixed lymphocyte reaction with patient-derived peripheral blood lymphocytes or with third-party cells, implying a specific GVL effect without a potential risk of GVHD (119).

In addition, in an eight-year follow-up study of the long-term survival of 153 patients it was shown that 18 patients with 5-year leukaemia-free survival (LFS) and better overall survival had an increased γδ T cells count. The remaining patients with low numbers of γδ T cells were 4.3 times more likely to have an unfavourable LFS. There was no observation of increased risk of acute GVHD in patients with high and low γδ T cells (120).

More recent studies have corroborated the potential benefit of γδ T cells in the aHCT setting. As innate-like effector cells and fast reconstitution after aHCT, the elevated γδ T cells concentrations significantly reduced the risk of disease progression and aGVHD after 2 months of transplantation (103). Moreover, a higher proportion of γδ T cells was correlated to the absence of bacterial infection and a significantly lower incidence of fungal and viral infection after aHCT in pediatric patients, indicating a protective function of these cells (121). A strong

association between increased γδ T cells percentage in peripheral blood after aHCT with better clinical outcomes in the aspect of fewer infections, prolonged survival and maintained continuous complete remission (CR) with lower incidence of GVHD has been confirmed by a recent meta-analysis from 11 studies (amongst 2414 studies) which consisting of 919 patients with 30 months median follow-up (13).

The poor or favourable outcome attributed to γδ cells in the incidence of GVHD after aHCT was further investigated by studying the role of the different subsets of γδ T cells. For instance, a distinct subset of regulatory γδ T cells (Foxp3+ γδ Treg) was significantly lower in patients with limited and extensive cGVHD. This could suggest an operational role for Foxp3+ γδ Treg in controlling cGVHD and maintenance of tolerance post-aHCT (122). Similarly, there was a negative correlation between the proportion of Foxp3 γδ cells including Foxp3+ Vδ1 and Foxp3+ Vδ2 subsets and the occurrence of aGVHD after aHCT (123). Furthermore, a lower fraction of CD27+ Vδ1 Tregs in the graft was also adversely associated with higher incidence of aGVHD, as patients who received grafts with fewer CD27 Vδ1 Tregs were more likely to develop aGVHD (124). This observation suggests that the presence of CD27- Vδ1 T cells in the graft may reflect their effector function, potentially contributing to aGVHD development or acting through a bystander effect.

As CD27 acts as a co-stimulatory molecule in γδ T-cell activation, it promotes cell proliferation, enhances IFN-γ production, and supports cell survival (125). Interestingly, another study demonstrated that a higher frequency of CD27+ γδ T cells in the graft composition was negatively correlated with the development of relapse (6.53 vs 2.79) and CMV reactivation (5.91 vs 4.57) post aHCT (126). Furthermore, the graft with a large proportion of CD8+ γδ T cells was associated with an increased prevalence of aGVHD, indicating a possible alloreactive role for this fraction (126). More recently, the increase expression of CXCR4 in γδ T cells alongside CD4 and CD8 T cells was observed in aHCT patients with GVHD grades II-IV. The authors narrowed down that the penitential function of CXCR4 γδ T cells to be involved in the recruitment of alloreactive CD4 T cells to GVHD target organs after aHCT suggesting the indirect mechanism of CXCR4 γδ T cells in the incidence of aGVHD (127).

Given the heterogeneity of γδ T cells and the contradictions between studies of the of γδ T cells role after aHCT, more studies are required to explore the contributory function of different γδ T cell subsets of post-transplantation and their association with clinical outcomes.

1.2.4 γδ T cell and CMV reactivation

Human cytomegalovirus (CMV) is a ubiquitous herpesvirus with a high prevalence of around 30-90% in the human population. Naturally, CMV can be transmitted through any infected body fluid (e.g. saliva and urine) (128). CMV mechanisms of adaptability constitutes of multiple-evolved immunomodulatory strategies that prevents viral clearance inside the host. HCMV creates a lifelong asymptomatic infection in immunocompetent individual, attributed to the host's vigorous and well-coordinated immune response (128).

In contrast, CMV infection and reactivation are a leading cause of morbidity and mortality in immunocompromised patients such as those undergoing either solid organ transplantation or HCT (128, 129). They are vulnerable to opportunistic pathogens such as CMV because of the conditioning regimen, lymphopenia and immunosuppressive therapy. Clinically, this pathogen can cause tissue-invasive disease, severe symptoms, and several sequelae which may lead to death in immunocompromised hosts. CMV escape the immune response, via different mechanisms, one of them is by generating an HLA class I homologue to interfere with antigen presentation on infected cells to avoid cytotoxic T lymphocyte recognition (128, 130).

Post-aHCT, γδ T cells appear to be an important player in controlling CMV infection/reactivation (**Figure 9**) (131). Remarkably, the Vδ2^{neg} γδ T cells which mainly express Vδ1+, Vδ3+, and in specific events Vδ5+ subsets are described to be the principal subsets for CMV control in healthy subjects and transplanted patients (40, 81, 132). Vδ1+ and Vδ3+ γδ T cell subsets are highly prevalent in epithelial tissues where CMV is actively replicating, supporting their responsibility in CMV control (128, 132). Following allogeneic transplantation, the Vδ2^{neg} γδ T cells are regarded as a first line of defence against CMV infection/reactivation alongside NK cells due to their rapid reconstitution (108, 133). Numerous studies have shown a notable Vδ2^{neg} γδ T cells expansion, particularly Vδ1+ and Vδ3+ subsets, in response to CMV infection/reactivation following solid organ transplantation and aHCT (108, 134, 135) . Interestingly, this increased expansion of Vδ2^{neg} γδ T was specific to CMV infection/reactivation while no such causality was observed in Epstein-Barr virus (EBV)-positive and CMV-negative groups (136). In another study, Vδ2^{neg} γδ T cells of CMV seropositive donors exhibited a long-memory response with a faster expansion to a second encounter with CMV-infected cells. These CMV-reactive Vδ2^{neg} γδ T cells displayed a restricted repertoire with increased terminally differentiated phenotype, which coincided with efficient CMV resolution in patients who experienced CMV infection. Altogether, these finding confirm the adaptative

antiviral immune response of Vδ2^{neg} γδ T cells in memory response and clonal selection, as well as resolution function of these subsets (132).

Noteworthily, a recent study has identified a small subset expressing Vδ2+ subset unpaired with Vγ9 (Vγ9-Vδ2+) involved in the CMV immune response. Unlike Vγ9+Vδ2+ γδ T cells, absolute numbers of Vγ9-Vδ2+ and Vδ2^{neg} γδ T cells were considerably more abundant in kidney transplant recipients with CMV+ serostatus than in the CMV- counterpart. The Vγ9-Vδ2+ presented a highly terminally differentiated phenotype in CMV+ patients while CMV- transplanted recipient displayed a high naïve one. The Vγ9-Vδ2+ γδ T cells responsiveness was specific to CMV-infected fibroblasts and no reactivity against cells infected with herpes simplex virus (HSV) or varicella-zoster virus (VZV) could be detected. Altogether, the Vγ9-Vδ2+ T cells are consistently compatible with adaptive like properties in the context of immunologic memory with clonal expansion during CMV infection (22).

Although numerous studies have investigated the protective role of γδ T cells in CMV infection and reactivation, the exact mechanism of CMV recognition by CMV-reactive cells are poorly known (131). One study has found that the EPCR could be a putative TCR ligand for Vγ4Vδ5+ cells exhibiting CMV recognition. The proposed recognition suggests a direct binding of the Vγ4Vδ5+ TCR to EPCR (40). Moreover, like NK cells, the frequency of NKG2C+ Vδ1+ γδ T cells are highly correlated with CMV reactivation, which could reflect their capacity to help control the infection. This is thought to be mediated by the interaction between the NKG2C receptor and HLA-E/CMV-derived-UL40 peptide expressed on CMV infected cells (134). Additionally, the upregulation of CD16 enables Vδ2^{neg} γδ T cells to identify IgG-opsonized virus and stimulate the generation of IFNγ during CMV infection (137).

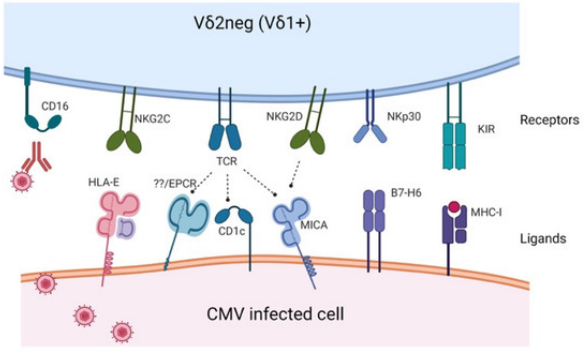


***Figure 9**. Illustration of the potential ligands and receptors interactions between CMV and γδ T cells. Reproduced from Gaballa et al. (131)*

1.3 How to improve γδ T cell immunotherapy

1.3.1 New strategies: Bispecific antibody

The extraordinary advances in immunotherapeutic antibody development during the past decades have generated multiple novel approaches to treat cancer patients. Indeed, monoclonal antibodies (mAbs) such as anti-PD-1 and anti-CTLA-4 mAbs treatment have been approved and now used in cancer treatment with a significant potency. Unfortunately, these antibodies have a limited effect in so called "cold" tumors (138). Cold tumors are characterized by a failure of T cells to infiltrate the tumor site (tumor parenchyma) and its surrounding stroma. Their microenvironment is enriched with immunosuppressive cells, including myeloid-derived suppressor cells (MDSCs), which release inhibitory cytokines and limit the response to immune checkpoint blockade (139). Thus, there is an unmet need to further develop novel immunotherapeutic antibodies (138).

Several bispecific antibodies (BsAbs) have emerged as promising candidates in cancer treatment. BsAbs are a second generation of immunomodulatory antibodies characterized by improved specificity and superior cytolytic capacity compared to mAbs (138). To date, various formats of BsAbs have been produced and tested in cancer treatment and many of them are now at different stages undergoing pre-clinical and clinical trials. Examples of BsAbs and their actions and structures in the clinical trial are listed in **Table 1**. Unlike mAbs, these antibodies comprise two distinct binding sites within one molecule against two different epitopes. The epitopes can be expressed on the same or separate targets (140-142)

There are two primary categories of BsAbs depending on whether they contain a constant fragment domain (Fc) or not. The asymmetrical IgG-like format has a structure comparable to the native form of natural antibodies. The presence of the Fc domain extends the half-life, improves stability and facilitate their purification. In addition, this format can be assembled with multiple domains at the Fc region for possible extra epitope targeting. The second BsAbs category, defined as non-IgG-like (no Fc region), is a single-chain variable fragment (scFv) BsAbs. The scFv is a fusion protein derived from two variable heavy (VH) and variable light (VL) domains of two antibodies connected via a flexible polypeptide linker. The scFv fragment preserves the binding activity of each antibody when assembled, supported with a short flexible linker for stabilizing and providing free rotation of the two arms during the interaction of the target and T cell. The absence of the Fc region in a non-IgG-like format makes it more permeable into tumor tissues because of decreased molecular weight (50 KDa vs 150 KDa) (138,

143). Their relatively short half-life (around 2h) requires a continuous administration to patients. Extension of the BsAb half-life was possible by fusing Fc domain to non-IgG-like structure such as BTE molecules (140, 141, 144). The "Half-life extended" (HLE) BTE molecules resulted in prolonged a half-life around one week (145). BTE molecule, one bispecific T cell engager molecule belonging to the non-IgG subtype, will be further discussed in the next section (141).

Table 1: Structure of bispecific antibodies in clinical trials.

Description	Target	Antibody format	Name	disease	Phase
Redirection of immune cells	CD19-CD3	DART	MGD011	B cell lymphoma	Phase II
	CD19-CD3	BiTE	Blinatumomab	ALL	Approved
	CD19-CD3	HLE-BiTE	AMG562	Lymphoma	Phase I
	CD33-CD3	BiTE	AMG330	AML	Phase I
	CD33-CD3	HLE-BiTE	AMG673	AML	Phase I
	CD33-CD3	Tand Ab	AMV-564	AML	Phase I
	CD20-CD3	DuoBody	GEN3013	Hematological malignancies	Phase I/II
	CD20-CD3	Xmab	Plamotamab	Hematological malignancies	Phase I
	BCMA-CD3	BiTE	AMG420	Multiple myeloma	Phase I
	FcRH5-CD3	BiTE	RG6160	Multiple myeloma	Phase I
	CD30-CD16	TandAbs	AFM13	Hodgkin's lymphoma	Phase II
	CEA-CD3	BiTE	AMG111	Gastrointestinal adenocarcinoma	Phase I
	CEA-CD3	CrossMAb	RG7802	Solid tumor	Phase I
	EpCAM-CD3	BiTE	Solitomab	Malignant ascites	Phase I
	EpCAM-CD3	Triomab	Catumaxomab	Malignant ascites	Approved
	EGFRvIII-CD3	BiTE	AMG596	Glioblastoma	Phase I
Blocking of immune checkpoint	PD1-CTLA4	DART	MGD019	Solid tumor	Phase I
	PD1-LAG3	DART	MGD013	Solid tumor	Phase II/III

1.3.2 Bispecific T cell engagers (BTE) recruit γδ T cells against tumor cells

BTEs are widely applicable to treat solid and haematological malignancies, they are small molecules (55-60 KDa) which can easily circulate and enter the tumor tissue (141). BTEs are scFv fragments that target tumor-specific antigen (e.g. CD19) and effector cell antigen such as the CD3 subunit on T cells. BTEs activate T cells as demonstrated by the increased expression of CD69 and CD25. Additionally, BTEs support the proliferation of T cells upon dual-binding interaction with the target cells (140). Cytolytic activity occurs when BTEs bind T cells to tumor cells: effector T cells release perforin and granzyme B, forming a pore on the tumor cells membrane to mediate target cell lysis (**Figure 10**) (140). The small size of BTEs allows them to cross the blood-brain barriers which opens up for treatment of brain malignancies. For example, AMG 596, a BTE that link CD3+ T cells to EGFRvIII-expressing Glioblastoma multiforme cells, showed antitumor activity in vitro and significantly increased the survival in a mouse model for brain tumor (146).

Blinatumomab was the first FDA-approved BTE antibody for the treatment of B-cell precursor acute lymphoblastic leukemia (B-ALL) in 2014 (147). It consists of binding moieties to CD19 and CD3 connected by a short peptide linker. The accelerated approval was supported by the phase II study (NCT01466179, 189 adult patients) from which 33% of patients achieved complete remission, and 10% obtained CRh with partial haematological recovery following two treatment cycles (148). In 2017, full approval to treat adults and children with B-ALL was granted (149).

Currently, various BTE antibodies are developed: for instance, CD33-CD3 and FLT3-CD3 compounds were produced for acute myeloid leukemia (AML), whereas PSMA-CD3 and EGFRvIII-CD3 were developed against solid tumors of prostate and glioblastoma, respectively. These molecules offer promising therapeutic potential in haematological malignancies and solid tumors (144).

The combination of *ex vivo* expanded Vγ9Vδ2 T cells and bispecific T cell engager molecules as a synergetic function can boost the durability and effectiveness of tumor cell killing (150-152). Chen et al. found that the B-lymphoblasts cell line lysis increased in the combination of expanded Vγ9Vδ2 T cells with blinatumomab rather than the expanded cells alone. Interestingly, the repeated infusion of this combination showed extended survival rate versus blinatumomab alone or expanded cell alone in the mice xenograft model. Additionally, the tumor size outside the bone marrow was reduced in response to the adoptive transfer of

expanded Vγ9Vδ2 T cells in the presence of blinatumomab, indicating a potential role of Vγ9Vδ2 cells in treating extramedullary disease (151).

Multiple BsAbs have been developed for the selective recruitment of the Vγ9Vδ2 T cells. For instance, a prototypic engager molecule (anti-TRGV9/anti-CD123) induced selective recruitment of Vγ9Vδ2 T cells to lysis AML blast cells without causing a cytokine storm (150). Another engager molecule CD1d-specific Vγ9Vδ2 selectively redirects the Vγ9Vδ2 to leukemic cells resulting in efficient tumor lysis with strong activation of Vγ9Vδ2 T cells (152). Oberg et al. demonstrated that the combination of Vγ9Vδ2 T cells infusion with bispecific antibody (Her2/γ9) led to significant regression of tumor growth in mice models of pancreatic cancer (153). This combination strategy can be therapeutic potential for pancreatic cancer that have a tumor microenvironment with low penetration (153). The dual binding of BsAbs facilitates penetration by engaging effector T cells and enhancing their infiltration toward cancer cells, thereby bypassing the tumor stroma of pancreatic tumors. With BsAbs, T cells can be recruited and activated independently of chemokines and antigen presentation (153, 154). All these findings highlight that the combination of these novel immune therapies i.e. bispecific T cell engager and γδ T cell-based therapy could be utilized for immunotherapeutic approach for patients with hematological or solid malignancies.

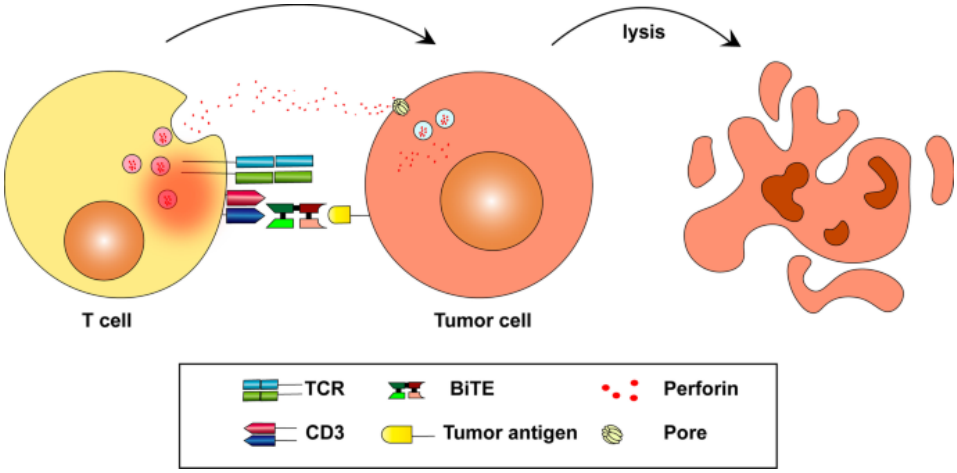


Figure 10. *BTE-induced T cell redirection and tumor cell lysis. Reproduced from Tian et al (140).*

1.4 Thymoquinone (TQ) as a promising anticancer candidate

Medicinal plants have been used in treatments of various diseases (e.g. inflammation, diabetes, gastrointestinal disorders, and many others) since ancient times (155, 156). These plants produce a wide range of bioactive compounds, known as phytochemicals, including secondary metabolites (155, 156). The production of these compounds is primary linked to the plants' adaptation to their environmental conditions and their defense mechanisms against pest invasions (155, 156). Due to their abundance of bioactive molecules, medicinal plants are considered valuable sources of novel therapeutic candidates, including anti-cancer agents. Notably, plant-derived compounds comprise about 25% of the active ingredients in synthetic drugs (155).

In this respect, Thymoquinone (TQ), is a promising anticancer agent, a naturally occurring phytochemical derived from the black seeds of the *Nigella sativa* plant (157). It is the main pharmacologically active compound in the volatile fraction of *Nigella sativa* seeds and form the most of the herb's medicinal benefits (157). TQ exhibits anticancer activity by inducing apoptosis through multiple mechanisms. It also presents several pharmacological activities, including anti-inflammatory, immunomodulatory, epigenetic, and antioxidant effects (157, 158). The combination of these properties underscores the potential of TQ in cancer therapy as discussed in the subsequent subsections.

1.4.1 Pharmaceutical characteristics of TQ

TQ was first isolated in 1960 with a chemical formula of $C_{10}H_{12}O_2$ and a low molecular weight of 164.20 g/mol (**Figure 11**). It is a yellow crystalline compound, characterized by high solubility in fats and lower in water, and consequently exhibits poor bioavailability (158). It has been reported that TQ bioavailability is ~58% with a lag time of approximately 23 min (159). A study revealed that the interactions of TQ with proteins and other blood components uncover significant impacts on its bioavailability, metabolism and elimination. The covalent binding of TQ with bovine serum albumin (BSA) leads to a reduction in its anticancer effect against the tested cancer cells. In contrast, the interaction of TQ with another blood protein (alpha/1 acid glycoprotein) did not affect its antitumor activity. The results highlight the chemical specificity of TQ, demonstrating its ability to form a covalent bond with BSA which reduces its bioavailability (160, 161). Moreover, TQ is a light-sensitive compound and thus it can be degraded by light exposure (162). The stability of TQ was decreased in the alkaline solution due to increased pH (162).

The administration of TQ has been investigated using different routes (163-165). TQ showed no toxicity in the rat model at an oral dose of 50 mg/kg, which reduced the inflammation and apoptosis caused by toluene compound exposure in the rat lungs (163). Another study also indicated that TQ displayed antioxidant role and no hepatotoxicity when administering intraperitoneal injection in mice (12.5 mg/kg) (164). TQ showed a safety profile in humans and was tolerated effectively when taken orally in 500 mg capsules, twice daily for 14 days (166). Another recent clinical trial demonstrated that TQ showed no significant toxic effects on clinical parameters (e.g. liver function and lipid profile) in 70 healthy subjects at an oral dose of 200 mg/day over 90 days, (165).

The median lethal dose (LD50) after oral administration was determined in mice and rats, 870.9 mg/kg and 794.3 mg/kg, respectively, whereas 104.7 mg/kg in mice and 57.5 mg/kg in rats after intraperitoneal injections (167).

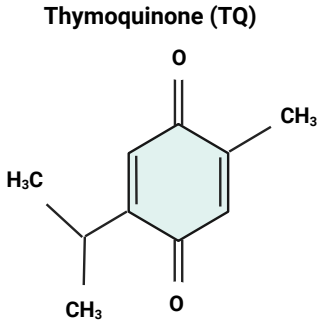


***Figure 11**. The chemical structure of thymoquinone (TQ). (created by BioRender).*

1.4.2 Benefits of Nanotechnology for TQ

The use of TQ in the clinical application is delayed due to its limited bioavailability. To overcome pharmacokinetics limitations, nanotechnology could address this issue by offering TQ nanoparticles compared to free TQ (157). Several studies have used different nanoparticles to enhance bioavailability of TQ. Amongst, Poly-lactide-co-glycolide (PLGA) is the most common used in nanomedicine (168). It is considered relatively safe, as it can be converted into lactic acid and glycolic acid, which can then be metabolized and eliminated as water and carbon dioxide from the body (168). TQ-PLGA nanoparticles can be made through dissolving the polymer in acetone in the presence of a stabilizer and an emulsifier (168). It has been demonstrated to be more effective than free TQ, with an IC_{50} of 0.03 mg/mL for TQ-PLGA nanoparticles compared to 0.13 mg/mL for free TQ against breast cancer cell lines (169). Another one, cyclodextrin is an effective polymeric nanoparticle for encapsulation due to its external hydrophilic surface and

hydrophobic interior cavity (170). TQ-conjugated with cyclodextrin showed enhanced anticancer activity in breast cancer cells, exhibiting a fivefold reduction in IC_{50} compared to free TQ (171). Like TQ, paclitaxel (PTX) is a phytochemical compound that faces similar challenges regarding their bioavailability. However, the formulation of albumin nanoparticles (e.g. Abraxane) enabled the transition of PTX into clinical use for advance metastatic cancers and obtaining FDA approval (172).

1.4.3 Mechanisms of TQ in cancers

Several studies have demonstrated the anticancer properties of TQ in solid cancers and hematological cancers (158). TQ are considered as a potent anti-cancer agent due to its capacity to act on multiple dysregulated cancer-specific targets (**Figure 12**): It can interfere with several carcinogenic signaling pathways such as PI3k/AKT, JAK/STAT, and p38 MAPK as well as apoptosis-related signaling, molecules which are related to metastatic factor, tumor growth, and anti-carcinogenesis. The dysregulation of the JAK/STAT signaling pathway is well known to promote tumorigenesis and epigenetic silencing of tumor suppressor genes (173, 174). TQ was found to suppress the enzymatic activity of the JAK/STAT signalling pathway and promote the re-expression of tumor suppressive genes (e.g. SHP-1) through hypomethylation in MV4-11 AML cell line (173). Interestingly, another study revealed that TQ can act as a histone acetylation inhibitor in leukemic and solid cancer cells, through downregulating the expression of histone deacetylase (HDAC) HDAC1, HDAC4, and HDAC9, in addition to its role in modulating DNA methyltransferases (DNMT) (175). Simultaneously, the tumor suppressor and pro-apoptotic genes are up regulated (175). Altogether, these observations indicate that TQ is a promising epigenetic drug for cancer therapy.

Given that the overexpression of WT1 and BCL2 genes are associated with inhibition of apoptosis and drug resistance in AML (176). TQ was described to trigger the intrinsic and extrinsic pathway of apoptosis, through activation of caspase-3, ultimately reducing the expression of WT1 and BCL2 and subsequent apoptosis (177). Furthermore, TQ has been reported to reduce the invasion and metastasis of cancer cells: TQ can inhibit the metastasis of glioblastoma cells via downregulation of Focal adhesion kinase (FAK), which leads to reduce the level of matrix metalloproteinases (MMP-2 and MMP-9), known to be implicated in cancer progression (157, 158) and ERK activation (178). Moreover, TQ was found to interfere with TWIST promoter activity in breast cancer cell lines, suppressing the cancer invasion and metastasis driven by epithelial-mesenchymal transition (179).

Intracellular reactive oxygen species (ROX) are crucial in various pathological process, including cancer cells (180). These ROX are exploited by cancer cells to maintain their proliferation and transformation by activating the MAPK and PI3K/AKT pathways (180). A study reported that TQ generates ROS and activates caspases 3 and 8 in leukemic cells (CEMss) (181) . Thus, TQ can work as a pro-oxidant in cancer cells, increasing the level of ROS inside the cancer cells, ultimately inducing apoptosis (157).

Combination therapy is justified by its ability to overcome drug resistance, improve efficacy, and reduce side effects through multiple mechanisms of action (182, 183). For example, Gemcitabine is known as the most chemotherapeutic used for treating patients with pancreatic cancer. However, its limited efficacy is mainly due to increased chemoresistance and its toxicity to normal tissue (158). TQ pretreatment improves the sensitivity of GEM against pancreatic cancer cell lines (182). This synergistic effect augments the apoptosis by inhibiting BCL-XI and BCL2 phosphorylation, upregulating Bax, releasing cytochrome C and cleaving caspase-3 and -9 in pancreatic cancer cell lines. It has been suggested that TQ pretreatment may block the activation of Notch1/NICD and AKT/mTOR signalling pathway, while upregulating PTEN (182). Glamoclija et al. further found that the combination of metformin (1.25 mM) and TQ (0.625 μM) synergistically suppressed Akt and NF-kB signaling in imatinib-resistant human leukemic cells (183). Importantly, oral TQ with dual immune checkpoint inhibitors (CTLA-4 and PD-1), demonstrated encouraging outcomes, achieving progression-free survival over two years in patients with metastatic extrapulmonary neuroendocrine carcinomas (184). These promising results have been translated into an ongoing clinical trial study (NCT05262556).

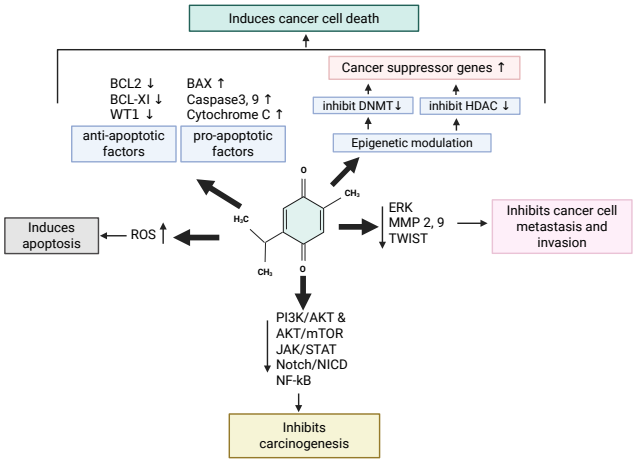


Figure 12. Major anticancer mechanisms of thymoquinone (TQ). *(created by BioRender).*

2 Research aims

The global aim of this thesis was directed toward the role of γδ T cells in aHCT and their potential use in the treatment of hematological malignancy. Better characterization of long-term γδ T cell reconstitution and their association with clinical outcomes will contribute to improving therapeutic interventions in the aHCT field. Owing to their unique biology, harnessing γδ T cell-based therapies, including allogeneic ex vivo-expanded cell products and their combination with bispecific antibody or chemotherapy, bring further individualized approaches for patients undergoing aHCT.

<u>The specific aims of doctoral projects were:</u>

1. To deeply analyze long-term γδ T cell reconstitution ≥ 8 years post-aHCT using next-generation sequencing of the TCR γ-chain and high-parameter flow cytometry. (**Study I**).
2. To address the association of long-term γδ T cell phenotype and repertoire with clinical outcomes. (**Study I**).
3. To explore the use of expanded γδ T cells in combination with a novel CD34/CD3 BTE in mediating cytotoxicity against CD34+ leukemia cell lines and augmenting selective lysis in CD34+ AML blasts. (**Study II**).
4. To further assess the biological activity of CD34/CD3 BTE on the γδ T cells proliferation and cytokine production in vitro. (**Study II**).
5. To investigate the targeting cytotoxicity of γδ T cell-based CD34/CD3 BTE approach on healthy cells such as endothelial blood-brain barrier cell line (hCMEC/D3) and CD45dim CD34+ HSC from bone marrow. (**Study II**).
6. To continue to explore the use of epigenetic drug TQ for its ability to sensitize the leukemia cell line and ehance expanded γδ T cells-mediated cytotoxicity. (**Study III**).
7. To evaluate the synergistic effect of TQ pretreatment of leukemia cell lines in combination with γδ T cell-based CD34/CD3 BTE approach. (**Study III**).

3 Materials and methods

3.1 Human material

All human samples in this thesis were obtained from Karolinska University Hospital, Huddinge, following ethical approvals and informed consent procedures.

- **Study I:** Mononuclear cells (MNCs) were isolated from the collected blood of patients undergoing aHCT and their respective donors.

- **Study II:** MNCs were isolated from the collected blood from patients diagnosed with AML and bone marrow (BM) cells from healthy donors. In addition, MNCs from healthy volunteers' buffy coats served as starting material for γδ T-cell expansion.

- **Study III:** γδ T cells expanded from MNCs isolated from healthy volunteers' buffy coats.

3.2 Ethical consideration

This thesis used human-derived material in accordance with the Helsinki declaration and relevant regulations and has been approved by the Regional ethical committee in Stockholm. The Swedish pharmaceutical insurance and the Swedish patient insurance (LFF) covered all subjects. It is crucial to maintain honesty; patients recognize the importance of the study outcome, which ultimately has a greater beneficial effect on future patients than directly benefits the participants. The patients have agreed to participate in the study but have the right to reject and withdraw their participation.

All studies aim to provide critical knowledge that can improve current aHCT treatment strategies or develop new therapeutic options, such as targeting γδ T cells against leukemic cells using a novel immune cell engager (CD34/CD3 BTE) with/without an anticancer drug (TQ).

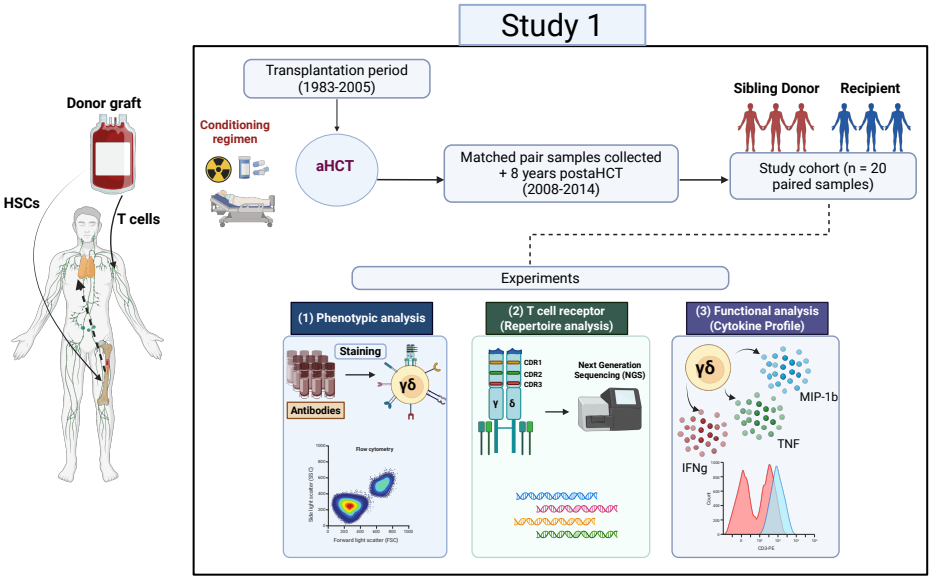
The best interest has been taken to avoid physical and social harm to all the participants. The preclinical part involves collecting blood samples from patients and healthy donors. Blood collection of less than 50 mL for each sample is safe and without side effects for all the subjects.

Study I: Patent data was managed in a quality registry at Karolinska University Hospital, with the registers consistently updated and controlled. All patients' information and personal numbers are kept highly confidential, and patients are

assigned codes upon publication. In addition to our original ethical application, we have been permitted to transfer samples abroad for NGS analysis.

3.3 Laboratory methods

This thesis employed a range of laboratory methodologies, with immunophenotyping by multicolour flow cytometry serving as the central technique throughout all studies. In addition, complementary state-of-the-art approaches, such as next-generation sequencing (NGS), were implemented to address specific research objectives. An overview of the experimental workflow, together with the materials and methods applied, is presented in **Figure 13**.



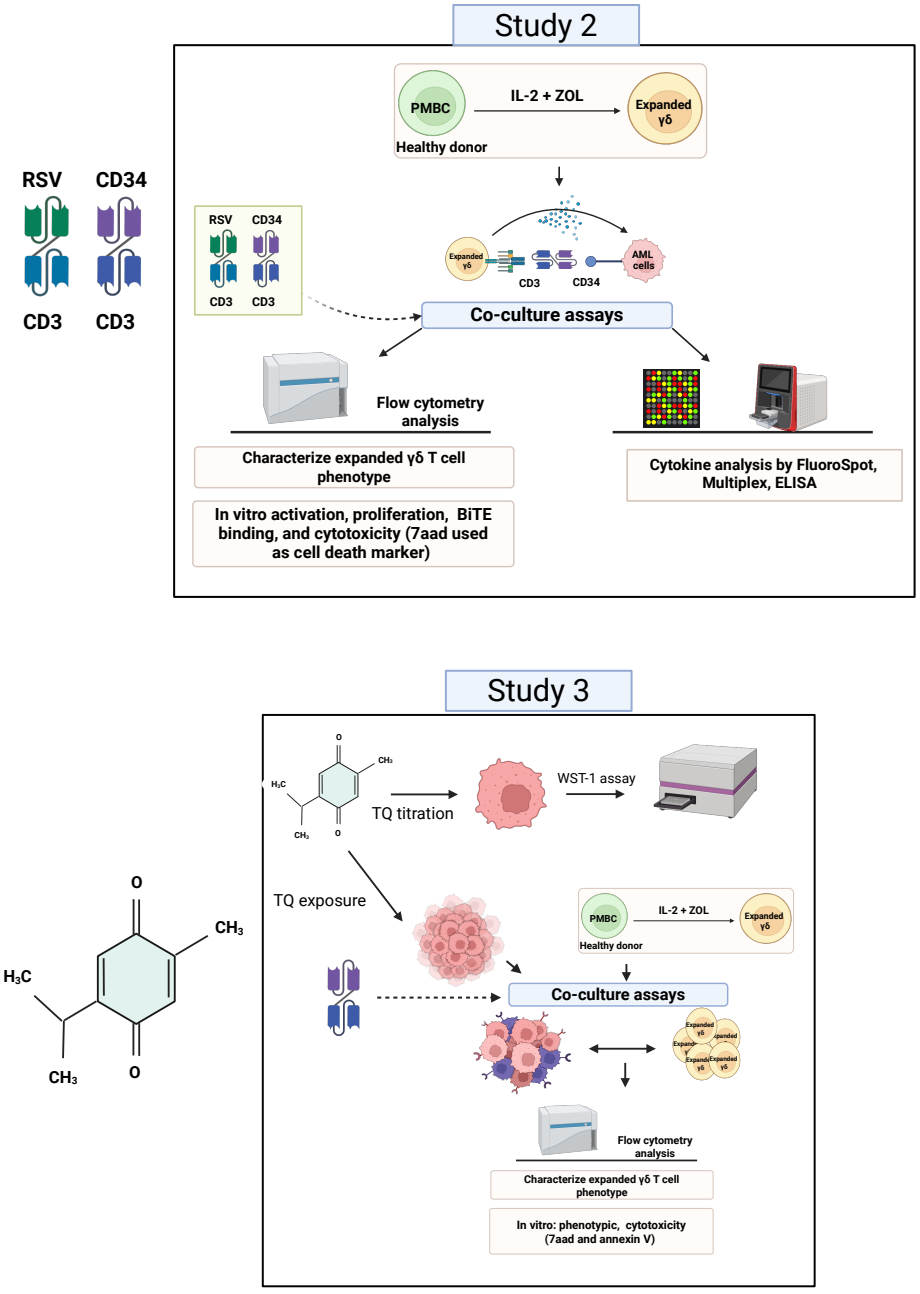


Figure 13. Schematic overview of the experimental workflow. *(created by BioRender).*

3.3.1 Cell lines and MNCs

In **Studies II and III**, Human leukemia cell lines were used as target cells. These included the CD34+ cell lines SUPB15, Kasumi, and KG1a, as well as the CD34- cell line NALM-6, all obtained from the American Type Culture Collection (ATCC). Cells were maintained in RPMI-1640 medium (Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone) and 1% penicillin/streptomycin (P/S; Gibco, Life Technologies) (complete medium) at 37°C in a humidified atmosphere containing 5% CO_2. hCMEC/D3 cells (Nordic biosite) were cultured in tissue culture flasks pre-coated flask with rat-tail collagen type I (Sigma) and maintained in EGM-2-MV BULLETKIT medium (Lonza, Fisher Scientific) supplement with 10 mM HEPES, 200 ng/mL bFGF, 1.4 μM hydrocortisone, 5μg/mL ascorbic acid, 5% FBS, and 1% P/S.

Mononuclear cells (MNCs) were obtained and isolated from the peripheral blood and in some cases BM of healthy volunteers or patients using density gradient centrifugation with Ficoll-Paque (GE Healthcare) at 800 × g for 20 minutes without brake, following the manufacturer's instructions. The buffy coat layer containing MNCs was subsequently collected and washed twice with phosphate-buffered saline (PBS) before counting. MNCs were either used fresh in subsequent assays or cryopreserved at -192°C in complete culture medium supplemented with 10% dimethyl sulfoxide (DMSO; Sigma) until further use.

3.3.2 γδ T cell in vitro expansion

MNCs were cultured at a cell density of 1 x 10^6 viable cells/mL in complete media (RPMI 1640 supplemented with 10% pooled FBS serum and 100 IU/mL penicillin G and 100 mg/mL streptomycin) in the presence of 5 μM zoledronate (Sigma) and 300 IU/mL recombinant IL-2 (Miltenyi Biotec). Cells were incubated at 37 °C in 5% CO_2 and culture media was exchanged every 2-3 days based on cell growth. At day 12 of cultivation, γδ T cells were harvested and purified if necessary, using negative selection of γδ T-cells by magnetic beads (TCRγ/δ+ T-Cell Isolation Kit; Miltenyi Biotec).

3.3.3 Flow cytometry

Flow cytometry is a widely recognized gold-standard technique for single-cell analysis, enabling the characterization of both intracellular and extracellular phenotypes. The principle relies on hydrodynamic focusing to create a laminar flow stream, allowing cells to pass individually through a laser beam. The detection system: comprising lasers, optical filters, and photomultiplier tube (PMT)

detectors measures light scatter in different directions: forward scatter (FSC), which correlates with cell size, and side scatter (SSC), which reflects cellular structural complexity (**Figure 14**). In addition, fluorescent dyes or fluorochrome-conjugated antibodies can be used to detect specific cell markers, enabling identification of immune cell subsets, evaluation of apoptosis, and measurement of cytokine production.

Cell surface staining was performed as a standard procedure in all studies. Briefly, MNCs were washed and resuspended in PBS at a concentration of 1–2 × 10^6 cells/mL. Cells were incubated with titrated volumes of fluorochrome-conjugated antibodies in PBS for 20–30 minutes at 4°C, washed with PBS, and subsequently stained with 7-aminoactinomycin D (7-AAD) for 10 minutes at room temperature. Data acquisition was performed on a CytoFLEX cytometer (Beckman Coulter), and analysis was conducted using FlowJo v10 software (BD Biosciences). Appropriate controls were included for each experiment, including single-color stained compensation beads and fluorochrome-minus-one (FMO) controls to ensure accurate gating. A complete list of monoclonal antibodies (mAbs) used is provided in **Table 2**.

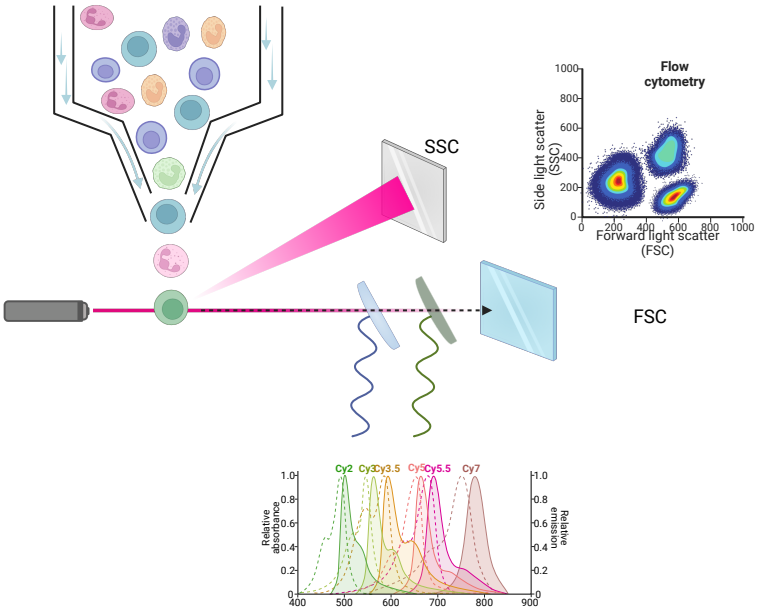


Figure 14. Basic principle of flowcytometry. *(created by BioRender)*.

Marker	Fluorochrome	Clone	Company	ECS/ICS (P= phenotyping) (F = Functional) (S = Sorting)	Cat No
Anti-Human CD3	Bv510	UCHT1	Biolegend	ECS (P, F, S)	300448
Anti-Human CD3	Bv510	UCHT1	BD Bioscience	ECS (P, F, S)	563109
Anti-Human γδ	PE	REA591	Miltenyi Biotec	ECS (P, F, S)	130-113-512
Anti-Human Vδ1	FITC	TS8.2	Thermofisher Scientific	ECS (P, F)	TCR2730
Anti-Human Vδ2	VioBlue	123R3	Miltenyi Biotec	ECS (P, F)	130-101-157
Anti-Human Vγ9	FITC	B3	Biolegend	ECS (P)	331306
Anti-Human CD4	AlexaFluor700	RPA-T4	BD Bioscience	ECS (P)	557922
Anti-Human CD8	APC Cy7	SK1	BD Bioscience	ECS (P)	557834
Anti-Human CD27	PE Vio770	M-T271	Miltenyi Biotec	ECS (P)	130-113-631
Anti-Human CD45RO	APC	UCHL1	BD Bioscience	ECS (P)	559865
Anti-Human CD192/CCR2	Bv650	1D9	BD Bioscience	ECS (P)	747849
Anti-Human CD195/CCR5	PE Cy7	2D7/CCR5	BD Bioscience	ECS (P)	557752
Anti-Human CD196/CCR6	PE-CF594	11A9	BD Bioscience	ECS (P)	564816
Anti-Human CD197/CCR7	PE-CF594	150503	BD Bioscience	ECS (P)	562381
Anti-Human CD199/CCR9	APC	112509	R&D Systems	ECS (P)	FAB179A
Anti-Human CD183/CXCR3	Bv785	G025H7	Biolegend	ECS (P)	353737
Anti-Human CX3CR1	Bv650	2A9-1	Biolegend	ECS (P)	341625
Anti-Human CD314/NKG2D	Bv650	1D11	BD Bioscience	ECS (P, F)	563408
Anti-Human CD226/DNAM-1	Bv785	11A8	Biolegend	ECS (P)	338321
Anti-Human CD159c/NKG2C	PE Vio770	REA205	Miltenyi Biotec	ECS (P)	130-120-589
Anti-Human CD159a/NKG2A	APC	REA110	Miltenyi Biotec	ECS (P)	130-113-563
Anti-Human CD336/NKP44	APC	P44-8	Biolegend	ECS (P)	325110
Anti-Human CD274/PDL1	APC	MIH1	BD Bioscience	ECS (P)	563741
Anti-Human CD279/PD1	PE	MIH4	BD Bioscience	ECS (P)	557946
Anti-Human CD366/TIM3	APC	F38-2E2	Miltenyi Biotec	ECS (P)	130-120-700
Anti-Human CD39	PE Vio770	REA739	Miltenyi Biotec	ECS (P)	130-110-790
Anti-Human CD69	Bv786	FN50	BD Bioscience	ECS (P, F)	563834
Anti-Human CD16	FITC	3G8	BD Bioscience	ECS (P)	555406
Anti-Human CD56	Bv421	NCAM16.2	BD Bioscience	ECS (P)	562751
Anti-Human CD86	AlexaFluor700	2331 (FUN-1)	BD Bioscience	ECS (P)	561124
Anti-Human HLA-DR	APC-H7	G46-6	BD Bioscience	ECS (P)	561358
Anti-Human CD103	PE	Ber-ACT8	BD Bioscience	ECS (P)	550260
Anti-Human CD137	VioBright FITC	4b4-1	Miltenyi Biotec	ECS (P)	130-119-971
Anti-Human CD154	APC	TRAP1	BD Bioscience	ECS (P)	560955
Anti-Human CD158b (KIR2DL2/L3 NKAT2)	PE Cy7	DX27	Biolegend	ECS (P)	312610
Anti-Human αβ	FITC	BW242/412	Miltenyi Biotec	ECS (S)	130-113-530
Anti-Human αβ	PE-Vio 770	BW242/412	Miltenyi Biotec	ECS (S)	130-113-532
Annexin V	APC	---	BD Bioscience	ECS (P)	550474
Anti-Human CD34	FITC	581 (RUO)	BD Bioscience	ECS (S)	555821
Anti-Human CD112 (Nectin-2)	PE	TX31	Biolegend	ECS (P)	337410
Anti-Human MICA/B	PE Cy7	6D4	Biolegend	ECS (S)	320918
Anti-human CD155 (PVR)	BV 421	SKII.4	Biolegend	ECS (P)	337632
Anti-Human CD274/PDL1	BV650	MIH1 (RUO)	BD Bioscience	ECS (P)	563740
Anti-Human TNF	APC	MAB11	BD Bioscience	ICS (F)	554514
Anti-Human IFNγ	PE Cy7	B27	BD Bioscience	ICS (F)	557643
Anti-Human IL-17A	PerCP Cy5.5	N49-653	BD Bioscience	ICS (F)	560799
Anti-Human CD107a	PE	H4A3 (RUO)	BD Bioscience	ICS (F)	555801
Anti-Human MIP-1β	AlexaFluor700	D21-1351	BD Bioscience	ICS (F)	561278
7-Amino-Actinomycin D (7AAD)	PerCP Cy5.5	---	BD Bioscience	Viability dye (P, S)	559925
Fixable Viability Stain 780	APC Cy7	---	BD Bioscience	Viability dye (F)	565388
LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit	Bv510	---	Thermofisher Scientific	Viability dye (F)	L34957

Table 2: List of antibodies used in this thesis.

3.3.4 Proliferation assay (CTV)

In **Study II**, we implemented a flow cytometry-based assay to assess γδ T cell proliferation response in Study II. Initially, γδ T cells were washed in PBS and centrifuged at 300 × g for 10 minutes. The resulting cell pellet was resuspended in 1 mL of PBS containing CellTrace™ Violet (Thermo Fisher Scientific) at a final concentration of 2 µM and incubated for 10 minutes at 37 °C in the dark. Labeled

cells were then diluted five-fold in complete medium, incubated for an additional 5 minutes, and washed with PBS prior to use in subsequent experiments.

Labeled γδ T cells were co-cultured with target cell lines at a 3:1 effector-to-target ratio in serial dilutions of bispecific T cell engager (BTE). Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 for 5 days. Proliferation was assessed using flow cytometry by quantifying the percentage of CellTrace Violet (CTV) low cells relative to unstimulated controls (**Figure 15**).

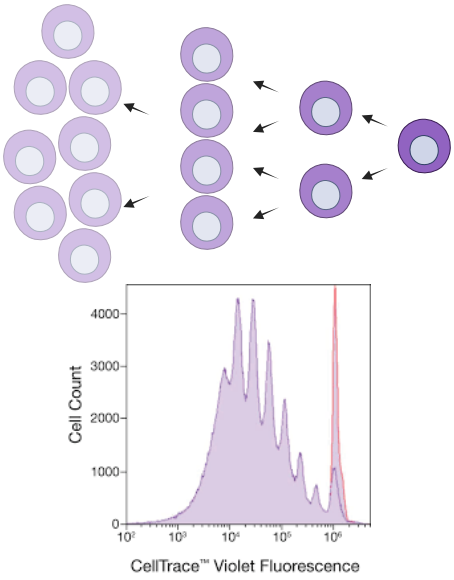


Figure 15. Evaluation of cell proliferation by cell trace violet (CTV). *(created by BioRender).*

3.3.5 TQ preparation

In **Study III**, TQ (Sigma) was dissolved in DMSO (WAK-Chemie Medical GmbH, Germany) to achieve a final stock concentration of 60.9 mM. The stock was kept in aliquots at – 80 °C until needed. The specified concentrations were diluted with the complete RPM-1 medium prior to each experiment.

3.3.6 Cell viability (WST-1)

In **Study III**, cell viability was assessed by WST-1 reagent. The method relies on the reduction of WST-1 tetrazolium salts into a colored formazan dye that is soluble in cell culture. Briefly, The MNCs and leukemia cells were exposed to different concentrations of TQ for 24 and 72 h in a 96-well plate incubated at 37°C. After

incubation, WST-1 reagent was added to the cells and incubated for an additional 3 h at 37°C. Finally, the plate was read at 450 nm using a SpectraMax i3x Multi-Mode Microplate Reader (American Laboratory Trading, USA). Measurements at a reference wavelength of 650 nm were conducted for the purpose of background correction. The percentage of cell viability is determined using this equation: (Absorbance of treated cells minus Absorbance of background) / (Absorbance of untreated cells minus Absorbance of background) x 100.

3.3.7 Cell purification and sorting

This thesis involved the implementation of two main cell purification techniques. In **Study I**, a higher purity of γδ T cells was required for the immunosequencing of TRG; thus, a Fluorescence Activated Cell Sorting (FACS) technique was employed (Sony MA900, Sony Biotechnology Inc.). In **Studies II and III** Magnetic Activated cell sorting (MACS) was sufficient to provide a high yield of purified γδ T cells after expansion if needed. For this purpose, the negative selection was performed using (human TCR γ/δ+ T Cell Isolation Kit, Miltenyi Biotec). For magnetic beads sorting, a selection buffer consisting of PBS containing 0.5% bovine serum albumin (BSA) and 2 mM EDTA was used.

3.3.8 Next generation sequencing (NGS)

Next-generation sequencing (NGS) has revolutionized genomics by enabling massively parallel sequencing at high speed, scale, and reduced cost compared with traditional Sanger sequencing. Among the available platforms, Illumina technology dominates the field and accounts for the majority of sequencing data worldwide. Illumina sequencing relies on sequencing-by-synthesis (SBS), in which DNA fragments are clonally amplified on a flow cell and fluorescently labeled nucleotides are incorporated during strand synthesis. Each incorporated base is identified by its emission spectrum, enabling highly accurate base calling.

In **Study I**, the NGS workflow comprised four key steps: (1) nucleic acid extraction, (2) library preparation, (3) sequencing, and (4) bioinformatic analysis. Library preparation and sequencing steps were performed by iRepertoire (Huntsville, AL, USA).

Step 1. Nucleic acid extraction

NGS can be applied to either DNA or RNA. In our study, RNA was used. Cells were sorted using the Sony MA900 cell sorter, achieving a median efficiency of 92%. RNA was immediately extracted with the RNeasy Micro Kit (Qiagen, Hilden,

Germany), quantified using a NanoDrop2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), and stored at –80 °C until shipment.

Step 2. Library preparation

Reverse transcription PCR was performed by iRepertoire using multiplexed V and J primers to amplify T-cell receptor (TCR) γ-chain CDR3 rearrangements. The resulting amplicons were processed into Illumina-compatible sequencing libraries.

Step 3. Sequencing

Paired-end sequencing was carried out by iRepertoire on an Illumina NGS platform, where millions of library fragments are read in parallel.

Step 4. Bioinformatic analysis

The large datasets generated by NGS require specialized bioinformatic workflows. Standard steps include quality filtering, adapter trimming, alignment to a reference genome, and repertoire characterization. Sequencing data were initially processed using the iRweb platform (iRepertoire). Raw data were subsequently downloaded and analyzed with the Immunarch package (https://github.com/immunomind/immunarch) to assess TRG CDR3 diversity, clonal space homeostasis, spectratype distribution, and V/J-segment usage. Only productive CDR3 rearrangements were included.

To minimize bias, datasets were normalized by down-sampling to the size of the smallest sample. Repertoire similarities were calculated using the Morisita–Horn and Jaccard indices. Recipient clonotypes that showed a fold increase >1.5 compared with the donor were extracted, aligned with Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/), and used to generate CDR3 amino acid motif logos via the WebLogo server (http://weblogo.threeplusone.com/create.cgi).

3.3.9 Functional assay

In Study I, MNCs from recipients were thawed overnight in complete medium. The following day, cells were stimulated for 6 h with PMA and ionomycin (Sigma-Aldrich) or with PepTivator CMV pp65 (premium grade, Miltenyi Biotec) in the presence of Brefeldin A (Sigma-Aldrich) and GolgiStop (monensin; BD Biosciences). After stimulation, cells underwent extracellular and intracellular staining and were analyzed using a CytoFLEX flow cytometer (Beckman Coulter).

3.3.10 Killing assay

In **Studies II and III,** we assessed the capacity of γδ T cells to lyse the leukemia cell lines. Briefly, in **Study II**, expanded γδ T cells (effector, E) were co-cultured with CTV-labelled leukemia cells (target, T) at an E:T ratio of (3:1) in the presence of different concentration of CD34/CD3 BTE and RSV/CD3 BTE serve as a control and incubated at 37 °C for 4h, 24h, 48h, 7h, and 120h. After incubation, cell viability was assessed using 7AAD and % of specific killing was calculated using the formula.

$$killing\ (\%) = \left(1 - \frac{\%\ of\ viable\ target\ cells\ in\ coculture\ with\ BTE}{\%\ of\ viable\ target\ cells\ \ in\ coculture\ no\ BTE}\right) * \ 100$$

In **Study III**, leukemia cells were pretreated with TQ 5 μM TQ, 0.007 % DMSO as vehicle control (VC), or left untreated in medium as control at 37 °C for 24 h. After incubation, all treated cells (target, T) were pre-labelled with CTV before co-culturing with expanded γδ T cells (effector, E) at an E:T ratio of (3:1) in the presence/absence of CD34/CD3 BTE (100 μg/mL) for an additional 24 h. Cell apoptosis was evaluated using dual staining with Annexin V and 7AAD. % of killing was determined separately for each condition (non-treated, TQ-treated, and DMSO-treated) by using the following formula:

$$killing\ (\%) = \left(1 - \frac{\%\ of\ alive\ target\ cells\ in\ culture}{\%\ of\ alive\ target\ cells\ alone}\right) * \ 100$$

The specific killing mediated by BTE was calculated individually for each condition as outlined below:

$$killing\ (\%) = \left(1 - \frac{\%\ of\ alive\ target\ cells\ in\ culture\ with\ BTE}{\%\ of\ alive\ target\ cells\ \ in\ culture\ no\ BTE}\right) * \ 100$$

3.3.11 FluoroSpot assay

In **Study II**, the FluoroSpot assay was used to analyze the secretion of IFN-γ, TNF-α, and granzyme B at a single level after co-culturing the γδ T cells (effector cells) with leukemia cells (target cells) for 24 h. During the incubation, released cytokines are captured directly by plate-bound antibodies. After incubation, cells are washed and then plate-bound analytes are visualized as a spot after adding the fluorescence-conjugated reagents.

3.3.12 Luminex multiplex assay

In **Study II**, we analyzed various cytokines and chemokines in cell culture supernatants from cytotoxicity experiments using a Luminex multiplex assay. This assay is a powerful method that allows the detection of multiple soluble factors in a single sample at once. In this assay, color-coded beads are conjugated with capture antibodies. When samples are added, the analytes bind to the antibody-coated beads. After that, the biotin-labelled detection antibodies are added. Finally, the addition of a fluorochrome-conjugated substrate generates a fluorescent signal. The plate is run on a multiplex reader machine to measure the fluorescent intensity.

3.3.13 ELISA

An enzyme-linked immunosorbent assay (ELISA) is a common technique for quantifying soluble factors based on light intensity measured with a spectrophotometer. The plate is coated with a capture antibody that targets the antigen. In **Study II**, supernatants from the cytotoxicity experiments were added to the plate, allowing IFN-γ to bind to the immobilized capture antibody. A biotin-labeled detection antibody was then applied, followed by enzyme-conjugated streptavidin, which binds to the biotin on the detection antibody. Finally, a substrate was added, which is cleaved by the enzyme, generating a color change proportional to the amount of IFN-γ in the sample.

3.4 Statistical methods

All statistical analyses were performed using GraphPad Prism (version 10 or later), with significance at $p \leq 0.05$. The dose-response data were assessed using a nonlinear regression fit, and LC50/IC50 were determined by interpolating 50% cytotoxicity from the fitted dose–response curve. We used the Wilcoxon matched-pairs signed-rank test to compare two paired samples. Comparisons across multiple groups were analyzed using the Friedman test and Dunn's multiple comparisons test. For unpaired samples involving two or more groups (e.g., GVHD or CMV status/reactivation), analyses were conducted using the Mann–Whitney U-test or Kruskal–Wallis test followed by Dunn's post-test. The assessment of differences in cytokine production (FluoroSpot, Luminex, ELISA) and proliferation assays was conducted using the Friedman test and Dunn's correction in relation to the appropriate BTE condition. For correlation analysis, we used Spearman's test.

4 Results and Discussion

For decades, allogeneic haematopoietic cell transplantation (aHCT) has been considered a curative approach for various blood disorders, particularly hematological malignancies (185). Despite the advancements in the field, there is a remaining knowledge gap in understanding the long-term homeostatic steady state of γδ T cells post-aHCT (101). Moreover, significant challenges persist in reducing the relapse rate and transplant-related mortality following aHCT and the adverse effects of the pre-transplant conditioning regimen can result in long-term side complication after aHCT. Thus, there is an urgent need for new treatment in addition to preserving the quality of the patient's life as well. (186-189).

This thesis aimed to provide an in-depth analysis of γδ T cell reconstitute ion in long-term survivors after aHCT and its association with previous clinical outcomes, an important aspect lacking in earlier studies (**Study I**). Furthermore, it introduces a novel CD34/CD3 BTE antibody to enhance the antileukemia effects of γδ T cells (**Study II**). It also demonstrates a promising anticancer compound, TQ, which acts as a sensitizer to the leukemic cells, making these cells susceptible to γδ T cell-based CD34/CD3 BTE therapy (**Study III**).

4.1 γδ T cell reconstitution in a long-term post aHCT (Study I)

4.1.1 Phenotype and TCR repertoire

Using multiparameter immunophenotyping and immune sequencing of TCR γ-chain (TRG), we compared a cohort of long-term survivors (recipients) who underwent aHCT to their corresponding sibling donors (n = 20) at a median sampling recipients/donors of 18/18.5 years post aHCT. Our phenotyping analysis revealed no significant differences in the frequencies of total γδ T cells and their subsets or the Vδ1/Vδ2 ratio between donors and recipients (**Figure 16**). Furthermore, unlike αβ T cells, we observed a comparable memory phenotype within γδ T cells and their subsets across both groups. This similarity extended to the expression of CD4+ and CD8+ markers, chemokine and NK cell receptors, as well as exhaustion and activation markers in the total γδ T cell population, as detailed in **Study I**.

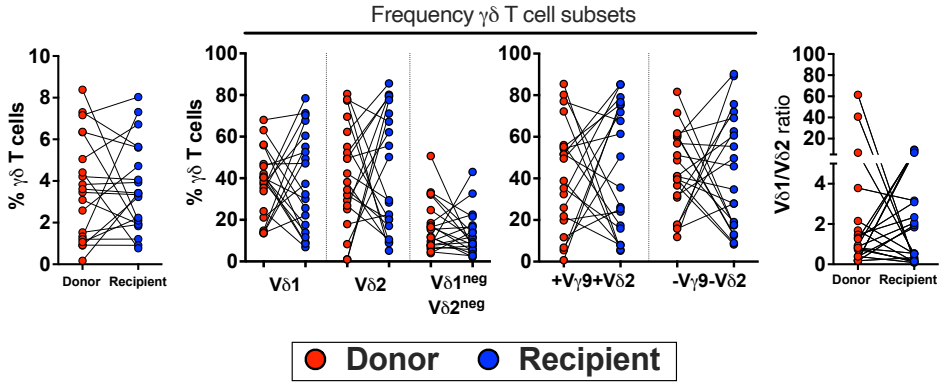


***Figure 16**. Phenotypic analysis of γδ T cells post-long-term aHCT, showing γδ T cell frequency and their subsets, and Vδ1/Vδ2 ratio in donors versus recipients (n = 20). Parts from Study I.*

TCR repertoire diversity is another important determinant for evaluating immune reconstitution (190): Talvensaari et al. found that the TCR repertoire was abnormal, with a lower T-cell receptor excision circle (TREC) value in patients who underwent cord blood or bone marrow transplants within the initial year. After two years, the TCR diversity had increased, alongside the TREC value, only in cord blood than in bone marrow transplant (191). The reconstitution of γδ T cell repertoire occurs rapidly and reestablished to be stable at 6 months after aHCT (108). However, the γδ T cell reconstitution and its repertoire can be influenced by viral infection/activation or another transplant-related factors (101). It has been shown that TRG γδ repertoire composition after aHCT was perturbed by CMV reactivation (108) or likely due to immunosuppressive therapies (192). These findings have been somewhat investigated in γδ T cell repertoires within a short-term after aHCT, whereas limited information/nothing is available on long-term γδ T cell repertoires.

Here, as the phenotypic profile indicated similarity between donors and recipients, we further quantified the overall TCR diversity according to the number of clonotypes, the Chao1 estimator, the Gini-Simpson index, and the inverse Simpson index. The results indicated that TRG diversity was comparable between donors and recipients (**Figure 17**), with no significant differences observed in repertoire space occupancy, CDR3 length distribution, or V–J gene segment usage, as presented in **Study I**. These findings indicate that the TRG repertoire composition is restored and remains stable long-term after transplantation.

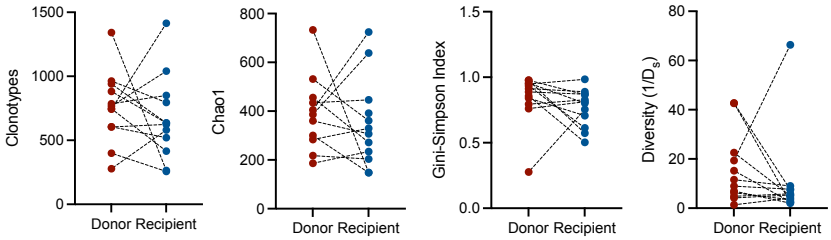


***Figure 17**. TRG repertoire diversity of γδ T cells between the donors and long-term aHCT recipients (n = 12 paired samples). Parts from **Study I**.*

After aHCT, a crucial question is the origin of the γδ TCR repertoire, whether it is generated from the donor graft or *de novo* generation in the recipient thymus (108, 110). the current consensus is that early γδ reconstitution in the recipient following aHCT is thought to be a chimera of cells, mainly from the donors and a minority from the recipients (107). For example, Hirokawa et al. demonstrated peripheral expansion of donor-derived γδ clonotypes. Nevertheless, certain γδ clones were identified in the recipient repertoire post aHCT that were not present before transplant or in the graft, suggesting a thymic and/or extrathymic differentiation (110).

In our analysis, recipients exhibited a high prevalence of private clonotypes compared to their matched donors, who presented more public clonotypes, indicating *de novo* generation of naïve γδ T cells within the host thymus or extrathymic differentiation (**Figure 18A**). Consistent with this, we observed a low number of shared clonotypes between donor-recipient pairs and minimal overlap across all samples, confirming the predominance of private clones in recipients (**Figures 18B and 18C**). These results align with a recent study where the most recipient γδ TCR repertoire displayed less shared clonotypes and minimal overlap of donor-recipient pairs identified as a private repertoire. Thereby, it was suggested that efficient de novo generation of γδ T cells from the thymus in the recipient γδ TCR repertoire after aHCT (108).

However, we observed three recipients (R4, R5, and R13) showing higher shared clonotypes with their matched donors and clear clustering (**Figure 18C**). Taken together, our phenotyping and NGS data suggest that long-term γδ T cell reconstitution reaches a homeostatic steady state with a normalized TRG repertoire following aHCT. A larger cohort of patients is needed to verify these findings.

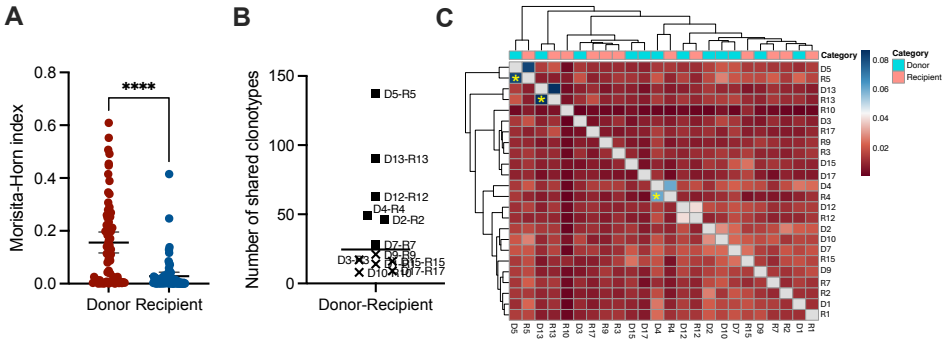


***Figure 18**. Analysis of public and private clonotypes between donor-recipient samples post-aHCT. (A) clonotype similarity by Morisita-Horn index. (B) Number of shared clonotype across donor-recipient pairs. (C) Hierarchical clustering of samples using Jaccard indices. Adapted from **Study I**.*

4.2 Associations with clinical outcomes (Study I)

4.2.1 Moderate to Severe Chronic GVHD (M/S cGVHD)

So far, the role of different subsets of γδ T cells in GVHD post-transplantation (193) or within the graft remains an outstanding question (126, 194). Several studies have described the early reconstitution of γδ T cells post-aHCT, correlating with improved clinical outcomes and a reduced risk of GVHD, likely due to their MHC-independent recognition mechanisms (13, 14, 102). Moreover, the co-infusion of γδ T cells and NK cells within CD34+ HSC in TCRαβ+/CD19+ depleted graft, has been shown to reconstitute early with a protective contribution against opportunistic infections, with no sign or less incidence of GVHD after aHCT (107, 195, 196). Nevertheless, a few studies have also indicated a potential association between some γδ T cell subsets and GVHD development, post-aHCT (127, 192) or within the graft (115, 126). These discrepancies between studies could originate from γδ T cells heterogeneity of, with some γδ T cell subsets having some phenotypes and functions (e.g. CD8+ γδ T cells in the graft) that may have alloreactivity roles (126) or act indirectly to aggravate aGVHD (e.g. CXCR4-expressing γδ T cells) (127).

This study focused on the potential association of clonotype dynamics and phenotypic profiles with clinical outcomes. To identify potentially clinically relevant clonotypes, we first tracked the top 10 donor-derived clonotypes (TRG clones) persistence and expansion in recipients post-aHCT. These top 10 clonotypes accounted for a median of 8.6 % of all recipients' TRG repertoire. Strikingly, three recipients (R4, R5, and R13) exhibited marked expansion of the top 10 clonotypes, reaching 24.17%, 59.25%, and 88.85% of their total TRG repertoires,

respectively. Other recipients, such as R10, showed a decline in the proportion of top 10 clonotypes to undetectable levels (**Figure 19A**).

These donor-derived clonotypes expanded with a notable increase of more than 1.5-fold in recipients (R4, R5, and R13), all of whom developed M/S cGVHD post-aHCT (**Figure 19B**). These findings suggest a possible association between expanded clonotypes in those recipients' repertoire and cGVHD development. This pattern of clonal expansion mirrors that of conventional T cells, where an increased number of expanded clones was described to be associated with aGVHD after aHCT (197, 198).

Moreover, CDR3 spectra typing revealed a distinct sequence pattern, indicating private clonal expansion restricted to the donor-recipient pair (**Figure 19C**). Importantly, R4 and R5 had ongoing M/S cGVHD during sampling. This finding aligns with recent evidence showing a possible association between aGVHD severity and skewed γδ T cell clonality after UCB transplantation (192). However, this expansion may also be conceivable due to the cell reconstitution process regardless of cGVHD status.

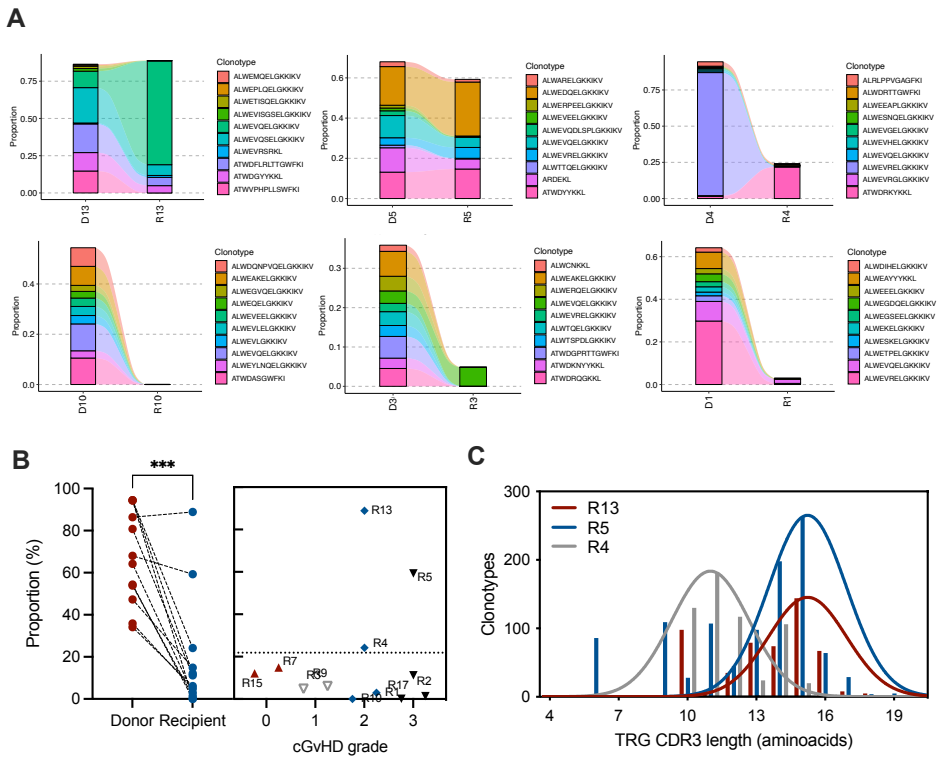


Figure 19. *(**A**) Tracking of the top 10 abundant clonotypes in the donor-recipient pairs based on cGVHD grade. Upper panels show recipients (R4, R5, and R13) who developed*

*M/S cGVHD have increased persistence of donor-derived clonotypes, while lower panels show recipients who developed mild cGVHD. (**B**) Left panel: Proportion of the top 10 clonotypes in donors and matched recipients. Right panel: individual recipient data showing the proportion of top 10 clonotypes according to cGVHD grade. (C) Distribution of unique CDR3 sequence lengths in recipients (R4, R5, and R13). Adapted from **Study I**.*

Next, we extended our analysis to include phenotypic data and included an additional cohort of patients with ongoing M/S cGVHD (n = 5) as positive control patients for further investigation. We observed an inverted Vδ1/Vδ2 ratio and increased expression of HLA-DR in Vδ1 T cells within the recipients (n =4; R2, R4, R5 and R12) with ongoing M/S cGVHD compared to recipients with a previous history of no/mild cGVHD, who preserved a normal Vδ1/Vδ2 ratio and regular levels of HLA-DR expression. This altered pattern was similarly observed in the positive control patients (**Figure 20**). HLA-DR expression on Vδ1 T cells is an activation marker, which is upregulated under infection conditions or various stimuli (e.g. CMV stimulation) (81, 199, 200). A study revealed that a high co-expression of CD38 and HLA-DR on Vδ1 T cells in acute HIV patients compared to healthy controls, reflecting their activation state against HIV. The frequency of CD38+ HLA-DR+ Vδ1 T cells decreased following anti-viral treatment, attributed to a reduced viral replication (199). Additionally, using cell-free CMV, the HLA-DR has been upregulated in Vδ1 T cells in response to CMV stimulation (200). However, we did not find any association of a high expression of HLA-DR by Vδ1 T cells in recipients who experienced CMV reactivation, as previously described in infants with primary CMV infection (81).

Overall, these findings, drawn from NGS and immunophenotyping in a small cohort, suggest that persistent cGVHD after long-term aHCT may significantly affect thymic-dependent reconstitution and alter the phenotypic composition. This effect is likely mediated by the overrepresentation of donor-derived peripheral clones and the inverted Vδ1/Vδ2 ratio. Although the mechanism is uncertain, a possible association of HLA-DR+ Vδ1 T cells with cGVHD development could be a therapeutic target. Further investigations with larger cohorts are needed to verify these findings.

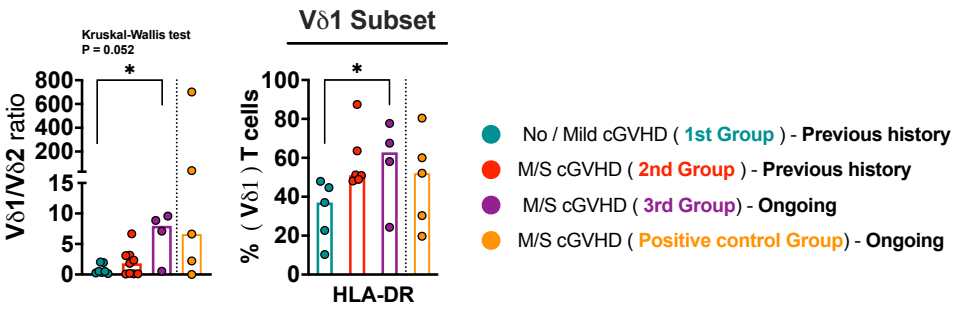


Figure 20. *The association between γδ T cell phenotypes and cGVHD in recipients, as indicated by the ratio of Vδ1/Vδ2 and HLA-DR expression levels in Vδ1 T cells. Data were analyzed based on the cGVHD status at the time of sampling. Statistical comparisons were performed to compare ongoing M/S cGVHD (3rd group: purple) and previous history of M/S cGVHD (2nd group: red) versus previous history of no/mild cGVHD (1st group: green). An additional cohort of patients with ongoing M/S cGVHD (orange) served as a positive control. Adapted from* ***Study I****.*

4.2.2 CMV

The pivotal role of Vδ2-γδ T cells against CMV infection/reactivation has been of interest in an immunocompromised setting and after aHCT (18, 135). In immunocompetent hosts, CMV can persist for life without any symptoms, due to the well-coordinated interaction between innate and adaptive immunity (128). However, it may result in morbidity and mortality in transplant patients or immunocompromised individuals who are vulnerable to CMV viral complications (128, 200). The enrichment of Vδ1 T cells is significantly associated with CMV infection/reactivation (81, 108, 131). Post-aHCT. Liu et al found that the proportion of Vδ1 T cells expanded significantly in patients with occasional CMV reactivation after haploHCT upon stimulation with an anti-TCR Vδ1 antibody, whereas this expansion capacity was absent in CMV-refractory patients (200). Furthermore, longitudinal monitoring of CMV DNAemia in plasma samples revealed that patients with occasional CMV reactivation exhibited elevated cytokine levels, particularly CXCL10 and IFN-β, compared to CMV-refractory patients at 60- and 90-days post-reactivation. Conversely, the levels of these cytokines were low/suppressed and comparable to those without CMV reactivation. These results underscore the anti-CMV activity of Vδ1 T cells after transplantation, suggesting IFN-β and CXCL10 play an important role during CMV reactivation. Similarly, treatment with IFN-β and CXCL10 may restore the functional responses in CMV-refractory patients (200).

In our analysis, we investigated the functional responses of recipient T cells to in vitro stimulation with CMVpp65 peptide, according to degranulation CD107a,

TNF-α, and IFN-γ production. Recipients' γδ T cells were classified as responders (n = 12) and non-responders (n = 3) according to their T cell response to CMV stimulation. We observed that the responder group displayed an increasing trend in the frequency of γδ T cells and the Vδ1/Vδ2 ratio versus the non-responder group. Interestingly, a positive correlation was found between the Vδ1/Vδ2 ratio and CD107a, TNF-α, and IFN-γ expressing T cells, indicating that CMVpp65-specific T-cell responses are associated with Vδ1 T cells (**Figure 21**). We further analyzed the association between the long-term γδ T cell phenotype and CMV history, including serostatus and reactivation. Consistent with prior studies based on CMV serostatus, our results found that CMVsero+ recipients had a higher frequency of Vδ1 subset and significantly elevated expression of NKG2C and CD158 within the total γδ T cells (132, 194), further supporting the protective association of the Vδ1 subset with CMV.

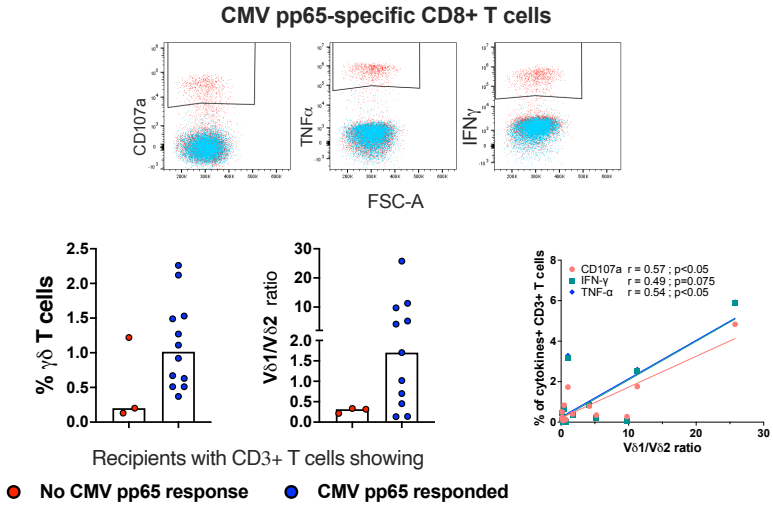


***Figure 21**. Frequencies of γδ T cells and the Vδ1/Vδ2 ratio in transplant recipients classified by their CD3+ T cell reactivity (blue dots) or lack of reactivity (red dots) to CMVpp65 stimulation. Parts from **Study I**.*

In the light of the adaptive immune response, several studies indicate that the differentiated phenotype of CMV-specific subsets, such as Vδ2- and Vγ9-Vδ2+ subsets, is associated with CMV infection/reactivation control (22, 135). A recent study demonstrated that Vδ1 γδ T cells experience effector differentiation, clonal expansion, and remain functional during persistent CMV viremia in common variable immunodeficiency. These observations suggest a persistent, adaptive-like response to CMV by Vδ1 γδ T cells, even in the absence of B cell immunity (18). Shortly after aHCT, the proportion of effector memory cells within the Vδ2- subset was significantly higher in patients experiencing CMV reactivation (CMV+)

compared to CMV- patients. Moreover, the repertoire of Vδ1 T cells in CMV+ patients was clonally restricted compared to CMV- patients. Thus, results suggest that CMV can drive the Vδ1 γδ T cells' expansion and reshape their memory phenotype differentiation (135).

Our memory phenotype analysis revealed a higher proportion of effector memory cells within Vδ1 and Vδ1-Vδ2- subsets among recipients experiencing CMV reactivation (median 55 days) post-aHCT compared to the no CMV reactivation group (**Figure 22**). These findings indicate that Vδ2- subsets, particularly the Vδ1 subset, exhibit adaptive immune response and play a critical role in long-term CMV control after aHCT. Moreover, higher levels of NKG2D expression were observed in the Vδ1 subset within the CMV reactivation group, suggesting an innate-like response against CMV post aHCT (**Figure 22**). These findings align with study demonstrating the innate-like immune response of Vδ1 T cells to CMV. It has found that both cell-free CMV virions and CMV-infected cells significantly upregulated NKG2D expression on Vδ1 T cells. Blocking TCRγδ and NKG2D signaling significantly diminished reduced their cytotoxicity against CMV-infected cells, indicating that these receptors pathways are essential for their antiviral role. In addition, Vδ1 T cells responded directly to cell-free CMV virions, with upregulation of TLR2.

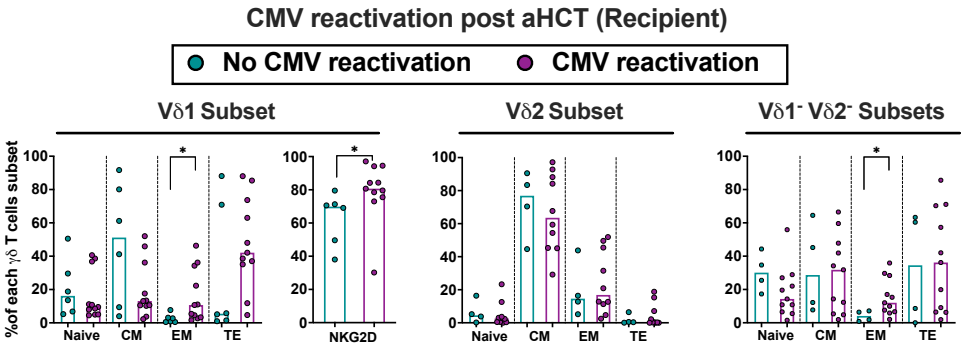


***Figure 22**. Lasting imprint between memory phenotype in γδ T cell subsets and NKG2D expression by Vδ1 γδ T cells with CMV reactivation post-aHCT. Parts from **Study I**.*

4.3 Synergistic strategy to boost γδ T cell antileukemia efficacy without aggravating GVHD (Study II & Study III)

High relapse rates remain a common adverse event following conditioning chemotherapy and aHCT (186, 187). Leukemic stem cells (LSCs) strongly contribute to relapsed disease because when in a quiescent state, they are able to evade chemotherapy by remaining unaffected (201, 202). Moreover, these cells

exhibit phenotypic and epigenetic plasticity during therapy stress, further increasing leukemia's multidrug resistance (201). LSCs commonly reside within the CD34+CD38- cell fraction, and highly express CD34 (202). Notably, elevated CD34 expression has been observed on AML blasts in patients exhibiting a high relapse rate and poor prognosis (203, 204). Since CD34 is also expressed on HSC, leukemia patients must achieve remission before proceeding to aHCT. Therefore, CD34 represents a suitable target antigen to eradicate AML blasts and CD34+ HSCc before aHCT. This treatment might be eligible for patients who cannot undergo high-dose pre-HCT conditioning chemotherapy.

To exploit this, we investigated two approaches including immune cell engager therapy (BTE) alone to target CD34 and; a combinatorial approach of BTE together with a novel epigenetic drug to sensitize leukemia cell lines to γδ T-cell-mediated cytotoxicity. **In Study II**, we demonstrated that a CD34/CD3 bispecific T-cell engager (BTE) selectively redirects expanded γδ T cells (effector cells) to target CD34+ cell lines and primary CD34+ AML blasts (target cells) in vitro. **In Study III**, we showed the pre-treatment of leukemia cell lines using low dose of thymoquinone (TQ) to induce immune sensitization by demonstrating enhanced susceptibility to rapid killing by expanded γδ T cells and supporting the CD34/CD3 BTE approach in vitro.

4.3.1 Expanded γδ T cells are appropriate as effector killers (Study II & Study III)

V γ9Vδ2 T cells are ideal effector cells for hematological malignancies therapy due to their abundance in peripheral blood (19). The MHC-independence of Vγ9Vδ2 T cells further enhances their potential for allogeneic immunotherapy. This characteristic can reduce the risk of alloreactivity and the need for immunosuppressive agents. These cells are highly responsive to PAgs expressed on cancer cells, allowing for a rapid anticancer effect. Beside their TCR engagement, Vγ9Vδ2 T cells can express a variety of NK receptors, which further augment their cytotoxicity (19). After interacting with their respective ligands via TCR and NK receptors, activated Vγ9Vδ2 T cells release IFN-γ and TNF-α, together with antitumor effector molecules such as perforin and granzymes. Unlike Vδ1 T cells, Vγ9Vδ2 T are strongly expressed CCR5 inflammatory homing receptor which can enhance their migration to leukemia microenvironment. These cells with APC-like function can also prime and activate the anticancer activity of other immune cells such as NK cells and αβ T cells. These anticancer mechanisms of Vγ9Vδ2 T cells place them as promising candidates cell in cancer immunotherapy (19).

Various approaches have been employed to polarize Vδ2 T cells into effector-like profile. For example, IL-2 acts as a T cell growth factor, essential for the survival and proliferation of Vγ9Vδ2 T cells (205). It is the frequently used cytokine in combination with PAgs compounds such as amino bisphosphonates (e.g. Zoledronate: ZOL) or synthetic phosphoantigen (e.g. BrHPP) for expanding Vγ9Vδ2 T cells in vitro. This common combination was widely used for generating a sufficient number of Vγ9Vδ2 T cells for adoptive cell immunotherapy with large amounts of IFN-γ and TNF-α (205).

Therefore, we focused our studies particularly on the predominant blood circulating Vδ2 subset as effector cells for hematological malignancies (15, 206). Clinically, expanded γδ T cell (Vγ9Vδ2)-based therapies have garnered increasing interest in hematological malignancies, with a demonstrated safety profile and promising outcomes (15, 16). **In Study II and III**, we expanded γδ T cells from healthy PBMC with IL-2 and zoledronate for 12 days, achieving a median purity above 90%. Moreover, we applied further purification using a negative selection if needed. Phenotypic characterization revealed a significant upregulation of multiple activation markers, NK receptors, and cytotoxic receptors in expanded γδ T cells, as reported in **Study II**. Collectively, these data indicate that expanded γδ T cells, which exhibit upregulation of cytotoxic and NK receptors, are highly suitable for allogeneic immunotherapy.

4.3.2 CD34: an optimal target for pre-transplant in hematological malignancies conditiong? (Study II)

Despite the promising outcomes of allogeneic expanded Vγ9Vδ2 T in patients with bone marrow blasts, the disease progression was observed by day 100 (16). Therefore, after allogeneic γδ T cell therapies, these cells require long-term persistence to enhance their functional activity (15, 16). BTEs are an attractive immunotherapy approach that aims to improve tumor targeting and boost T cell efficacy, including γδ T cells. The administration of BTEs relies on dose- and time-dependent manner, which also offer a tolerated method to control overactivation and mitigate cytokine release syndrome (207). **Study II** serves as a proof-of-concept study, with the objective to investigate the potential use of combining expanded γδ T cells with CD34/CD3 BTE as an adjuvant therapy before aHCT. The hypothesis is expanded γδ T cells have a safe and potent anticancer activity via innate and adaptive mechanisms against hematological malignancy cells without potentially aggravating GVHD, and their combination with CD34/CD3 BTE could enhance their anticancer functionality. This approach can also be a treatment option for patients who are ineligible for high-dose chemotherapeutics (186, 187, 207). We first confirmed the specific binding of CD34/CD3 BTE to CD3 expressed

on expanded γδ T cells and to CD34+ cell lines (KG1a, SUPB15, and Kasumi) but not to CD34- cell line NALM6.

4.3.3 CD34/CD3 BTE-induced γδ T cells cytotoxicity in CD34+ leukemia (Study II)

To further strengthen the concept, we assessed specific cytotoxicity induced by γδ T cells in combination with CD34/CD3 BTE. CD34+ cell lines (targets) co-cultured with expanded γδ T cells (effectors) at an E: T ratio of 3:1, using increasing concentrations of CD34/CD3 BTE or a control RSV/CD3 BTE at different time points in vitro. In agreement with previous findings using conventional T cells (208), our results demonstrate that CD34/CD3 efficiently elicits γδ T cell-mediated cytotoxicity against CD34+ cell lines (KG1a, SUPB15, and Kasumi) in a dose-dependent manner at 24 h and 48 h, indicating the specific targeting of CD34/CD3 BTE (**Figure 23**).

Although the specific killing rate was minimal at 4 h of co-culture, no significant differences were found between the 24 h and 72 h time points, suggesting that most of the specific cytotoxicity by γδ T cells occurred during the first 24h, with early killing observed after 4 h (**Figure 23**). Furthermore, limited cytotoxicity was noted in CD34- subpopulations of Kasumi cells and the NALM6 (CD34- cells) when treated with CD34/CD3 BTE (1000 ng/mL). This effect may result from their inherent sensitivity to activated γδ T cells post-expansion.

In contrast to αβ T cells, γδ T cells combine the immunological characteristics of both innate and adaptive immunity. The innate-immune characteristics of γδ T cells can contribute to achieving more killing in the tumor cells (209). Through a direct comparison, we demonstrated that γδ T cells in combination with CD34/CD3 BTE exhibited superior antileukemic effect compared to pre-stimulated αβ T cells or resting αβ T cells alone, suggesting γδ T cells as promising T cell subset for cell-based therapy in combination with CD34/CD3 BTE before proceeding to aHCT in future.

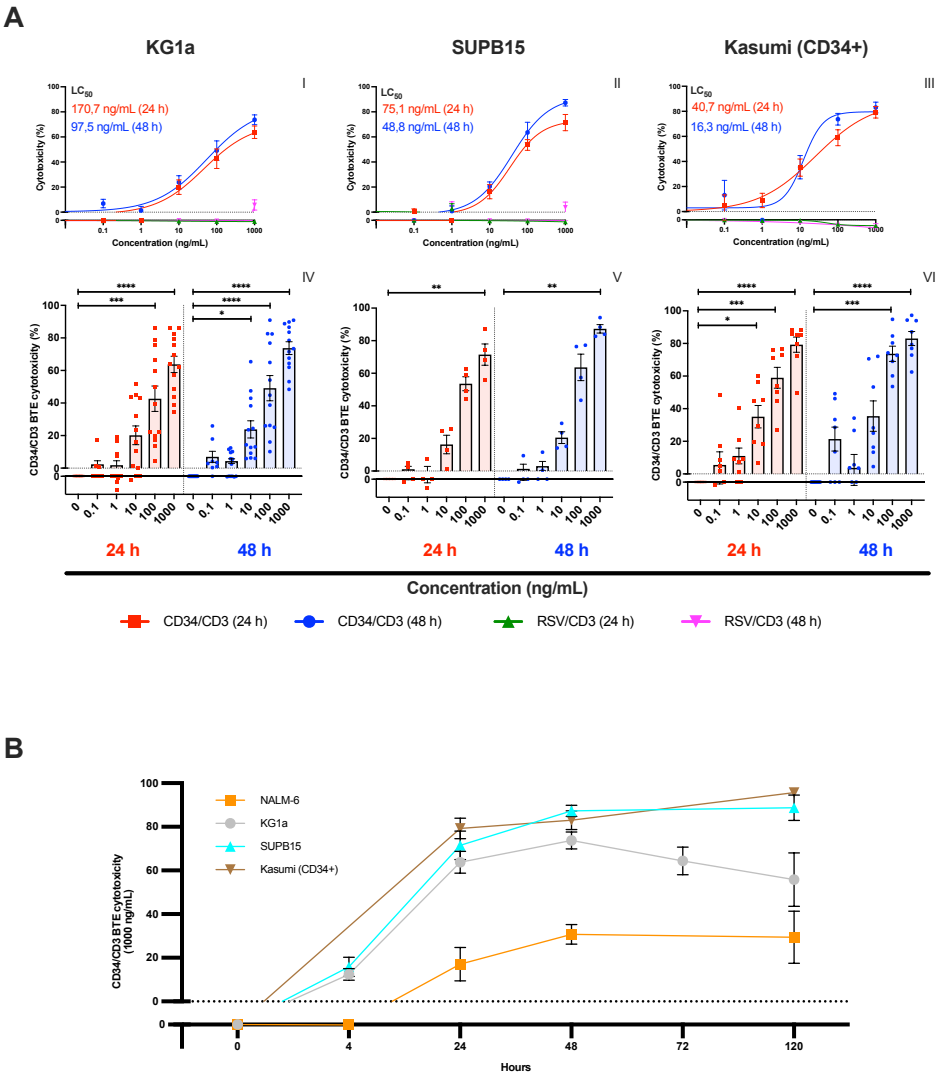


Figure 23. *(**A**) CD34/CD3 BTE-induced γδ T cytotoxicity in (**A–I**) KG1a (n = 13), (**A–II**) SUPB15 (n = 4), (**A–III**) CD34⁺ population of Kasumi (n = 8) in co-culture assay at 24 h and 72 h. (**A–IV, A–V, and A–VI**) Comparison of cytotoxicity relative to the no BTE condition at 24 h and 72 h of co-culture for each CD34+ cell line. (**B**) The cytotoxicity analysis of the CD34/CD3 BTE at 1000 ng /mL in CD34+ cell line (KG1a, SUPB15, and Kasumi) and CD34- cell line (NALM6) across multiple time points following co-culture with γδ T cells. Adapted from **Study II**.*

4.3.4 CD34/CD3 BTE-induced cytokine release and γδ T cell proliferation

We next aimed to analyze the cytokine production by γδ T cells in response to BTE. Using FluoroSpot for a single-cell level definition, high concentrations of CD34/CD3 BTE (100 and 1000 ng/mL) could induce high levels of cytokines (IFN-γ, TNF-α, and granzyme B) by γδ T cells when co-cultured with KG1a for 24 h. In contrast, we found a minimal cytokine release in NALM6 (CD34- cells) or when using RSV/CD3 BTE (**Figure 24**).

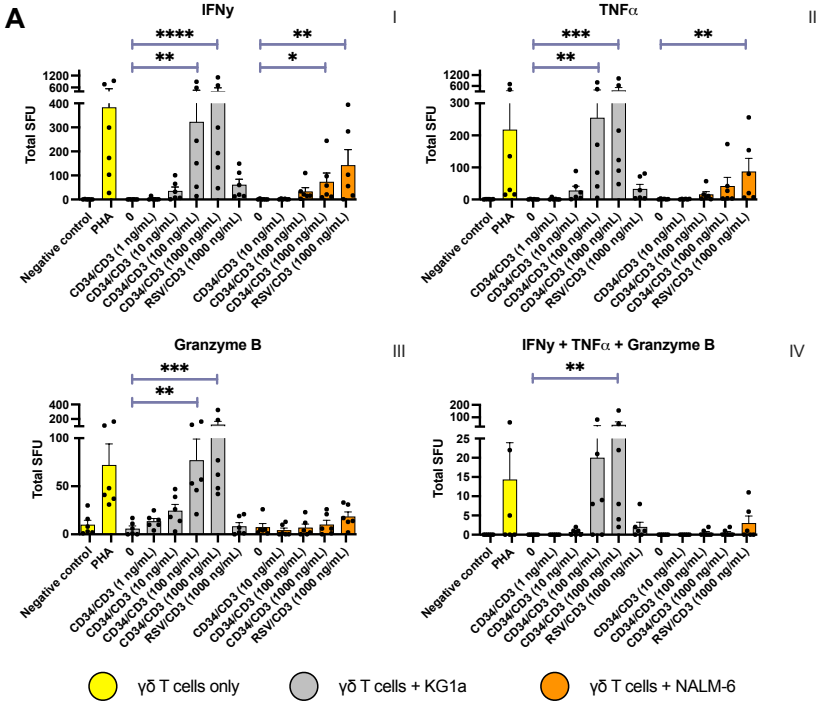


Figure 24. *(**A**) Cytokine analysis in FluoroSpot plate after 24 h of co-culture. (**A-I, A-II, A-III, and A-IV**) γδ T cells released a high level of cytokines when co-cultured with KG1a under (100 and 1000 ng/mL) of BTE, a limited release of cytokines in NALM6. Cytokine responses were quantified as spot-forming units (SFU). Aparts from **Study II**.*

We further measured cytokine levels in the co-cultured supernatant at 24 h and 48 h after the cytotoxicity assay using both the Luminex and ELISA platforms. The Luminex assay revealed increased levels of IFN-γ, TNF-α, MIP-1b, and FASL in KG1a cells under high concentrations of CD34/CD3 BTE. In NALM6 (CD34- cells), although cytotoxicity remained minimal, both BTE-induced detectable cytokine release compared to the no BTE control. Further validation using ELISA confirmed a dose-dependent increase in IFN-γ production from γδ T cells co-cultured with

KG1a at 24 h in response to BTE, consistent with the pattern observed in other cytokine analyses.

The different plate setups and cell numbers between the different immunoassay (Fluorospot vs. Luminex/ELISA) may explain the disparity observed in the NALM6 condition. Moreover, the detectable cytokine release with RSV/CD3 in KG1a or both BTE in NALM6 (CD34- cells) is most likely due to CD3-targeting BTE. Speculatively, this could be induced with other CD3-targeting BTE.

In addition to BTE's role in redirecting γδ T cells toward tumor cells, it markedly increased their cytokine production. We then demonstrated whether γδ T cells co-cultured with any target cells for 5 days using a high concentration of both BTE could promote γδ T cell proliferation. Notably, both BTE provoked γδ T cell proliferation, highlighting the role of CD3-targeting BTE in γδ T cell activation, irrespective of target cell cytotoxicity. Surprisingly, this differs from our previous finding, in which conventional T cell proliferation occurred only in response to CD34/CD3 BTE and CD34+ target cells (208). These data suggest a clinical promise for the in vitro expansion of γδ T cells, preserving proliferative capacity in vivo after re-infusion with BTE.

4.3.5 CD34/CD3 BTE induced γδ T cell-mediated lysis of CD34+ AML blasts (Study II)

The CD34 expression on leukemic blasts in AML is commonly associated with resistance to apoptosis and poor responsiveness to chemotherapeutic drugs (203, 204). In our study, although the primary leukemia cells from AML patient samples were limited (n = 3), we further evaluated the ability of CD34/CD3 BTE to eliminate CD34+ AML blasts. In line with CD34+ cell line data above, γδ T cells significantly induced dose-dependent killing of CD34+ blasts when co-cultured with primary leukemia cells for 72 h in the presence of CD34/CD3 BTE compared to RSV/CD3 BTE (**Figure 25**). These results support further investigation into the efficient and selective CD34/CD3 BTE targeting for γδ T cell-mediated cytotoxicity.

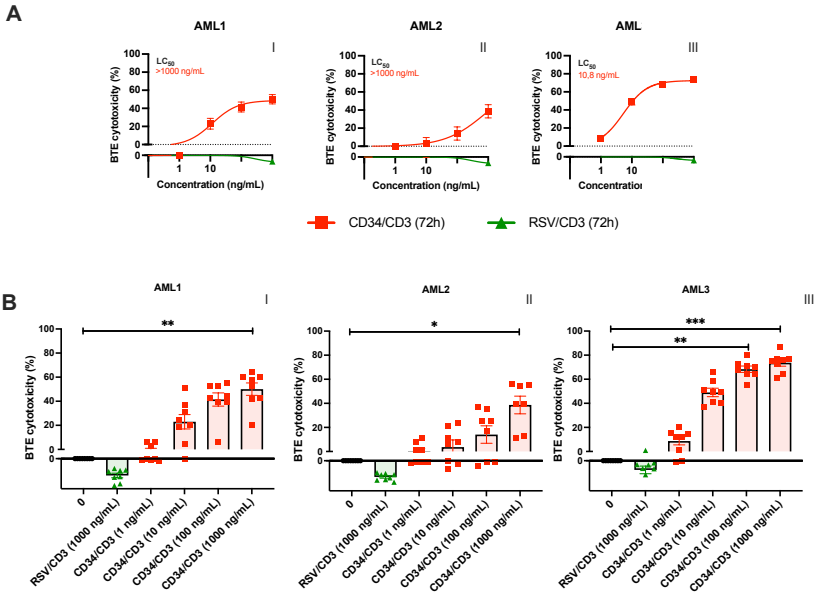


Figure 25. *(**A–B**) CD34/CD3 BTE-induced lysis of patients CD34+ AML blasts by γδ cells after 72 h of co-culture. Adapted from **Study II**.*

Furthermore, CD34 is also highly expressed in healthy hematopoietic stem and progenitor cells and, to a moderate extent, in endothelial cells (210). Therefore, it is crucial to assess their potential targeting effect by γδ T cells in combination with CD34/CD3 BTE. In our study, neither the healthy CD34-intermediate endothelial blood-brain barrier cell line (hCMEC/D3) at 24 h nor CD45dim CD34+ HSCs from healthy bone marrow (BM) samples (n = 3) at 48 h of co-culture were affected by γδ T cell in the presence of CD34/CD3 BTE, suggesting that γδ T cell-based CD34/CD3 approach might be safe for in vivo assays.

Overall, **Study II** highlighted that CD34/CD3 BTE induced significant cytotoxicity and robust cytokine release by γδ T cells against CD34+ primary blasts and CD34+ leukemia cell lines while sparing healthy cells. These findings suggest a promising adjuvant therapeutic strategy for targeting LSCs, which could be translated clinically in future. Nevertheless, these results warrant further research with larger sample sizes and In vivo models to extend this concept.

4.3.6 TQ is a novel anticancer candidate in leukemia therapy (Study III)

Targeting distinct LSC states is challenging due to their plasticity, which is likely patient-specific. Additionally, epigenetic modification and/or phenotypic plasticity in LSCs may play a role in chemotherapy resistance and immune evasion (201, 202, 211). The epigenetic modifications, including the re-expression of tumor suppressive genes can be reversible by epigenetic drugs, which may lead to apoptosis. Thus, the epigenetic drug could provide a therapeutic opportunity to diminish LSCs' activity (201, 212). Yet, single use of epigenetic drug such as azacitidine or decitabine alone, showed limited efficacy in treating patients with? AML (213). Currently, a combinatorial approach has been initiated to overcome relapse-dependent LSCs in AML patients (201). For instance, the talazoparib drug acts as an inhibitor for PARP1 enzymes, resulting in the re-expression of NKG2DL on LSCs, thereby sensitizing these cells for NK cell cytotoxicity in vivo (211) . This strategy has been moved to a clinical trial (NCT05319249) that involves PARP1 inhibition with subsequent infusion of alloreactive NK cells.

With the current rise of the combinatorial approaches, TQ, a multitarget epigenetic agent that acts as a hypomethylating agent and exerts a histone deacetylase (HDAC) inhibitory effect in various cancers, including leukemia (173, 175) could be a promising candidate. It demonstrated anticancer effects as monotherapy and/or synergistically with chemotherapies and chemoimmunotherapy with a safely tolerated profile in preclinical models and clinical trials (184, 214, 215). Based on the promising findings in **Study II**, we aimed to enhance γδ T cell multifunctionality by increasing tumor ligand expression on leukemia cell lines: in **Study III**, we introduced a combinatorial approach in which low-dose TQ pre-treatment sensitizes leukemia cells to enhance their recognition by expanded γδ T cells. This conditioning step may allow the low dose of CD34/CD34 to further augment the antileukemic activity of γδ T cells in vitro.

Initially, we assessed the anti-proliferative effects of TQ on several leukemia cell lines using the WST-1 assay. NALM6, KG1a, and Kasumi cells were exposed to increasing concentrations of TQ ranging from 0.1 to 50 μM for 24 and 72 h. TQ significantly reduced the cell viability across all treated cell lines in a concentration- and time-dependent manner, with a consistent reduction in cell viability observed after 72 h of treatment. Markedly, NALM6 cells were more sensitivity than KG1a and Kasumi, which displayed increased resistance, as reflected by higher IC_{50} values presented in **Study III**.

4.3.7 TQ-induced apoptosis and immunomodulation in leukemia cells

To alleviate the drug cytotoxicity (189), we specifically investigated apoptosis and immunomodulatory responses in leukemia cell lines treated with six selected concentrations of TQ (all below 10 μM) at 24 h and 72 h under high cell density in vitro. Our findings revealed that low-dose TQ (5 μM) triggered apoptosis in leukemia cell lines after 24 hours, with a notable increase in apoptotic cells, particularly in NALM6 and Kasumi (**Figure 26A & 26B**). These findings are aligned with prior studies demonstrating TQ-induced apoptosis at concentrations ranging from 3–10 μM in different leukemia models (173, 215). In contrast, KG1a exhibited fewer apoptotic cells at 5 μM (**Figure 26C**), reflecting their resistance to chemotherapy and immunotherapy (216).

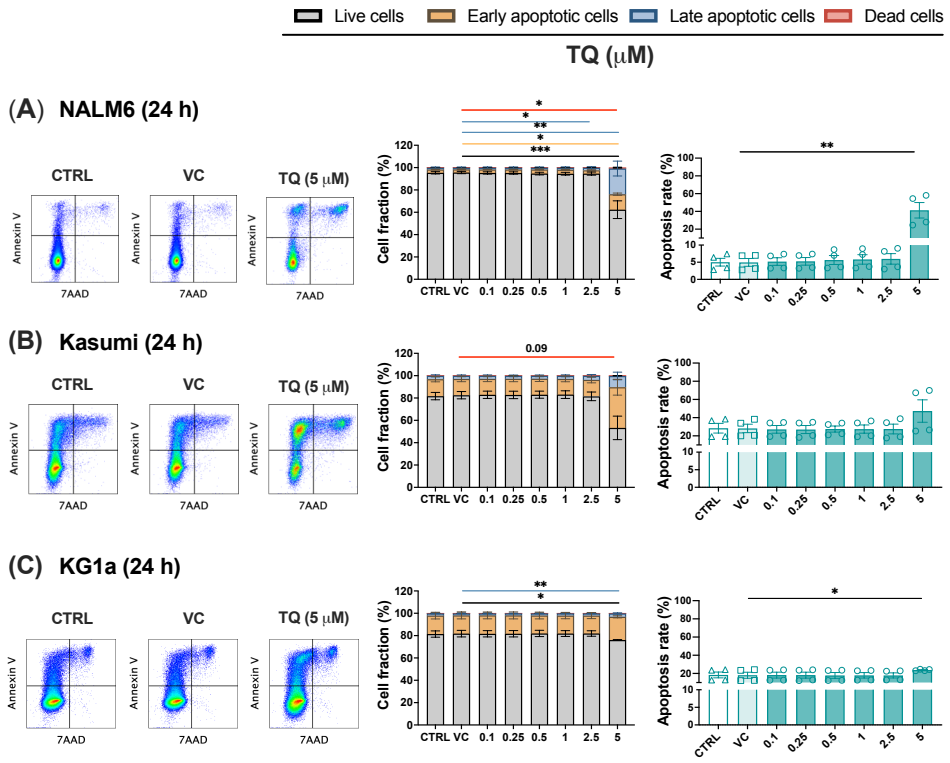


Figure 26. *(**A–C**) TQ at 5* μM *induced apoptosis in leukemia cell lines (A) NALM6, (B) Kasumi, and (C) KG1a at 24 h. Adapted from **Study III**.*

The modulation of NKG2D ligands (MICA/B) and DNAM-1 ligands (PVR, Nectin-2) on cancer cells is a promising approach to enhance γδ T-cell cytotoxicity (212, 217). For instance, bortezomib, an epigenetic drug, has been shown to upregulate the NKG2D ligands ULBP2/5/6 in AML and ALL cell lines and enhance γδ T-cell–mediated killing (217). This suggests that γδ T cells may achieve more robust cytotoxicity when combined with drugs that induce the expression of stress ligands.

Next, we evaluated the immunomodulatory effects of TQ after confirming that 5 μM TQ is sufficient to induce apoptosis, albeit with varying sensitivity responses among leukemia cell lines. At the same dose, we found significantly increased Nectin-2 and MICA/B expression in NALM6 and Kasumi cells, respectively, following TQ treatment compared to the vehicle control (VC) (**Figure 27A** and **27B**). Moreover, we observed a trend of upregulated PVR expression in KG1a cells (**Figure 27C**) compared to the vehicle control (VC). However, this was not statistically significant (p = 0.06), possibly attributable to the limited number of replicates. While the frequency of CD34 remained unchanged in the CD34+ population in both KG1a and Kasumi at 24 h and 72 h of TQ exposure. Importantly, no significant changes were observed in PDL1 expression across all leukemia cell lines or time points.

Consistent with other immunomodulatory agents, these findings indicate that a low dose of TQ may increase the antigen recognition between the leukemia cells and γδ T cells (217, 218).

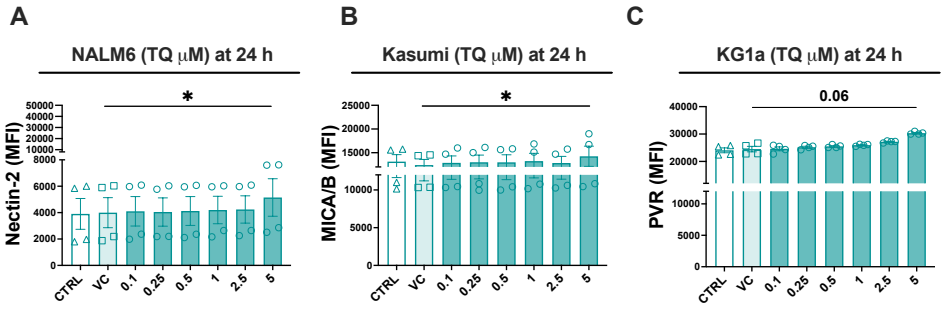


Figure 27. *(**A–C**) TQ at 5* μM *induced apoptosis in NKG2Dand DNAM-1 ligands in leukemia cell lines (**A**) NALM6, (**B**) Kasumi, and (**C**) KG1a at 24 h. Adapted from **Study III**.*

4.3.8 Is TQ a sensitizer for leukemia cells in γδ T cell-based BTE therapy?

To investigate how TQ would contribute to γδ T cell-based BTE therapy, we then pretreated the leukemia cell lines (targets) with 5 μM TQ for 24 h prior to co-culturing them with expanded γδ T cells (effectors) at a ratio E: T (3:1) for an additional 24 h. Our results indicated that TQ enhanced the susceptibility of leukemia cells to γδ T cell-mediated cytotoxicity, as evidenced by rapid killing with increased proportions of apoptotic cells in the TQ-pretreated condition compared to vehicle (VC) and untreated control in KG1a, Kasumi, and NALM6 cells (**Figure 28)**.

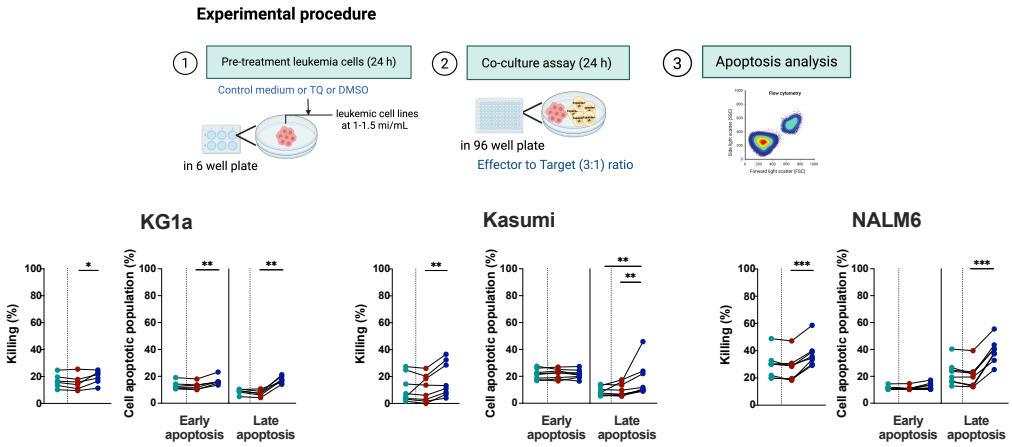


Figure 28. *TQ pre-treatment enhanced the sensitivity of leukemia cells to* γδ T cell-mediated cytotoxicity. *Adapted from* ***Study III****.*

Given that TQ offered a synergistic effect in combination with chemotherapies and immunotherapy in several cancers (183, 184) and having confirmed the immune-sensitizing properties of TQ in these leukemia cell lines, we explored whether this immune-sensitization effect could support the condition of γδ T cell-based CD34/CD3 BTE approach: in the presence of CD34/CD3 BTE (100 ng/mL), TQ pretreatment markedly enhanced γδ T cell-mediated killing in both the total KG1a cells and their CD34+ populations compared to vehicle (VC) and untreated control, as accompanied by a significant increase in apoptotic cells (**Figure 29)**. Conversely, we observed an almost similar level of killing in both the total Kasumi and their CD34+ populations across all the conditions, which is likely due to a small fraction of CD34+ expressed in Kasumi, and most CD34+ populations might be affected within the first 24 h of TQ pretreatment.

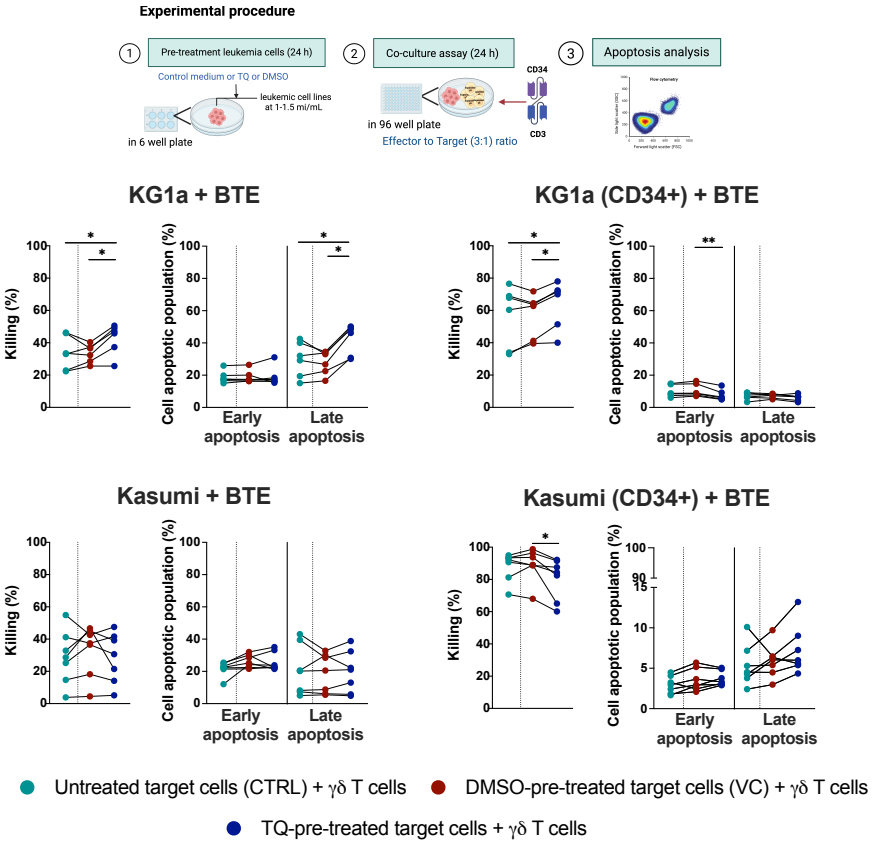


Figure 29. *The synergistic effect between the TQ pre-treatment and CD34/CD3 BTE enhanced* γδ T cell-mediated cytotoxicity against CD34+ *leukemia cells. Adapted from* ***Study III****.*

Overall, **Study III** in vitro indicates that a low TQ (5 μM) pretreatment can enhance γδ T cells mediated-cytotoxicity against leukemia cell lines. Following TQ pretreatment, lower CD34/CD3 BTE concentration is sufficient to provide additive cytotoxicity for further γδ T cells-mediated killing in CD34-expressing leukemia. This combination offers a promising synergistic therapeutic approach in leukemia therapy. However, further investigation is needed to assess the effect of repeated TQ exposure, the use of nanoformulations to enhance TQ bioavailability. More mechanistically, it would be also interesting to assess the impact of the changes of Nectin-2 and MICA/B expression by using anti-NKG2D/DNAM-1.

5 Conclusions

This thesis has investigated the role of γδ T cells in the context of aHCT and therapeutic applications in hematological malignancies. Understanding long-term γδ T cell reconstitution via powerful NGS and high-parameter flow cytometry provides insights into the homeostatic steady state at the phenotypic and clonotypic level, with a particular focus on clonotype dynamics and the role of different subsets of γδ T and their association with clinical outcomes. We also brought further knowledge on the antileukemic efficacy of γδ T cells in combination with CD34/CD3 BTE and epigenetic drug such as TQ. These combinations may provide a synergistic therapeutic strategy, paving the way to improve the current approaches for patients with relapsed disease or those ineligible for intensive conditioning regimens due to advanced age or comorbidities.

Concluding remarks and key points of these studies:

- γδ T cell characterization in long-term survivors (recipients) post-aHCT (**Study I**)
  - Long-term γδ T phenotype composition in long-term survival was comparable to that of their matched donors.
  - There were no significant differences in the regrade of diversity, clonality, and gene segment usage between the recipients and donors, indicating that TRG CDR3 composition is restored.
  - The recipients' γδ T cell repertoire exhibited a private clonotypes with a limited number of shared clonotypes compared to their matched donors, suggesting a de novo generation of naïve γδ T cells in the thymus.
  - γδ T-cells remain functional and provide long-term protection against CMV after aHCT after long-term aHCT.
  - cGHVD has a long-term effect on γδ T cell reconstitution, which is likely mediated by the overrepresentation of donor-derived peripheral clones and the inverted Vδ1/Vδ2 ratio in a small cohort of recipients.
  - Upregulating HLA-DR in Vδ1 T cells, which is possibly associated with ongoing M/S cGVHD, could be a therapeutic target.

Overall, homeostasis is achieved in long-term γδ T cells reconstitution with a normalised repertoire observed in TRG CDR3 composition.

- In our work about expanded γδ T cells in combination with CD34/CD3 BTE and TQ (**Study II** & **Study III**)

- CD34/CD3 BTE boosted γδ T-specific cytotoxicity against the CD34-expressing leukaemia cell line and CD34+ AML blasts in a dose-dependent manner (**Study II**).
- γδ T cells exhibited stronger killing capacity compared to pre-stimulated αβ T cells or resting αβ T cells (**Study II**).
- CD34/CD3 significantly enhanced the cytokine production by γδ T cells (**Study II**).
- γδ T cells showed a further proliferative capacity in response to a high CD34/CD3 BTE concentration (**Study II**).
- γδ T cells did not exert specific cytotoxicity against healthy cells in the endothelial blood-brain barrier cell line (hCMEC/D3) and CD45dim $CD34^+$ HSCs (**Study II**).

- TQ displayed antiproliferative effects on leukaemia cell lines in a concentration- and time-dependent manner (**Study III**).

- A low dose of TQ (5 μM) induced apoptosis in leukemia cell lines at higher cell density (**Study III**).
- A low dose of TQ (5 μM) modulated the expression of NKG2D and DNAM-1 ligands in leukaemia cell lines, particularly upregulating Nectin-2 expression in NALM6 cells, MICA/B expression in Kasumi cells, and PVR expression in KG1a (**Study III**).
- Pre-treatment TQ at (5 μM) increased the sensitivity of leukemia, leading to enhanced γδ T cells-mediated cytotoxicity against leukemia cell lines (**Study III**).
- Finally, pre-treatment TQ (5 μM) in leukemia cells exhibited a synergistic effect in combination with γδ T cell-based CD34/CD3 approach against CD34+ expressing leukemia cells (**Study III**).

6 Points of perspective and Future direction

Parts of this thesis presented in **Study I** advance the current understanding of long-term γδ T cell reconstitution after aHCT, particularly its association with clinical outcomes. As these findings are generated from a limited sample size of heterogeneous cohorts in terms of graft source, conditioning regimens, and GVHD prophylaxis, further studies are needed to generalize and strengthen these findings. Despite these limitations, our results provide a valuable foundation for future research by raising several important questions:

Our repertoire analysis was restricted to the TCR γ (TRG) chain without separating the Vδ1 and Vδ2 repertoires. Given the observed association between expanded donor-derived TRG clonotypes and inversion of the Vδ1/Vδ2 subset ratio in some recipients with ongoing M/S cGVHD, future studies should analyze Vδ1 and Vδ2 repertoires independently to gain detailed insights into their diversity, clonality, and reconstitution dynamics. Furthermore, using single-cell TCR sequencing would be interesting to further interrogate the simultaneous clonal expansion with the gene expression in both TCR γ and δ chains at the single-cell level (219). The single-cell TCR analysis will help to identify specific expanded clonotypes relevant to cGVHD, emphasizing bulk RNA sequencing data as utilized in cancer models (219, 220). Additionally, we observed upregulated HLA-DR expression in Vδ1 T cells associated with cGVHD severity; however, the role of these cells is still ambiguous. Therefore, performing mixed lymphocyte reactions with HLA-DR blockade would provide a valuable approach to better understanding the functional role of HLA-DR+ Vδ1 T cells in cGVHD development.

Due to the limited sample size **in Study I**, as well as the absence of intermediate timepoint samples (e.g. pre- and 1–2 years post-aHCT) and the challenge of obtaining long-term samples from survivors and their corresponding donors, future studies will require large-scale, to overcome these limitations and provide solid conclusions.

In Study II, CD34/CD3 BTE has shown a promising result as adjuvant therapy with expanded γδ T cells targeting CD34+ leukemia cell lines and depleting CD34+ AML blasts. Based on these findings in **Study II** and **Study III**, the next step is to explore the potential role of expanded γδ T cells with CD34/CD3 BTE within an *in vivo* xenograft model in the presence or absence of TQ given as oral gavage. Additionally, our CD34/CD3 BTE construct can be re-engineered with multiple targeting to improve tumor targeting (207). For instance, the C-type lectin-like molecule-1 (CLL-1) is consistently expressed on the surface of CD34+ leukemic blasts and LSCs across all stages at diagnosis and relapse, indicating a

surveillance marker (221, 222). Moreover, CLL-1 expression is restricted to the leukemic compartment, is associated with quick relapse and is almost negligible/ absent in normal HSC (221, 222). Thus, adding this would improve the targeting with off-target toxicity against the normal cells.

With **Study III**, we have observed that TQ at (5 μM) induced the expression of NKG2D and DNAM-1 ligands on leukemia cell line, thereby sensitizing them and enhancing the anti-leukemic activity of γδ T cells. To strengthen these findings, it would be interesting to perform a blocking assay for these ligands, confirming the involvement of these pathways. Repeated exposure of leukemia cells to TQ at doses below 5 μM would be of interest to determine whether it can provide a more substantial induction effect on the expression of NKG2D and DNAM-1 ligands.

It would be interesting to examine the immune sensitizing effect of TQ on primary leukemic samples in future studies. Additionally, performing RNA sequencing would be valuable to evaluate TQ's epigenetic mechanisms on these leukemia cell lines (175). TQ exhibits moderate oral bioavailability ~58% in the rabbit model (159); thus, future studies should also investigate TQ-loaded nanoparticles in vitro and in vivo. Since nanoparticle formulations are widely used to improve the bioavailability of several drugs. They are smaller than cells, characterized by low or non-toxicity, and can prevent the degradation of conjugated drugs, as illustrated earlier in the case of TQ nanoparticles (157).

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8 Declaration about the use of generative AI

I take full responsibility for the content of the “kappa”/comprehensive summary of the thesis. The writing process did not involve the use of generative AI.

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