Ana Carolina Grais Condeço

Degree in Biology – Evolutionary and developmental biology

Tumour-infiltrating NK cells and γδ T-cells for cellular immunotherapy

Dissertation to obtain master's degree in
Molecular Genetics and Biomedicine

Supervisor: Prof. Markus Maeurer, MD PhD FRCP (London)

Jury:

President: Prof. Dr. Maria Alexandra Núncio de Carvalho Ramos Fernandes
Arguer: Prof. Dr. Dário José Pirralha Ligeiro
Vowel: Prof. Dr. Markus Joseph Maeurer

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Acknowledgments

“A felicidade não é um destino é uma viagem.”

Osho

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“Nenhum obstáculo será grande se a sua vontade de vencer for maior.”

Abstract

The frequent downregulation or complete loss of major histocompatibility complex (MHC) expression by tumour cells often compromises patients' response to standard cancer treatments or αβ T-cell-based therapies. Thus, NK cell and γδ T-cell-based cancer immunotherapies emerged as complementary treatments. Both immunotherapies have been successfully used to target haematological malignancies but their effectiveness in patients with solid tumours has limited their therapeutic application. Most NK cell and γδ T-cell-based immunotherapies use cells isolated and expanded from peripheral blood mononuclear cells, which may result in a cellular product with low homing capacity and/or anti-tumour reactivity. We aimed therefore to explore the feasibility of isolating and expanding tumour-reactive NK cells or γδ T-cells directly from the tumour tissue of patients with cancer.

Different expansion protocols using cytokine combinations of IL-2, IL-15, IL-7 or IL-1β along with molecularly defined targets for γδ T-cells were tested for their capacity to preferentially isolate and expand γδ T-cells from patients' tumour, while IL-2, IL-15 and stimulation with the K-562 cell line was used for NK cell isolation and expansion. The protocols' efficiency was assessed by evaluating the total cell number, cell viability and NK cells or γδ T-cells' frequency. Cells' functional capacity was determined by their ability to produce IFN-γ in response to autologous tumour cells and/or allogeneic cancer cell lines. We demonstrate that NK cells and γδ T-cells can be directly isolated from patients' tumour tissue and are able to recognize autologous tumour cells, producing IFN-γ. Tumour-infiltrating γδ T-cells revealed inclusive to use their T-cell receptor to target autologous tumour cells *in vitro*.

In summary, our data provide the first evidence that tumour-infiltrating NK cells and γδ T-cells can be expanded from cancer lesions and may be used in cancer immunotherapy for patients with solid tumours.

Keywords: γδ T-cells; NK cells; tumour tissue; MHC negative tumour cells; γδ T-cell receptor; anti-tumour reactivity

Resumo

A diminuição ou perda completa da expressão do complexo principal de histocompatibilidade (MHC) pelas células tumorais compromete frequentemente a resposta dos pacientes aos tratamentos 'standard' e terapias com células T αβ. Assim, as imunoterapias com células NK e T γδ surgiram como terapias complementares. Ambas têm sido bem-sucedidas no tratamento de cancros hematológicos, contudo a reduzida eficácia em pacientes com tumores sólidos tem limitado a sua aplicação terapêutica. A maioria destas imunoterapias usa células isoladas e expandidas das células mononucleares do sangue periférico, o que pode resultar num produto celular com baixa capacidade de infiltração e/ou reatividade anti-tumoral. Assim, o objetivo deste estudo foi explorar a exequibilidade de isolar e expandir células NK e T γδ com atividade anti-tumoral a partir do tecido tumoral de pacientes com cancro.

Protocolos combinando alvos molecularmente definidos para as células T γδ com várias combinações das citoquinas IL-2, IL-15, IL-7 ou IL-1β foram testados para isolar e expandir preferencialmente estas células do tumor dos pacientes, enquanto para as células NK foi usada IL-2, IL-15 e estimulação com a linha celular K-562. A eficácia dos protocolos foi avaliada através do número total de células, viabilidade celular e frequência das células NK ou T γδ. A capacidade funcional foi determinada através da produção de interferão-gama em resposta a células tumorais autólogas e/ou linhas celulares cancerígenas alogénicas. Este estudo demonstra que células NK e T γδ podem ser isoladas diretamente do tecido tumoral de pacientes e possuem capacidade de reconhecer células tumorais autólogas, produzindo interferão-gama. As células T γδ infiltradas no tumor revelaram inclusivamente usar o recetor de células T para reconhecer *in vitro* células tumorais autólogas.

Estes resultados fornecem a primeira evidência que células NK e T γδ infiltradas no tumor podem ser expandidas de lesões cancerígenas e usadas em imunoterapia para pacientes com tumores sólidos.

Palavras-chave: células T γδ; células NK; tecido tumoral; células tumorais MHC negativas; recetor das células T γδ; reatividade anti-tumoral

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World Health Organization	WHO

1. Introduction

1.1. Cancer

Cancer is the second leading cause of death worldwide. According to the World Health Organization (WHO), in 2018, approximately 18.1 million people worldwide had cancer, and 9.6 million died from this disease (WHO, 2018). The future of cancer is not encouraging since by 2040, the global burden is expected to increase to 29-37 million new cases of cancer (WHO, 2020). Although several anti-cancer therapies have been developed, tumour cells usually persist and lead either to a local relapse or metastases, since cancer cells exhibit a high adaptation capacity and explore different mechanisms to evade immune surveillance.

1.2. Immune evasion mechanisms

The most well-studied immune evasion mechanism explored by tumour cells is the downregulation or complete loss of major histocompatibility complex I (MHC-I) molecules (Maeurer *et al*., 1996a), which was already reported in several tumour types, such as breast (Cabrera *et al*., 1996), colorectal (Cabrera *et al*., 1998) and cervical cancers (Van Driel *et al*., 1996). Beyond this mechanism that prevents the tumour antigen presentation to T-cells avoiding tumour cell recognition, tumour cells may also hinder their elimination by excluding immune cells from the tumour, preventing T-cell priming and/or impairing immune cell functions. Immune cells are usually excluded from the tumour because tumour cells impair their recruitment by promoting the downregulation of adhesion molecules involved in T-cell migration, such as CD34, intercellular adhesion molecule 1 (ICAM1) and 2 (ICAM2) (Griffioen *et al*., 1996) or vascular cell adhesion protein 1 (VCAM1) (Piali *et al*., 1995), and/or by downregulating the expression of chemoattractant molecules through epigenetic silencing (Peng *et al*., 2015) or mutations in signalling pathways (Spranger *et al*., 2015). On the other hand, tumour cells ensure that tumour-infiltrating lymphocytes (TIL) are not able to target them by hamper their effector function through different mechanisms. The immunosuppressive microenvironment established by tumour cells along with cancer-associated fibroblasts (CAFs), tumour-associated macrophages (TAMs), dendritic cells (DCs), myeloid-derived suppressor cells (MDSCs) and regulatory T-cells (Tregs) (Kerkar and Restifo, 2012) is one of the main mechanisms responsible for the impairment of immune cell functions and consequently anti-tumour responses. For example, the transforming growth factor beta (TGF-β) produced by tumour cells (Tada *et al*., 1991), CAFs (Tauriello *et al*., 2018) and Tregs (Chen *et al*., 2005) acts on T-cells impairing their effector functions. Vascular endothelial growth factor (VEGF) is another cytokine found in the tumour microenvironment (TME), that beyond other functions, induces T-cell exhaustion by promoting the expression of inhibitory receptors in T-cells, *e.g.* programmed cell death protein-1 (PD-1), cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and T-cell immunoglobulin and mucin protein 3 (TIM-3) (Voron *et al*., 2015). Another mechanism contributing to T-cell exhaustion is for example the release by tumour cells of exosomes expressing programmed cell death-ligand 1 (PD-L1) that target PD-1^{+} CD8^{+} T-cells (Chen *et al*., 2018). All these mechanisms are just few examples of the strategies employed by tumour cells to evade tumour immune surveillance, several more exist and are reviewed in (Bhatia and Kumar, 2014).

1.3. Immunotherapy

Standard cancer treatments, such as surgery, chemotherapy and radiotherapy, have demonstrated limited efficacy for the treatment of patients with solid cancer. Thus, many cancer therapies have been explored over the years. Immunotherapy emerged as a path to circumvent the immune evasion mechanisms and potentiate immune cells to exert their anti-tumour functions. Several immunotherapies, from the least specific to the more tumour-directed, have been designed to overcome different tumour escape mechanisms. The least specific immunotherapies, *i.e.* oncolytic viral therapy, immunostimulatory cytokines and checkpoint blockade therapy, do not target tumour cells directly, they stimulate the immune system in a more general way. For example, oncolytic viruses preferentially target tumour cells inducing their death and consequently increasing the anti-tumour directed inflammation (Choi *et al*., 2016), while the administration of immunostimulatory cytokines, such as interferon (IFN) or IL-2, preferentially promote the activation of tumour-specific T-cells (Abdolvahab *et al*., 2020; Choudhry *et al*., 2018). On the other hand, checkpoint blockade immunotherapy focuses on the restoring of T-cell effector functions using monoclonal antibodies designed to interrupt coinhibitory signalling pathways that act to maintain immune tolerance and that are explored by tumour cells to evade immune-surveillance (Pardoll, 2012). The most common targets for immune checkpoint inhibitors are CTLA-4, PD-1 and PD-L1. In contrast with these non-specific therapies, tumour-directed immunotherapies, *i.e.* therapeutic vaccines and adoptive cell therapy/active cell therapy (ACT), were designed to take advantage of the antigens expressed in tumour cells as a consequence of the genomic instability and mutations that enabled the cell to acquire the cancer hallmarks (Hanahan and Weinberg, 2011). Therapeutic cancer vaccines stimulate antigen-specific immune responses through the immunization of the patient with one or multiple tumour antigens (Chen and Mellman, 2013), whereas *ex vivo* expanded tumour-reactive effector cells are used for ACT (Chen and Mellman, 2013; Met *et al*., 2019; Rosenberg and Restifo, 2015).

Among the different types of immunotherapy, immunomodulators, such as PD-1 or PD-L1 inhibitors (checkpoint blockade therapy), have been one of the most explored immunotherapies since last year, presenting the largest number of active clinical trials (Upadhaya *et al*., 2020). However, it is ACT that accounts for the largest number of new therapies under development since last year (Upadhaya *et al*., 2020). This is probably explained because, contrarily to the other immunotherapies, the *ex vivo* cell expansion and manipulation allows to generate a molecularly defined T-cell product with defined anti-tumour specificity.

1.4. ACT

ACT involves the isolation of immune cells from the patient or a donor, their *ex vivo* expansion, which may be preceded by a genetic modification, and their subsequent infusion into the patient. ACT comprises different approaches and may be classified according to the cell source (autologous or allogeneic) and the effector cell type used. There are three types of ACT: TIL therapy, T-cell receptor (TCR) gene therapy and chimeric antigen receptor (CAR)-T-cells (Met *et al*., 2019). TIL therapy isolates lymphocytes, mainly T-cells, that naturally infiltrate tumours and expand them *ex vivo* before reinfusion into the patient (Christofi *et al*., 2019). In contrast, TCR gene therapy and CAR-T-cells isolate T-cells

from the peripheral blood mononuclear cells (PBMCs) and engineer them to express a transgene encoding an antigen-specific receptor (Christofi *et al*., 2019). While in TCR gene therapy T-cells are engineered with an exogenous tumour-specific TCR, in CAR-T-cells ACT, T-cells are engineered with a CAR, *i.e.* an artificial receptor that combines the specificity of antibody-like recognition with the cytotoxic capacity of T-cells using as an extracellular domain a tumour-specific antibody fragment (Christofi *et al*., 2019). Thus, in contrast with TCR gene therapy, the use of CAR-T-cells enables the recognition of tumour cells in a non-MHC restricted manner (Gross *et al*., 1989; Kuwana *et al*., 1987). The three types of ACT have already demonstrated encouraging results, however in a long-term perspective TIL therapy is possibly the most promising because in contrast with TCR gene therapy and CAR-T-cells, it uses a polyclonal cell product to treat the patient (Met *et al*., 2019; Rohaan *et al*., 2019), which results in a lower chance of inducing tumour immunoediting and consequently prevents therapy resistance. TIL may also survive in a patient for a longer period and may therefore offer a more long-term protection against cancer cells. TIL therapy also has the advantage of not requiring the genetic engineering of the cells, which allows to save time and money.

αβ T-cells have been the focus of tumour immunology due to their potent tumour-killing capacity and extraordinary ability to detect mutated proteins, which offers a high specificity for the targeting of transformed cells. Until recently, αβ T-cells have been considered the effector cells for ACT, however, the fact that their activation depends on the recognition of tumour antigens presented by MHC molecules has been shown to compromise the effectiveness of ACT in the complete eradication of the tumour. Since tumour cells usually downregulate or lose MHC expression as a mechanism of immune evasion, ACT using αβ T-cells allows the escape of these MHC negative tumour cell clones, which may result in cancer recurrence. Therefore, the need to complement the standard ACT with new therapies that target MHC negative tumour cells emerged.

1.4.1. Non-αβ T-cell therapies

The pitfalls of antigen-specific therapies may be avoided with antigen-non-specific therapies. Thus, innate immune cells might represent promising platforms for innovative immunotherapy approaches. Due to their role in tumour immune surveillance, NK cells and γδ T-cells have been the ones receiving more attention.

1.4.1.1. NK cells

NK cells are a subset of innate lymphoid cells that act as the first line of defence against virus- and malignant-transformed cells. These cells are divided into two main subsets according to their CD56 expression: $CD56^{bright}$ and $CD56^{dim}$ NK cells. These two NK cell subsets differ in their main localization and effector function. While $CD56^{bright}$ NK cells are predominant in secondary lymphoid organs and are more efficient in the production of proinflammatory cytokines, such as interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α), $CD56^{dim}$ NK cells are abundant in peripheral blood, lung and spleen, and are more effective in the induction of cytolysis due to their increased expression of perforin and granzyme A (Jacobs *et al*., 2001; Tomasello *et al*., 2012). The activation mechanism of NK cells was first described by the 'missing-self theory' (Ljunggren and Kärre, 1990), which hypothesized that NK cells distinguished 'self' from 'non-self' through the MHC-I expression in cells' surface, being

activated when a loss or reduction of MHC-I expression was detected. This mechanism is partially responsible for the recognition of tumour cells by NK cells since tumour cells usually exhibit low expression of MHC-I and NK cells are able to recognize and eliminate them (Kärre *et al*., 1986; Ljunggren and Kärre, 1985). Nowadays, the activation mechanism of NK cells is known to be more complex than the 'missing-self theory', despite it is not yet fully understood. Now, NK cells are also known to express activating receptors, and the balance between the signals received from the activating and inhibitory receptors is considered the driving force that determines the NK cell activation status (Meza Guzman *et al*., 2020). Upon NK cell-to-cell interaction, the inhibitory and activating signalling simultaneously occur. Thus, the activation of NK cells requires either the break of the inhibitory signalling, as occur with tumour cells that lose or downregulate MHC-I expression, or an activating signal outweighing the inhibitory signal (**Figure 1.1**) (Meza Guzman *et al*., 2020).

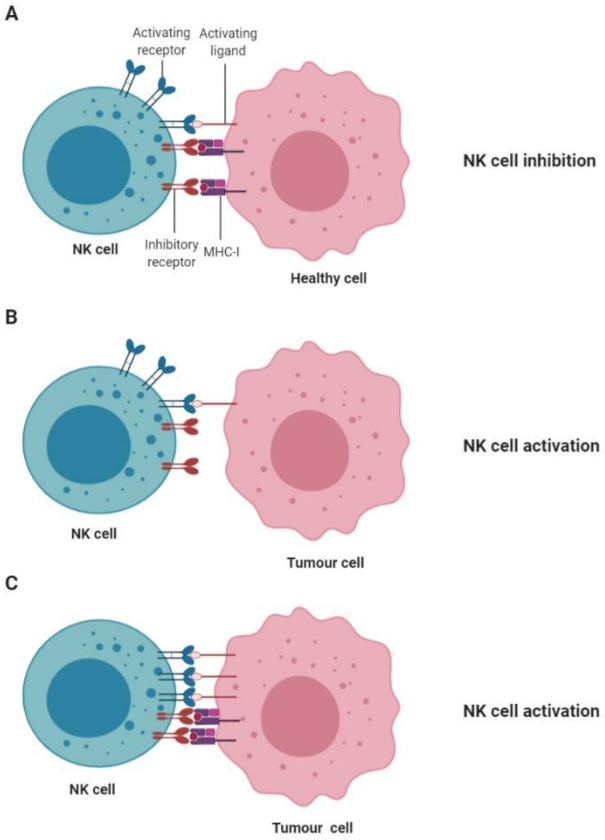


Figure 1.1 – NK cell activation. NK cells are tolerant to 'self'-healthy cells because the MHC-I molecules expressed on healthy cells' surface interact with the NK inhibitory receptors dampening the activating signals that NK cells receive **(A)**. When cells lose the expression of MHC-I molecules, as it often occurs in tumour cells, NK cells become activated since they do not receive the inhibitory signalling provided by the MHC-I molecules – 'missing-self theory' **(B)**. In addition, NK cells may also be activated due to the upregulation of activating ligands in 'stressed' cells, which leads to the delivery of an activating signalling that overcome the inhibitory signalling provided by the MHC-I molecules **(C)**. Illustration created with BioRender.com.

NK cells express a broad repertoire of inhibitory and activating receptors, which are grouped in three main families: killer cell immunoglobulin-like receptors (KIRs), natural killer group 2 (NKG2) members and natural cytotoxicity receptors (NCRs). KIR family comprises inhibitory (Burshtyn *et al*., 1996; Campbell *et al*., 1996) and activating receptors (Burshtyn *et al*., 1996) that are distinguish through the

presence of intracellular immunoreceptor tyrosine-based inhibitory motifs (ITIM) or activating motifs (ITAM), respectively. Like KIR family, the NKG2 members may also be activating (NKG2 member C, NKG2C, and member E, NKG2E) or inhibitory receptors (NKG2 member A, NKG2A), however, while they recognize a non-classical MHC molecule, the human leukocyte antigen (HLA)-E (Braud *et al*., 1998), KIRs specifically bind to HLA-A (Hansasuta *et al*., 2004), HLA-B (Peruzzi *et al*., 1996) and HLA-C (Zappacosta *et al*., 1997). Distantly correlated with the NKG2 family, NKG2 member D (NKG2D) is an NK cell activating receptor that in contrast with those previously mentioned recognizes a broad spectrum of ligands, namely the stress-inducible MHC-I chain-related gene A (MICA) and B (MICB) (Steinle *et al*., 2001), and the UL16 binding proteins (ULBP) 1 to 4 (Cosman *et al*., 2001). In contrast to KIR and NKG2 families, the NCR family only includes NK cell activating receptors, namely NKp46 (Sivori *et al*., 1997, 1999), NKp44 (Vitale *et al*., 1998) and NKp30 (Pende *et al*., 1999). NK cells also express other molecules that function primarily as co-receptors, being responsible for the amplification of the triggering induced by the NKG2D or NCRs. Some examples are 2B4 (Sivori *et al*., 2000), NK-T-B-antigen (NTB-A) (Bottino *et al*., 2001), DNAX accessory molecule 1 (DNAM1) (Shibuya *et al*., 1996), CD59 (Marcenaro *et al*., 2003) and NKp80 (Vitale *et al*., 2001).

The wide variety of receptors expressed by NK cells offers them the ability to recognize a broad spectrum of ligands in an MHC-independent manner. Moreover, some of these receptors demonstrated to play a very relevant role in tumour immunity not only because tumour cells express their ligands but also because their absence compromises tumour immune surveillance. NKG2D is an example since, on one hand, the expression of its ligands MICA and MICB revealed to render hepatocellular carcinoma tumour cells susceptible to NK cell killing (Jinushi *et al*., 2003), and on the other hand, its absence resulted in a defective tumour surveillance (Guerra *et al*., 2008). The NCRs NKp46 and NKp30 are other examples once the NKp46 deletion showed to lead to a reduction of NK cell ability to lyse target cells *in vivo* (Halfteck *et al*., 2009; Sivori *et al*., 1999) and the NKp30 ligand B7-H6 (Brandt *et al*., 2009) was shown to be absent in healthy cells but to be frequently expressed in different types of tumour cells, through a Myc-dependent mechanism (Textor *et al*., 2016). Besides the studies focusing on NK cell receptors and ligands have evidenced the role of NK cells in tumour immunity, other studies proved it directly even demonstrating that NK cells have the ability to recognize and target cancer stem cells from different types of tumours, such as breast cancer (Ames *et al*., 2015; Yin *et al*., 2016), pancreatic adenocarcinoma, sarcoma (Ames *et al*., 2015), colorectal cancer (CRC) (Tallerico *et al*., 2013) and glioblastoma (Castriconi *et al*., 2009).

Altogether, the capacity to recognize several ligands in an MHC-independent manner associated with the role of NK cells in tumour immunity has led to a boost in the field of NK cell-based immunotherapies. Several clinical trials have been developed during the last decade. Most of the completed clinical trials (ClinicalTrials.gov) focused on the infusion of allogeneic or autologous NK cells and demonstrated to have a higher success rate in the treatment of haematological cancers in comparison with solid tumours. Although some success was reported in clinical trials aiming to treat neuroblastoma and non-small cell lung cancer (NSCLC), in other types of solid cancers, such as metastatic melanoma, renal cell carcinoma (RCC) or advanced gastrointestinal tumours (Parkhurst *et al*., 2011; Sakamoto *et al*., 2015), the effectiveness of NK cell-based immunotherapies appeared to be

limited. Currently, several ongoing clinical trials (ClinicalTrials.gov) are exploring other approaches, such as CAR-NK cells. These new developed CAR-NK cells are mainly directed against antigens of haematological cancers, *e.g.* CD19, CD22, CD33 and CD7, and only some target solid tumours, using for example mucin 1 and mesothelin as target molecules. This broader focus on haematological cancers is not surprising since besides these therapies have been showing more promising results in these types of cancers, the safety of CAR-NK cells was already demonstrated in the treatment of patients with haematological cancers, such as acute myeloid leukaemia (AML) (Tang *et al*., 2018) and non-Hodgkin's lymphoma or chronic myeloid leukaemia (CML) (Liu *et al*., 2020). Of note that most of the currently recruiting clinical trials continue to focus on the infusion of allogeneic or autologous NK cells to treat not only haematological malignancies, but also several types of solid tumours.

1.4.1.2. γδ T-cells

γδ T-cells represent a non-conventional T-cell subset that exhibit innate- and adaptive-like properties thus performing the bridge between innate and adaptive immunity (Melandri *et al*., 2018). This is evidenced by the combination of TCR-mediated and NK-characteristic mechanisms to recognize and kill target cells. γδ T-cells express a TCR composed of γ and δ chains and are divided into several subsets according to their TCR usage of Vδ chains, being the most common Vδ1$^+$ and Vδ2$^+$ γδ T-cells. While Vδ1$^+$ γδ T-cells are predominantly found as tissue-resident cells, most Vδ2$^+$ γδ T-cells express the Vγ9 chain and constitute the main γδ T-cell population in peripheral blood (Bonneville *et al*., 2010). Besides their tissue tropism, these two γδ T-cell subsets exhibit different phenotypes: Vδ2$^+$ γδ T-cells are more inflammatory, whereas Vδ1$^+$ γδ T-cells present a more regulatory phenotype (Kress *et al*., 2006). Despite their differences, both γδ T-cell subsets have capacity to target tumour cells, performing an important role in tumour immunity. Vδ1$^+$ γδ T-cells, due to their tissue tropism, have a biologically relevant role in immune responses directed against tumour cells of epithelial origin, such as skin (Girardi et al., 2001), colon and rectal cancers (Rong *et al*., 2016; Zocchi *et al*., 2017). In contrast, in addition to their ability to respond to pathogen-derived pyrophosphates (Tanaka *et al*., 1995), Vγ9$^+$Vδ2$^+$ γδ T-cells recognize the isopentenyl pyrophosphate (IPP) that frequently accumulates in tumour cells due to the dysregulation of the mevalonate pathway (Gober *et al*., 2003).

γδ T-cells are equipped with a TCR and activating NK cell receptors that allows them to recognize surface antigens without depending on antigen presentation by MHC-I molecules (Schild *et al*., 1994). Although the ligands for the different γδ T-cell receptors are not yet fully unravelled, it is known that these cells recognize molecules expressed at the cell surface in a stress-induced manner. Some TCR ligands were already described, despite were shown to be recognized by specific γδ T-cell subsets. For example, while Vγ4$^+$Vδ5$^+$ and Vγ8$^+$Vδ3$^+$ γδ T-cells recognize endothelial protein C receptor (EPCR) (Willcox *et al*., 2012), and annexin A2 (Marlin *et al*., 2017), respectively, Vδ1$^+$ γδ T-cells detect lipids presented by the surface glycoprotein CD1d (Luoma *et al*., 2013; Uldrich *et al*., 2013) and Vγ9$^+$Vδ2$^+$ γδ T-cells recognize the heat shock protein 60 (Laad *et al*., 1999), F1-ATPase (Scotet *et al*., 2005) and phosphoantigens presented by butyrophilins (BTN), such as butyrophilin subfamily 3 member A1 (BTN3A1) (Benyamine *et al*., 2016). In addition to γδ TCR antigens, γδ T-cells are activated by many other ligands as they also express activating NK cell receptors, such as NKp30, NKp46, NKp44 (Correia

et al., 2011; Mikulak *et al*., 2019) and NKG2D (Wrobel *et al*., 2007). Some of the ligands recognized by γδ T-cells through the activating NK cell receptors were not yet described, however, ligands such as ULBP1 (Lança *et al*., 2010), ULBP3 (Poggi *et al*., 2004), ULBP4 (Kong *et al*., 2009), MICA and MICB (Bauer *et al*., 1999; Das *et al*., 2001; Wu *et al*., 2002) have already been reported to activate γδ T-cells through NKG2D. Some of these ligands, namely ULBP4 (Kong *et al*., 2009), MICA and MICB (Bauer *et al*., 1999; Wu *et al*., 2002), have also been demonstrated to activate γδ T-cells via the TCR. In fact, NKG2D and TCR seem to cooperate and provide γδ T-cells the capacity to recognize cancer stem cells since γδ T-cells already showed to target colon cancer stem cells via TCR and to a lesser extent by NKG2D (Todaro *et al*., 2009). Besides activating NK cell receptors and the TCR, γδ T-cells also express inhibitory KIRs as NK cells, which provides them another mechanism through which they can detect tumour cells since they can be activated by cells that lose or downregulate MHC-I expression (**Figure 1.2**)(Dolstra *et al*., 2001; Halary *et al*., 1997).

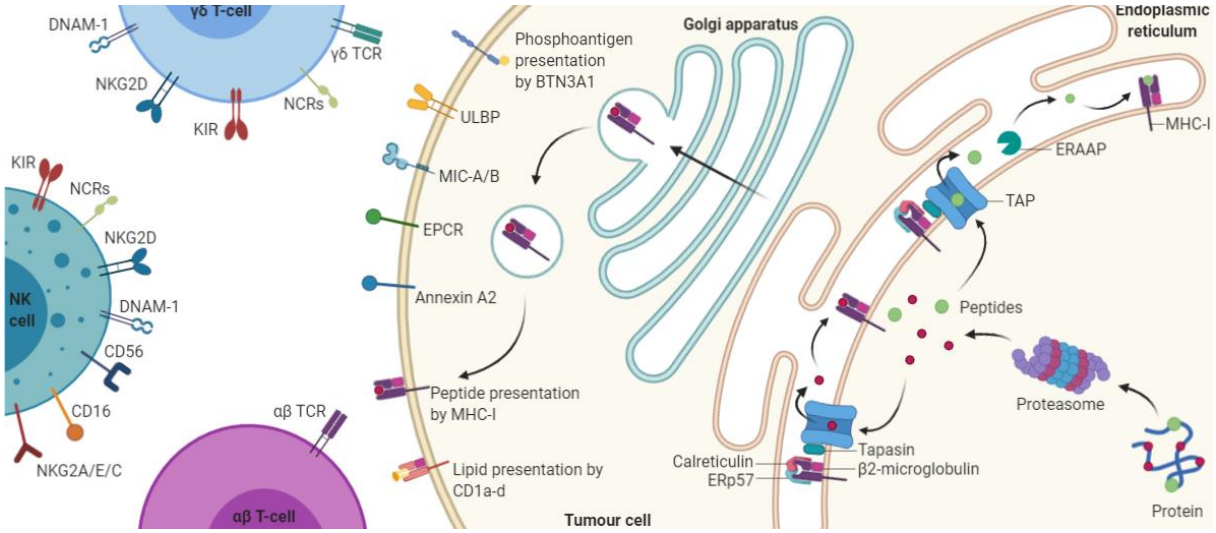


Figure 1.2 – MHC-I antigen presentation pathway and receptor diversity in NK cells and γδ T-cells. MHC-I molecules are constituted by a polymorphic heavy chain and a β2-microglobulin chain. Prior their association with peptides, MHC-I molecules are stabilized in the endoplasmic reticulum (ER) by calreticulin, Erp57 and tapasin. Tapasin interacts with the transporter protein associated with antigen presentation (TAP), which is responsible for the translocation of peptides from the cytoplasm to the ER. Peptides loaded on MCH-I molecules derive from the degradation of viral or self-proteins by the proteasome and are translocated into the ER by TAP. If the peptides continue to be too long after proteasome degradation, before binding to MHC-I molecules, they are trimmed in the ER by the ER aminopeptidase (ERAAP). The binding of peptides to MHC-I molecules results in the release of calreticulin and Erp57. Peptide-MHC-I complexes leave the ER, passing through the Golgi apparatus and being released from it in vesicles that are going to fuse with cells' membrane, allowing peptide-MHC-I complexes to be expressed at cells' surface. Tumour cells may express mutated peptides on their surface through the previously mentioned pathway, which allows αβ T-cells to target them. The lost (*e.g.* via loss of the short arm of the chromosome 6) or downregulation of MHC-I expression by tumour cells enables them to escape αβ T-cell recognition. However, they may still be targeted by γδ T-cells and NK cells, which recognize MHC-I non-restricted ligands, such as ULBP, MICA and MICB. γδ T-cells and NK cells share not only activating receptors, such as NCRs, NKG2D and DNAM1, but also inhibitory KIRs. In addition, γδ T-cells also present a γδ TCR that allows them to recognize ligands such as EPCR, annexin A2, phosphoantigens presented by butyrophilins (*e.g.* BTN3A1) and lipids presented by CD1a-d. Of note that CD1a-d have a similar structure to MHC-I molecules, being also

dependent of β2-microglobulin expression. Defects in β2-microglobulin may therefore also lead to the loss of CD1-restricted antigen-presentation. Illustration created with BioRender.com.

γδ T-cells present a broad spectrum of effector capacities, which are triggered after γδ T-cell activation according to the receptors involved in target cell recognition. For example, the engagement of NKG2D activates cytolytic γδ T-cell responses (Bauer *et al*., 1999), resulting in the killing of target cells through the granzyme-perforin axis as it was already reported to occur in response to tumour cells of RCC (Viey *et al*., 2005), squamous cell carcinoma (SCC) (Alexander *et al*., 2008), CRC (Todaro *et al*., 2009) and CML (D'Asaro *et al*., 2010). The induction of the death receptor signalling pathway in target cells through the production of TNF-related apoptosis-inducing ligand (TRAIL) (Dokouhaki *et al*., 2013; Tawfik *et al*., 2019) and Fas ligand (FasL) (Li *et al*., 2011) is another effector capacity of γδ T-cells that was also already described to be responsible for the killing of tumour cells. γδ T-cells are also able to produce cytokines (*e.g.* TNF-α, IFN-γ and IL-17) (Christmas and Meager, 1990; Lo Presti *et al*., 2017) and to perform antibody-dependent cellular cytotoxicity (ADCC) (Couzi *et al*., 2012). In addition to all these mechanisms that promote target cell death, γδ T-cells can induce innate and adaptive immune responses by functioning as antigen-presenting cells (APCs) of tumour antigens to αβ T-cells (Muto *et al*., 2015) or by co-stimulating the 4-1BB of NK cells, promoting their cytotoxic activity (Maniar *et al*., 2010).

γδ T-cells capacity to recognize surface antigens through different receptors in an MHC-independent manner (Schild *et al*., 1994), bypassing the most common immune evasion mechanism developed by tumour cells, the downregulation of MHC-I expression, and their ability to induce target cell death through various mechanisms called the attention for their use in cancer immunotherapy. Several clinical trials exploring γδ T-cell-based immunotherapies have been developed, specially over the last decade. Most of them are now completed (**Table 9.1** and **Table 9.2** in appendix) and were based in two different strategies: administration of a chemical compound to stimulate γδ T-cell expansion and activation *in vivo* or infusion of *ex vivo* expanded and activated γδ T-cells. Several chemical compounds boost or mimic the expression of phosphoantigens and stimulate directly $Vγ9^{+}Vδ2^{+}$ γδ T-cells. Some examples are the aminobiphosphonates, *e.g.* pamidronate and zoledronate/zoledronic acid, or the synthetic phosphoantigen analogues, such as bromohydrin pyrophosphate (BrHPP) and 2-methyl-3-butenyl-1-pyrophosphate (2M3B1PP), which increase the expression of endogenous phosphoantigens in APCs, such as monocytes, or in tumour cells (Eberl *et al*., 2003; Gu *et al*., 2018; Moulin *et al*., 2017). Despite the variability, most completed clinical trials used zoledronic acid to stimulate γδ T-cells *in vivo* (**Table 9.2** in appendix) or administered it in combination with *ex vivo* expanded γδ T-cells (**Table 9.1** in appendix). No severe toxicity was observed in any of both approaches, however, no substantial anti-tumour activity could be detected (Abe *et al*., 2009; Aoki *et al*., 2017; Bennouna *et al*., 2008, 2010; Kobayashi *et al*., 2011; Lang *et al*., 2011; Nakajima *et al*., 2010; Nicol *et al*., 2011; Pressey *et al*., 2016; Sakamoto *et al*., 2011; Wilhelm *et al*., 2003). Currently, at least 13 clinical trials (**Table 9.3** and **Table 9.4** in appendix, and **Table 1.1**) are exploring γδ T-cells in cancer immunotherapy. Although some of them are exploring technologies as αβ TCR transfer or CAR γδ T-cells since both already demonstrated promising results in pre-clinical studies (Harrer *et al*., 2017), the current recruiting clinical trials are focused on the infusion of allogeneic or autologous γδ T-cells (**Table 1.1**). In contrast with most

completed clinical trials, which were mainly developed for patients with solid tumours, the current recruiting clinical trials are more focused on haematological malignancies (**Table 1.1**), probably due to the higher success of these therapies in this type of cancers.

Table 1.1 - Recruiting clinical trials using γδ T-cell-based ACT for cancer treatment.

Cancer type	Phase	Year	Treatment	Enrolled patients	Clinical trial ID or reference
Leukaemia and myelodysplastic syndrome (MDS)	I	2020	Allogeneic γδ T-cells following hematopoietic stem cell transplant and post-transplant cyclophosphamide	38	NCT03533816
Glioblastoma multiforme	I	2020	Drug-resistant immunotherapy with activated gene-modified γδ T-cells infusion following standard therapy with radiation and chemotherapy (temozolomide)	12	NCT04165941
Hepatocellular carcinoma	I	2020	Allogeneic γδ T-cells	8	NCT04518774
Relapsed or refractory B-cell non-Hodgkin's lymphoma, chronic lymphoblastic leukaemia (CLL) and peripheral T-cell lymphoma	I	2019	Autologous γδ T-cells	6	NCT04028440
Relapsed or refractory AML	I	2019	Allogeneic γδ T-cells	38	NCT04008381
AML	N/A	2018	N/A (**Observational study:** Generation of CD33-CD28 CAR-γδ T-cells)	20	NCT03885076
Relapsed or refractory AML, high-risk MDS or multiple myeloma (MM)	I	2017	Autologous TCR αβ depleted cells expressing heterologous TCR $Vγ9^{+}Vδ2^{+}$	18	NTR6541; (Straetemans *et al*., 2018)

1.5. Aim of the project: tumour-infiltrating NK cells and γδ T-cells for cellular immunotherapy of solid tumours

Compared to αβ T-cells, NK cells and γδ T-cells offer: a) the recognition of multiple antigens instead of a single one, which may prevent tumour immune escape associated with single antigen loss; b) MHC-independent recognition of target cells, which allow their application as an “off-the-shelf” cellular therapy with low to absent risk of alloreactivity and graft-versus-host disease (GvHD) (Godder *et al*., 2007; Perko *et al*., 2015; Rubnitz *et al*., 2010; Ruggeri *et al*., 2002); and c) the simultaneously activation of innate and adaptive anti-tumour responses, since γδ T-cells may promote NK cell cytotoxic activity (Maniar *et al*., 2010) and function as APCs, priming antigens for T-cells (Brandes *et al*., 2005) and inducing a strong $CD8^{+}$ αβ T-cell response (Brandes *et al*., 2009). All these characteristics together with the fact that the infiltration of both cell types into tumours have been correlated with a better prognosis in different types of cancer (Gentles *et al*., 2015; Zhang *et al*., 2020) have led to the development of several NK and γδ T-cell-based immunotherapies. Although cellular therapies using NK cells or γδ T-cells have been

successful in targeting haematological malignancies, their clinical effectiveness in patients with solid tumours has not been very promising. Besides other factors, the appropriate homing into the tumour has been considered one of the most critical factors limiting better clinical outcomes.

Immune cell infiltration into tumour sites depends on interactions between chemokine ligands and receptors. Different NK cell subsets have distinct chemokine receptor repertoires (Berahovich *et al*., 2006; Campbell *et al*., 2001) and are differently distributed in blood and tissues. For example, NK cells expressing C-X-C motif chemokine receptor 3 (CXCR3) accumulate preferentially in tumours (Wendel *et al*., 2008). The same is verified for γδ T-cells: while peripheral blood-derived $V\delta2^+$ γδ T-cells express C-C motif chemokine receptor type 5 (CCR5) (Glatzel *et al*., 2002), and $V\delta1^+$ γδ T-cells are CCR2-positive and respond *in vitro* to C-C motif chemokine ligand 2 (CCL2) activation (Lança *et al*., 2013), tumour-infiltrating $V\delta1^+$ γδ T-cells express CXCR3 and are activated by C-X-C motif chemokine ligand 10 (CXCL10) (Ye *et al*., 2013). Thus, the limited clinical effectiveness of NK and γδ T-cell-based immunotherapies in patients with solid tumours may result from the cell source used to isolate and expand the effector cells. Most of these therapies use PBMCs to isolate and expand their effector cells, which in fact may result in a cell product with low anti-tumour reactivity and/or homing capacity due to the expression of a chemokine receptor repertoire that does not correspond to the ligands expressed by tumour cells. Therefore, there is an urgent need to explore new cell sources that allow the development of a therapy for solid tumours that provides an anti-tumour reactive cellular product, especially against MHC negative tumour cells, and that have capacity to properly infiltrate into the tumour. Based on the assumption that tumour-infiltrating NK cells and γδ T-cells are enriched for tumour antigen-specific target recognition, the isolation and expansion of these cells from the patients' tumour tissue to further use in therapy may allow to have an anti-tumour reactive cell product expressing the appropriate chemokine receptor repertoire to properly infiltrate into the tumour.

In this sense, the main goal of this work was to ascertain the feasibility of isolating and expanding tumour-reactive NK cells and γδ T-cells directly from patients' tumour tissue. For that, the tumour tissue of four patients with cancer was used in one hand to isolate and expand NK cells using IL-2, IL-15 and stimulation with the K-562 cell line, and on the other hand to test the capacity of different expansion protocols using cytokine combinations of IL-2, IL-15, IL-7 or IL-1β along with molecularly defined targets for γδ T-cells to preferentially isolate and expand γδ T-cells. The protocols' effectiveness was assessed through the evaluation of the total cell number, cell viability and NK cells or γδ T-cells' frequency. Finally, the effector function of the isolated NK cells and γδ T-cells was determined through their capacity to produce IFN-γ in response to autologous and/or allogeneic tumour cells.

Overall, this study offers a new prospect for the development of powerful cancer immunotherapies for solid tumours.

2. Materials and methods

2.1. Cell lines, primary cultures and culture conditions

A total of eight cancer cell lines were used in this study as targets for the recognition assays (**section 2.8**) performed to evaluate the effector capacity of the isolated and expanded NK cells and γδ T-cells. These cancer cell lines can be grouped into three types: lymphoblastoid (K-562 and Daudi), pancreatic (PANC-1, Panc 10.05 and CFPAC-1) and colorectal cell lines (HKe-3, SW480 and LoVo).

K-562 (ATCC® - American Type Culture Collection - CCL-243) is a cell line that was established in 1975 by Lozzio and Lozzio (Lozzio and Lozzio, 1975) from a pleural effusion of a 53-year-old female with CML in terminal blast crisis. K-562 cells are known as a standard NK cell target due to their lack of MHC-I expression. Thus, they were used not only as an NK cell target in the recognition assays, but also as feeder cells to stimulate and activate NK cells during their *in vitro* expansion.

Daudi (ATCC® CCL-213™) is a B-cell line that was established in 1967 by E. Klein *et al* (Klein *et al*., 1968) from a 16-year-old African male with Burkitt lymphoma, that is also known to lack MHC-I expression. As K-562 cells, Daudi cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium with GlutaMAX-I™ (Gibco™, Carlsbad, USA) supplemented with 10% (v/v) heat inactivated foetal bovine serum (FBS; Gibco™) and 1% (v/v) penicillin/streptomycin (Thermo Fisher Scientific, Massachusetts, USA).

PANC-1 (ATCC® CRL-2547™) is a cell line established from a pancreatic ductal adenocarcinoma of a 56-year-old Caucasian male. This cell line was maintained in Dulbecco's Modified Eagle Medium (DMEM) with high glucose content (4500 mg/mL) (Gibco™) supplemented with 10% (v/v) heat inactivated FBS (Gibco™) and 1% (v/v) penicillin/streptomycin (Thermo Fisher Scientific).

Panc 10.05 (ATCC® CRL-2547™) is a cell line derived from a pancreatic adenocarcinoma (PDAC) of a Caucasian adult male. Panc 10.05 cells were maintained in RPMI 1640 medium with GlutaMAX-I™ (Gibco™) supplemented with 15% (v/v) heat inactivated FBS (Gibco™), 10 U/mL of human recombinant insulin and 1% (v/v) penicillin/streptomycin (Thermo Fisher Scientific).

CFPAC-1 (ATCC® CRL-1918™) is a cell line established from a ductal adenocarcinoma metastasis in the liver of a 26-year-old Caucasian male with cystic fibrosis. This cell line was maintained in Iscove's Modified Dulbecco's Medium (IMDM) with L-glutamine, 25 mM HEPES without α-thioglycerol and β-mercaptoethanol (Corning Life Sciences, Tewksbury, MA, USA) supplemented with 10% (v/v) heat inactivated FBS (Gibco™) and 1% (v/v) penicillin/streptomycin (Thermo Fisher Scientific).

HKe-3 was donated by Dr. Angela Relogio (Institute for Theoretical Biology, Berlin) and it is a cell line derived from the HCT 116 cell line, which was established from a CRC of a 48-year-old adult male. The HKe-3 cell line was generated through the knockout of the mutated KRAS allele present in HCT 116 cell line.

SW480 (ATCC® CCL-228™) is a cell line established from a 50-year-old Caucasian male with colorectal adenocarcinoma. As HKe-3 cells, this cell line was maintained in DMEM with high glucose content (4500 mg/mL) (Gibco™) supplemented with 10% (v/v) heat inactivated FBS (Gibco™) and 1% (v/v) penicillin/streptomycin (Thermo Fisher Scientific).

LoVo (ATCC® CCL-229™) is a cell line derived from a colon adenocarcinoma metastasis in the left supraclavicular region of a 56-year-old male. These cells were maintained in Ham's F-12K Nutrient Mixture (F-12K) with L-glutamine (Corning Life Sciences) supplemented with 10% (v/v) heat inactivated FBS (Gibco™) and 1% (v/v) penicillin/streptomycin (Thermo Fisher Scientific).

Five types of primary cell cultures derived from patients were used in this study, namely: one B-cell line, TIL, PBMCs, tumour cells and CAFs. The B-cell line was maintained in RPMI 1640 medium with GlutaMAX-I™ (Gibco™) supplemented with 10% (v/v) human serum (HS; Sigma-Aldrich, St. Louis, MO, USA) and 1% (v/v) penicillin/streptomycin (Thermo Fisher Scientific). TIL and PBMCs were maintained in CellGenix® GMP DC medium (CellGenix GmbH, Freiburg, Germany) supplemented with 10% (v/v) HS (Sigma-Aldrich), antibiotics - 1% (v/v) penicillin/streptomycin (Thermo Fisher Scientific), 0.1% (v/v) clindamycin (1 mg/mL; Sigma-Aldrich), 0.5% (v/v) ciprofloxacin (Quinox 2 mg/mL solution for infusion; Fresenius Kabi Italia S.r.l., Verona, Italia) and 0.4% (v/v) amphotericin B (250 µg/mL; VWR, Radnor, PA, USA) (hereafter referred to as culture medium) - and the respective cytokine cocktail suitable for the cell type that was being expanded (details provided in the sections below). Tumour cells and CAFs were cultivated in Connaught Medical Research Laboratories (CMRL, Gibco™) medium supplemented with 0.1% (v/v) GlutaMAX (Gibco™), 20% (v/v) heat inactivated FBS (Gibco™) and antibiotics - 1% (v/v) penicillin/streptomycin (Thermo Fisher Scientific), 0.1% (v/v) clindamycin (1 mg/mL; Sigma-Aldrich), 0.5% (v/v) ciprofloxacin (Quinox 2 mg/mL solution for infusion; Fresenius Kabi Italia S.r.l.) and 0.4% (v/v) amphotericin B (250 µg/mL; VWR). The procedure to establish tumour and CAFs cell cultures is described in detail on **section 2.3**.

All cell lines and primary cell cultures were maintained at 37ºC in a humidified atmosphere containing 5% CO_2. Pancreatic and colorectal cancer cell lines, as well as primary cultures of tumour cells and CAFs were cultured under adherent conditions, while lymphoblastoid cell lines, patient B-cell line and primary cultures of TIL and PBMCs were cultured under suspension conditions. Adherent and suspension cell lines were cultured on tissue culture polystyrene and Nunclon™ Delta surface treated flasks (T-flasks; Thermo Fisher Scientific), however, T-flasks containing suspension cells were maintained in an upright position. TIL and PBMCs were cultured in polystyrene treated 24- or 96-well plates flat bottom (Corning Life Sciences).

For cell expansion and maintenance, adherent cells were split when 70-80% confluence was reached. Cells were washed with phosphate-buffered saline solution (PBS; Corning Life Sciences), incubated 5 min at 37ºC with TrypLE Express (Gibco™) and split through their dilution in pre-warmed medium. Suspension cells were split by dilution and separation of the cell suspension in new T-flasks or wells of a 24-well plate (Corning Life Sciences). TIL and PBMCs medium was refreshed every 3–4 days through half-media changes.

To establish working cell banks, cell lines and primary cell cultures were frozen in a cryopreservation solution of heat inactivated FBS (Gibco™) containing 10% (v/v) of dimethyl sulfoxide (Thermo Fisher Scientific) and stored in liquid nitrogen.

2.2. *Ex vivo* isolation and expansion of NK cells and γδ T-cells

2.2.1. Expansion of NK cells and γδ T-cells directly from patients' tumour tissue

Four patients with cancer (detailed description provided in **Table 2.1**), who were admitted to the Oncology Department at Champalimaud Clinical Center (CCC), provided written informed consent and were enrolled in the present study after approval by the institution's ethics committee. Tumour tissue from clinical biopsies or surgery were cut with a scalpel into 1–2 mm^3 tumour fragments, which were placed in groups of 2–3 in each well of a 24-well plate (Corning Life Sciences). Culture conditions varied according to the cell type to be expanded.

Tumour fragments from which NK cells were expanded were maintained in culture medium supplemented with IL-2 (1000 IU/mL; Miltenyi Biotec, Bergisch Gladbach, Germany) and IL-15 (180 IU/mL; Miltenyi Biotec). Since it was not possible to define the number of NK cells present in the cultured tumour tissue, feeder cell stimulation was performed every 10 days with $0.10x10^6$ K-562 cells irradiated at 55 gray (Gy).

To determine the best expansion conditions for γδ T-cells, tumour fragments were maintained in culture medium supplemented with different cytokine combinations: a) IL-2 (1000 IU/mL; Miltenyi Biotec), IL-15 (180 IU/mL; Miltenyi Biotec) and IL-1β (1000 IU/mL; Miltenyi Biotec); b) IL-2 (1000 IU/mL; Miltenyi Biotec) and IL-7 (1000 IU/mL; Miltenyi Biotec) and c) IL-7 (1000 IU/mL; Miltenyi Biotec) and IL-1β (1000 IU/mL; Miltenyi Biotec). A pool of 55 Gy irradiated PBMCs from three healthy donors was used as allogeneic feeder cells to stimulate γδ T-cells every 10 days. A total of five stimulations were performed by adding *per* well $1x10^6$ feeder cells treated with 5 μM of zoledronic acid (Aclasta 0.05 mg/mL solution for infusion; Novartis, Basiléia, Switzerland) or along with 30 ng/mL of anti-human CD3 antibody (clone OKT3, BioLegend, CA, USA). The first two stimulations were performed with feeder cells treated with zoledronic acid, while the others alternated between addition of feeder cells along with anti-human CD3 antibody and feeder cells treated with zoledronic acid.

Tumour fragments were removed from all conditions after 10 days of culture. The total cell number, cell viability and the NK cells or γδ T-cells' frequency started to be evaluated after the second stimulation with feeder cells. From that point on, these parameters were always assessed between stimulations, except the NK cells or γδ T-cells' frequency that was only evaluated when there was a reasonable number of cells to be removed to perform flow cytometry. After five stimulations with feeder cells, NK cells and γδ T-cells (from the best expansion condition(s)) were sorted by fluorescence-activated cell sorting (FACS) (**section 2.7**) and their effector capacity was determined through the evaluation of IFN-γ production against autologous (when available) and/or allogeneic tumour cell lines (**section 2.8.2**).

2.2.2. γδ T-cell expansion from TIL and PBMCs

In addition to the tumour tissue obtained from patients undergoing routine surgical procedures at CCC, we had access to tumour cell lines, TIL and/or PBMCs from two patients (D82 and D83, detailed description in **Table 2.1**) treated at a collaborating hospital (outside Portugal) with TIL therapy followed by high-dose IL-2 infusion. TIL used to perform the first cell infusion of patient D82, PBMCs collected after patient D83 first TIL infusion, as well as TIL from patient D126 (detailed description in **Table 2.1**) before receiving any treatment at the CCC were used to *ex vivo* expand γδ T-cells using the protocol

that showed best results in the expansion of γδ T-cells directly from patients' tumour tissue. Of note that PBMCs from patient D83 were isolated and freshly frozen, while TIL from patients D82 and D126 were expanded from tumour pieces using the optimal conditions for αβ T-cell growth (medium supplemented with IL-2, IL-15 and IL-21 and stimulation with allogeneic PBMCs and anti-human CD3 antibody) and then frozen. Moreover, after this first expansion, TIL from patient D126 were thawed and before being frozen again were cultured for 14 days under good manufacturing practice conditions to expand αβ T-cells.

Cells were thawed and cultured in a 24-well plate (Corning Life Sciences) with culture medium supplemented with IL-2 (1000 IU/mL; Miltenyi Biotec), IL-15 (180 IU/mL; Miltenyi Biotec) and IL-1β (1000 IU/mL; Miltenyi Biotec). Cells were stimulated every 10 days with allogeneic feeder cells (pool of PBMCs from three healthy donors) irradiated at 55 Gy. A total of five stimulations were performed by adding *per* well $1x10^6$ feeder cells treated with 5 μM of zoledronic acid (Aclasta 0.05 mg/mL solution for infusion; Novartis) or along with 30 ng/mL of anti-human CD3 antibody (clone OKT3, BioLegend). The first two stimulations were performed with feeder cells treated with zoledronic acid, while the others alternated between addition of feeder cells along with anti-human CD3 antibody and feeder cells treated with zoledronic acid. The total cell number, cell viability and the γδ T-cells' frequency started to be evaluated after the second stimulation with feeder cells. From that point on, these parameters were always assessed between stimulations, except the γδ T-cells' frequency that was only evaluated when there was a reasonable number of cells to be removed to perform flow cytometry. After five stimulations, γδ T-cells were negatively sorted by FACS (**section 2.7**) and their effector capacity was determined through the evaluation of IFN-γ production against allogeneic cancer cell lines and autologous tumour cells (patients D82 and D83) or autologous Epstein-Barr virus (EBV)-transformed B-cells (patient D126) (**section 2.8.2**).

Table 2.1 – Patient clinical data.

Patient ID	Gender	Age	Sample type	Tumour type	Treatment
D155	Male	82	Tumour tissue	Rectal cancer	-
D157	Female	72	Tumour tissue	Ovarian cancer metastasis in colon	-
D162	Male	81	Tumour tissue	Colon adenocarcinoma	-
D171	Male	43	Tumour tissue	Rectum adenocarcinoma	-
D82	Male	76	TIL	Glioblastoma	TIL therapy followed by high-dose IL-2 infusion
D126	Female	63	TIL	PDAC	-
D83	Male	39	PBMCs*	Pancreatic cancer, lung metastasis, peritoneal metastasis, lymphatic metastasis	TIL therapy followed by high-dose IL-2 infusion

* TIL were no longer available.

2.3. Establishment of patients' tumour cell lines

Four of the seven samples from patients with cancer used in this study correspond to tumour tissue obtained from clinical biopsies or surgeries performed at the CCC. To establish an autologous tumour cell line for each patient, samples were cut with a scalpel into 1–2 mm^3 tumour fragments and then groups of 2–3 fragments were placed in each well of a 24-well plate (Corning Life Sciences). Cells were

maintained in CMRL (Gibco™) medium supplemented with 0.1% (v/v) GlutaMAX (Gibco™), 20% (v/v) heat inactivated FBS (Gibco™) and antibiotics. Tumour fragments were removed after 10 days in culture and when confluence was achieved, cells were washed with PBS (Corning Life Sciences), harvested with TrypLE Express (Gibco™) and sub-cultured. Only for patient D157 was possible to establish an autologous tumour cell line since for patients D155, D162 and D171 only CAFs adhered. However, CAFs were maintained in culture under the same conditions that tumour cells. Tumour cells and CAFs were clearly distinguished by morphological observation since CAFs present an elongated morphology, while tumour cells have a more "cobblestone" appearance.

2.4. Determination of cell concentration and viability

Cell concentration and viability were determined using the trypan blue exclusion assay. This method is based on the concept that dead cells lose their membrane integrity being not able to exclude certain dyes, such as trypan blue, becoming stained in their presence. Cells were diluted in 0.4% (w/v) Trypan Blue in PBS (Corning Life Sciences) and the counting was performed in a haemocytometer (Neubauer improved bright-line chamber; Sigma-Aldrich), using an inverted microscope (ZEISS Primovert inverted microscope, ZEISS, Oberkochen, Germany). Cell concentration and viability were calculated according to **Equation 1** and **Equation 2**, respectively, while the total cell number was obtained by multiplying the cell concentration *per* the total volume of cell suspension.

Equation 1

$$\text{Cell concentration (cells/mL)} = \frac{\text{Number of viable cells}}{\text{Number of squares}} \times \text{Dilution factor} \times 10^4$$

Equation 2

$$\text{Cell viability (\%)} = \frac{\text{Number of viable cells}}{\text{Total number of cells}} \times 100$$

2.5. Phenotypic analysis

To monitor the expansion of NK cells and γδ T-cells during their time in culture, a phenotypic analysis by flow cytometry was performed. For NK cells, the LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (Thermo Fisher Scientific) was used with the following antibodies: anti-human CD45 APC Alexa Fluor 700 (clone HI30, BioLegend), anti-human CD3 PE-Cy™7 (clone UCHT1, BD Biosciences, CA, USA), anti-human CD56 PE (clone NCAM16.2, BD Biosciences) and anti-human CD16 BV785 (clone B73.1, BioLegend). For γδ T-cells, the LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (Thermo Fisher Scientific) was only used with the anti-TCR PAN γδ antibody PC5.5 (clone GL3, Beckman Coulter, Brea, CA, USA). Briefly, cells were washed with PBS (Corning Life Sciences) and incubated for 20 min with a master mix containing the viability dye and the respective antibodies. After the staining, cells were washed with PBS (Corning Life Sciences) with 2% heat inactivated FBS (Gibco™) - hereafter referred to as FACS buffer -, resuspended in 200 µL of FACS buffer and analysed by flow cytometry (BD LSRFortessa™ X-20, BD Biosciences). Data analysis was performed using FlowJo software version 10 and gating strategies are shown in **Figure 9.3 in Appendix**.

2.6. Immunohistochemistry (IHC)

Paraffin-embedded tumour tissue samples from patients enrolled in this study were provided by the Biobank of the Champalimaud Foundation to evaluate γδ T-cell infiltration in the tumour tissue. Five-µm-thick sections were cut from the paraffin blocks by the Histopathology Platform of the Champalimaud Foundation and were used for IHC of TCR δ chain. As a positive control, sections from a paraffin-embedded tissue of a normal colon were used. Briefly, paraffin sections were deparaffinized, washed in PBS (Corning Life Sciences) and incubated at room temperature for 15 min in PBS with 3% hydrogen peroxide (30% v/v, Thermo Fischer Scientific) to block internal peroxidases activity to avoid unspecific signals, since it was used a horseradish peroxidase (HRP) detection system. Sections were sequentially washed in PBS (Corning Life Sciences), distilled water and then placed, during 30 min, into a vegetable steamer cooker in a previously heated (30 min) Epitope Retrieval Solution 1x concentrate (Epitope Retrieval Solution, 10x concentrated, pH=9; Novocastra™ Leica Biosystems, Newcastle, United Kingdom). After cooling down, sections were sequentially washed in distilled water, PBS (Corning Life Sciences) and then incubated for 30 min at room temperature in PBS with 2% bovine serum albumin (Thermo Fisher Scientific) – hereafter referred as blocking solution. The staining of TCR δ chain was performed using the anti-TCR δ monoclonal antibody (H-41, 100 µg/mL, Santa Cruz Biotechnology, Texas, USA) diluted 1:50 in blocking solution and incubated with the tumour sections overnight in a wet chamber at 4ºC. Next day, tumour sections were sequentially washed with distilled water, PBS (Corning Life Sciences) and then incubated for 1 h inside a wet chamber at room temperature with a goat anti-mouse IgG HRP-conjugated secondary antibody (Thermo Fischer Scientific) diluted 1:500 in blocking solution. Samples were sequentially washed in PBS (Corning Life Sciences), PBS with 0.5% (v/v) Triton® X-100 (Fisher Scientific) for 1 min and then developed for 15 min with the chromogen 3,3'-Diaminobenzidine (Alfa Aesar, Thermo Fisher Scientific). Sections were counterstained with Harris haematoxylin solution, modified (Sigma-Aldrich) for 10 sec, washed with water and then dehydrated and mounted. Next day, images of the slides were acquired with magnifications of 10x/20x and 40x using a slide scanner (Philips Ultra fast scanner 1.6, Amsterdam, Netherlands).

2.7. FACS

For the functional assays described in **section 2.8**, effector and/or target cells were sorted by FACS (FACSAria™ Fusion, BD Biosciences). Two different types of effector cells were sorted: the NK cells and the γδ T-cells. NK cells expanded from the PBMCs of healthy donors and used to optimize the functional assays (CD107a assay and IFN-γ production assay) were obtained through a positive sorting using the LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (Thermo Fisher Scientific) and the following antibodies: anti-human CD3 PE-Cy™7 (clone UCHT1, BD Biosciences), anti-human CD56 PE (clone NCAM16.2, BD Biosciences) and anti-human CD16 BV785 (clone B73.1, BioLegend). NK cells isolated from patients' tumour tissue were obtained through a negative sorting using the LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (Thermo Fisher Scientific) and the anti-human CD3 PE-Cy™7 antibody (clone UCHT1, BD Biosciences). γδ T-cells were negatively sorted using the LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (Thermo Fisher Scientific) and the anti-human αβ TCR APC-Cy™7 antibody (clone IP26, BioLegend). Gating strategies to sort effector cells are summarized in **Figure 9.2 in Appendix**.

Two main types of target cells were used in the functional assays, namely autologous cells and/or allogeneic cancer cell lines. Allogeneic cancer cell lines and the autologous EBV-transformed B-cells were only stained with the LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (Thermo Fisher Scientific), while autologous tumour cells and CAFs were additionally stained with the anti-human epithelial cell adhesion molecule (EpCAM) APC/Fire™ 750 antibody (clone 9C4, BioLegend), which allowed to distinguish tumour cells ($EpCAM^+$) from CAFs ($EpCAM^-$).

Briefly, cells were washed with PBS (Corning Life Sciences) and incubated for 20 min with a master mix containing the respective antibodies (according to each cell type) and/or the viability dye. After the staining, cells were washed with FACS buffer, resuspended in FACS buffer and sorted by FACS (BD FACSAria™ Fusion, BD Biosciences).

2.8. NK cells and γδ T-cells functional assays

2.8.1. Degranulation assay

T lymphocytes and NK cells present cytolytic granules that contain different types of cytolytic proteins, such as granzyme and perforin, which are released after activation of these cells to induce target cell death. Vesicles that contain these cytolytic proteins present lining their membranes the lysosomal-associated membrane protein-1, LAMP-1 or CD107a, whose expression can be evaluated to assess the cytotoxic ability of an effector cell during/after its stimulation.

The degranulation assay was used to determine NK cell functionality after their *in vitro* expansion. CD107a expression was assessed after NK cell stimulation with the MHC-I negative cell line K-562. Different effector:target cells ratios were tested - 1:1, 3:1 and 10:1 -, using in all conditions $2.5x10^4$ or $3x10^4$ target cells and adjusting the number of effector cells according to the ratio being tested. Briefly, K-562 cells were sorted and simultaneously distributed into FACS tubes (Falcon™, Corning Life Science) (**section 2.7**), while NK cells after 14 days of expansion were washed with PBS (Corning Life Sciences), resuspended in medium supplemented with IL-2 (1000 IU/mL, Miltenyi Biotec) and IL-15 (180 IU/mL, Miltenyi Biotec), counted and manually distributed into the respective FACS tubes. Cells were co-cultured for 1 h at 37ºC, in a humidified atmosphere containing 5% CO_2 and in the presence of the anti-CD107a PE antibody (clone H4A3, BD Biosciences). After 1 h, protein transport inhibitor containing monensin (BD GolgiStop™, BD Biosciences) was added to the medium and incubated for 5 h to inhibit trans-Golgi apparatus function. Tubes with only effector cells were used as negative controls, whereas tubes with effector cells in the presence of 1 µg/mL of phorbol-12-myristate-13-acetate (PMA; 25 ng/mL; Sigma-Aldrich) for maximal stimulation were used as positive controls. Negative and positive controls were incubated simultaneously with anti-CD107a PE antibody (clone H4A3, BD Biosciences) and a transport inhibitor containing monensin (BD GolgiStop™, BD Biosciences) for 2 h at 37ºC in a humidified atmosphere containing 5% CO_2. After incubation, cells from all conditions were stained with the LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (Thermo Fisher Scientific) and the anti-human CD3 PE-Cy™7 (clone UCHT1, BD Biosciences), which did not allow to directly determine the $CD107a^+$ NK cells' frequency in the degranulation assay due to a colour incompatibility between CD107a and CD56 antibodies. To define the percentage of NK cells that were $CD107a^+$, a sample of cells was removed before starting the degranulation assay and stained with LIVE/DEAD™ Fixable Aqua Dead Cell Stain

Kit (Thermo Fisher Scientific), anti-human CD3 PE-Cy™7 (clone UCHT1, BD Biosciences), anti-human CD56 PE (clone NCAM16.2, BD Biosciences) and anti-human CD16 BV785 (clone B73.1, BioLegend). In both staining procedures, cells were washed with PBS (Corning Life Sciences) and incubated 20 min at room temperature with the master mix containing the viability dye and the respective antibodies. After the staining, cells were washed with FACS buffer, resuspended in 200 µL of FACS buffer and analysed by flow cytometry (BD LSRFortessa™ X-20, BD Biosciences). Data analysis was performed using FlowJo software version 10. To determine the $CD107a^+$ NK cells' frequency, the NK cell percentage inside the $CD3^-$ gate obtained in the routine flow cytometry analysis and the percentage of $CD107a^+$ cells inside the $CD3^-$ gate obtained in the degranulation assay were considered and the calculations were performed according to **Equation 3**. Once donor K did not present enough NK cells, this assay was only performed with NK cells from donors J and L. Experiments were conducted in triplicates. A response was considered positive when the frequency of $CD107a^+$ cells following stimulation with the target cell line was at least three-fold higher than the unstimulated control.

Equation 3

$$\text{CD107a}^+\text{ NK cells} = \frac{\text{CD107a}^+\text{cells inside CD3}^-\ (\%) \times \text{NK cells inside CD3}^-(\%)}{100}$$

CD107a data was adjusted to reflect the $CD107a^+$ NK cells when using $3x10^4$ effector cells.

2.8.2. IFN-γ production analysis

The effector capacity of the expanded cells was also analysed through their ability to produce IFN-γ when co-cultured with target cells. This assay was performed in two different contexts: a) to determine the optimal effector cell:target cell ratio for assays with NK cells and b) to evaluate the effector capacity of NK cells and γδ T-cells expanded directly from the tumour tissue.

The IFN-γ production assay was optimized using NK cells expanded from PBMCs of donors J, K and L as effector cells and the MHC-I negative cell line K-562 as target cells. When the final number of NK cells was sufficient, three different effector cell:target cell ratios were tested - 1:1, 3:1 and 10:1. A total of $2.5x10^4$ or $3x10^4$ viable target cells were used, and the respective number of effector cells was adjusted according to the ratio being tested. Briefly, K-562 cells were sorted and simultaneously distributed in a 96-well plate round bottom (Corning Life Sciences) (**section 2.7**), while NK cells after 14 days of expansion were washed with PBS (Corning Life Sciences), resuspended in medium supplemented with IL-2 (1000 IU/mL, Miltenyi Biotec) and IL-15 (180 IU/mL, Miltenyi Biotec), counted and manually distributed into the 96-well plate round bottom (Corning Life Sciences).

In the IFN-γ production assays performed to analyse the effector capacity of the NK cells and γδ T-cells expanded directly from patients' tumour tissue, different autologous (a) and allogeneic (b) target cells were used: a) tumour cells, CAFs and/or EBV-transformed B-cells (when available) and b) lymphoblastoid (K-562 and Daudi), pancreatic (PANC-1, Panc 10.05 and CFPAC-1) and colorectal (HKe-3, SW480 and LoVo) cancer cell lines. Different expansion conditions for γδ T-cells were tested, however only cells expanded in culture medium supplemented with a) IL-2 (1000 IU/mL, Miltenyi Biotec), IL-15 (180 IU/mL, Miltenyi Biotec) and IL-1β (1000 IU/mL, Miltenyi Biotec) and/or b) IL-2 (1000 IU/mL, Miltenyi Biotec) and IL-7 (1000 IU/mL, Miltenyi Biotec) were used. Independently of the effector cell type

being tested, an effector:target cells ratio of 1:1 was applied and a total of $1x10^3$ or $2x10^3$ effector cells/well was used, according to the number of effector cells available. The only exception was the γδ T-cells from patient D82, which were tested with an effector cell:target cell ratio of 4:1 due to a technical error. Target cells were sorted and simultaneously distributed in a 96-well plate round bottom (Corning Life Sciences) (**section 2.7**), while effector cells were sorted, washed, resuspended in the respective medium and manually distributed. According to the effector cell type being tested, the culture medium used was supplemented with different cytokines: a) IL-2 (333 IU/mL, Miltenyi Biotec) and IL-15 (60 IU/mL, Miltenyi Biotec) for NK cells and b) IL-2 (333 IU/mL, Miltenyi Biotec), IL-15 (60 IU/mL, Miltenyi Biotec) and IL-1β (333 IU/mL, Miltenyi Biotec) for γδ T-cells. To determine whether the γδ T-cell response against the target cell lines tested was generated through TCR recognition, their co-culture was also performed in the presence of a) an anti-human γδ TCR blocking antibody (5 µg/mL, clone B1, BioLegend) or b) a monoclonal mouse IgG1 κ isotype control antibody (5 µg/mL, clone MOPC-21, BioLegend). When the number of cells obtained was not sufficient, not all test conditions were performed for all target cell lines, as indicated in the corresponding figure(s).

For both assays, wells with only effector cells were used as negative controls, whereas wells with effector cells in the presence of 5 µg/mL of phytohaemagglutinin (PHA, Sigma-Aldrich) for maximal stimulation were used as positive controls. For γδ T-cells assays, two additional controls were performed: a) only effector cells in the presence of the anti-human γδ TCR blocking antibody (5 µg/mL, clone B1, BioLegend) and b) only effector cells in the presence of the monoclonal mouse IgG1 κ isotype control antibody (5 µg/mL, clone MOPC-21, BioLegend). In all conditions, cells were incubated for 5 days, at 37ºC in a humidified atmosphere containing 5% CO_2. Supernatants were harvested at day 5 and tested for the presence of IFN-γ using the Enzyme-Linked Immunosorbent Assay (ELISA) for IFN-γ (Mabtech, Nacka Strand, Sweden) according to manufacturer instructions. Biological triplicates were performed for all conditions, whenever possible.

The IFN-γ data correspondent to the optimization of the effector cell:target cell ratio to be used in the NK cell assays were adjusted to $1x10^4$ target cells and is reported after medium subtraction as IFN-γ pg/mL/5 days, while the IFN-γ data correspondent to the evaluation of the expanded NK cells and γδ T-cells effector capacity were adjusted to $1x10^4$ effector cells and is reported after medium subtraction as IFN-γ pg/mL/10^4 cells/5 days. The results are shown as the mean of the biological triplicates or duplicates (when possible). A response was considered positive when the IFN-γ production following stimulation with target cells was at least three-fold higher than the unstimulated control.

2.9. Statistical analysis

The statistical analysis presented in this master thesis was kindly performed by “Consultório Estatístico” from Centro de Estatística e Aplicações da Universidade de Lisboa (CEAUL). To determine the factors influencing the NK cell expansion, a model was fit to data using as response variables a) the cytokine combination, b) the presence or absence of K-562 cell stimulation and c) the time point to sort NK cells. A generalized linear mixed model was implemented to fit with function glmmTMB (Brooks *et al*., 2017) in the R environment for statistical computing (R Core Team, 2020). Two random effects were considered, one to allow for different intercepts *per* patient, and another to account for temporal

autocorrelation within group, modelled as a 1st order autoregressive process. The same analysis was performed for γδ T-cell data in order to determine the impact of the different cytokine combinations (response variable) in the a) total cell number, b) cell viability and c) γδ T-cells' frequency.

3. Results

3.1. Optimization of the conditions to expand NK cells *in vitro*

The main goals of expanding NK cells *ex vivo* for adoptive immunotherapy are to yield high cell numbers and to obtain an NK cell product with increased cytotoxic capacity. For this purpose, during *in vitro* expansion, NK cells need to be cultured with appropriate stimuli. Cytokines play a very important role in NK cell maturation, survival, proliferation and cytotoxicity, though additional stimulatory signals, such as the presence of monocytes (Miller *et al*., 1992) or B-lymphoblastoid cells (Igarashi *et al*., 2004; Rabinowich *et al*., 1991), are also required to sustain NK cell proliferation. Thus, before addressing the feasibility of isolating and expanding NK cells directly from patients' tumour tissue, different expansion protocols to grow NK cells *in vitro* were tested. PBMCs from healthy donors (donor G, H and I) were used and four different cytokine combinations were tested (IL-2+IL-15+IL-21; IL-2; IL-2+IL-15 and IL-15) in the presence or absence of K-562 cell stimulation. The $CD3^-$ population was obtained from PBMCs and cultured for 7 days under the eight expansion protocols, after which NK cells were sorted and maintained at the same conditions for more 7 days. To compare the expansion protocols' efficiency the total cell number, cell viability and NK cells' frequency were assessed over time (**Figure 8.1 in Supplementary material**). The stimulation with K-562 cells resulted in higher total cell numbers (**Figure 8.1 A in Supplementary material**), cell viabilities (**Figure 8.1 B in Supplementary material**) and NK cells' frequencies (**Figure 8.1 C in Supplementary material**). IL-2+IL-15 appeared to be the most effective cytokine combination. However, irrespective of the expansion protocol, the total cell numbers obtained were generally low. Thus, the influence of different time points to sort NK cells (at day 0, after a 7- or 14-day expansion of the $CD3^-$ population) was evaluated using all the previously tested culture conditions. However, since the use of K-562 cell stimulation enhanced NK cell expansion, only these results are shown in **Figure 8.2 in Supplementary material**. Almost no differences were detected in the NK cell percentage-fold increase (approximately 3-fold, **Figure 8.2 C in Supplementary material**), however, the time point chosen to isolate NK cells influenced the cell viability (**Figure 8.2 B in Supplementary material**) and the total cell number-fold increase (**Figure 8.2 A in Supplementary material**). The highest cell viabilities (98-99%) were obtained when NK cells were sorted from the $CD3^-$ population after a 14-day expansion, whereas the highest total cell number-fold increase was obtained when the sorting was performed at day 0. A model was fit to data to determine the variables (cytokine combination, presence or absence of K-562 cell stimulation and time point to sort NK cells) that were affecting each assessed parameter (total cell number, cell viability and NK cells' frequency). The total cell number was statistically significantly higher with the K-562 cell stimulation ($p<0.001$) and when NK cells were sorted after a 14-day expansion of the $CD3^-$ population ($p<0.001$). Cell viability also demonstrated a statistically significant increase with the K-562 cell stimulation ($p<0.01$) and the use of IL-2+IL-15 ($p<0.05$), however, the sorting of NK cells after the 14-day expansion of the $CD3^-$ population contributed to its decrease ($p<0.05$). Finally, the NK cells' frequency only showed to be affected by the time point in which NK cells were sorted, decreasing when cells were obtained following the culture of the $CD3^-$ population ($p<0.001$). In conclusion, isolation of NK cells at day 0 and subsequent culture with

IL-2+IL-15 and K-562 cell stimulation for 14 days appeared to result in the best trade-off between total cell number, cell viability and NK cells' frequency.

3.2. *Ex vivo* expanded NK cells express CD107a and produce IFN-γ when co-cultured with the MHC-I negative cell line K-562

Although different expansion protocols were tested to grow *in vitro* NK cells, all conditions resulted in low total cell numbers, ranging from $0.003x10^6$ (IL-2+IL-15+IL-21) to $0.182x10^6$ total cells (IL-2+IL-15+K-562 cell stimulation, **Figure 8.1 A in Supplementary Material**). The most suitable protocol (IL-2+IL-15 and K-562 cell stimulation) was used a) to determine the influence of different initial cell concentrations in the total cell numbers obtained and b) to evaluate the phenotype and the effector capacity of the NK cells grown under these conditions. For this purpose, NK cells were isolated from PBMCs of three healthy donors (donor J, K and L) and co-cultured for 14 days with irradiated K-562 cells (at a 10:1 immune cell:target cell ratio) in the presence of IL-2+IL-15 at different cell densities ($0.5x10^6$, $1x10^6$ and $2x10^6$ cells/mL). Cell number and cell viability were evaluated at different time points and showed that the initial cell concentration did not influence cell viability and is not a determinant factor in the fold-expansion reached by NK cells, although, as expected, higher initial cell densities result in higher cell numbers at the end of the expansion (**Figure 8.3 in Supplementary data**). The frequency of the different NK cell subsets was determined at the beginning (day 0) and end of the experiment (day 14, **Figure 3.1**). Irrespective of donor-dependent differences, the $CD56^{dim}$ was the main NK cell subset detected at day 0 in NK cells from donors J (26.2%) and L (28.31%), whereas donor K presented more $CD56^{bright}$ (28.45%) than $CD56^{dim}$ NK cells (9.70%). After a 14-day expansion, NK cells showed an increase in the CD56 expression and $CD56^{bright}$ NK cells represented the main NK cell subset in cells from all donors, comprising between 88.8 (donor L) and 96.1% (donor J) of all NK cells. In the beginning of the expansion, $CD16^+$ NK cells ($CD56^{dim}CD16^+$ and $CD56^{bright}CD16^+$) were the most frequent within each NK cell subset. However, after 14 days in culture, $CD56^{dim}$ NK cells lost their CD16 expression and the $CD56^{dim}CD16^-$ became the main $CD56^{dim}$ subset, whereas $CD56^{bright}CD16^+$ continued to represent the majority of the $CD56^{bright}$ NK cells.

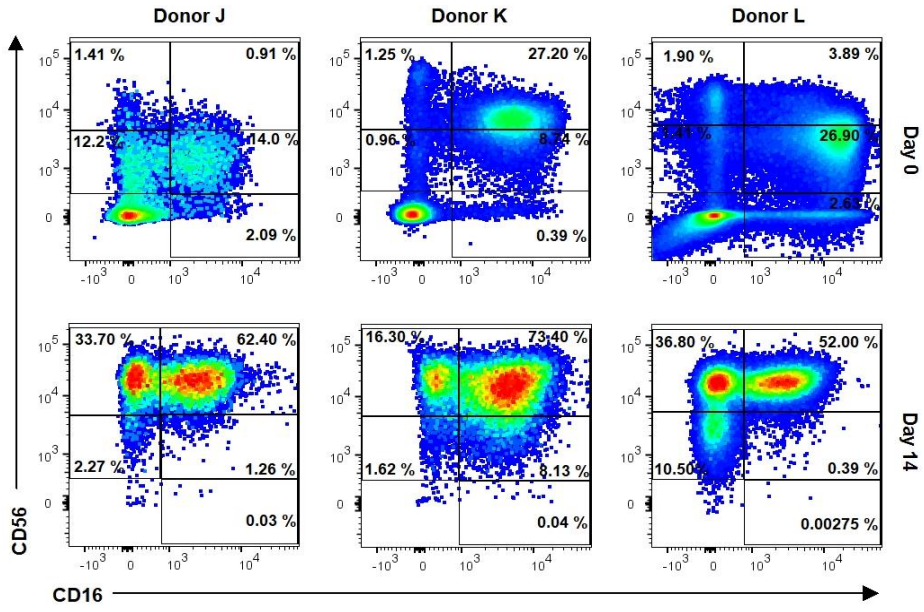


Figure 3.1 – *Ex vivo* expansion of NK cells with IL-2+IL-15 and K-562 cell stimulation promotes the CD56^{bright} phenotype. NK cells isolated from the PBMCs of three healthy donors (donor J, K and L) through a positive sorting were cultured in the presence of IL-2+IL-15 and stimulated with irradiated K-562 cells at an immune cell:target cell ratio of 10:1. The frequency of the NK cell subsets was determined by flow cytometry at the beginning (day 0) and end of the culture (day 14).

NK cells contribute to the anti-tumour immunity by promoting tumour cell death through the death ligand TRAIL, the release of cytolytic molecules, such as perforin and granzymes, or by secreting the pro-inflammatory cytokine IFN-γ (Pegram *et al*., 2010). Any of the previously mentioned mechanisms can be used as an indicator of the effector capacity of NK cells. Here, the functionality of the NK cells after 14 days of *ex vivo* expansion was evaluated by assessing the expression of the CD107a and the IFN-γ production after NK cells co-culture with the MHC-I negative cell line K-562. NK cells from all donors were isolated through a positive sorting and co-cultured with the K-562 cell line at different effector cell:target cell ratios (1:1, 3:1 and 10:1), when the total cell number obtained was sufficient. The expression of CD107a was evaluated by flow cytometry, whereas the IFN-γ produced by NK cells in response to different target cell lines was evaluated through an ELISA for IFN-γ (**Figure 3.2**).

CD107a is a protein expressed in the cytolytic granules that contain perforin and granzymes. The fusion of the cytolytic granules with the immune cell membrane upon cell activation leads CD107a to be located in the cell surface. The evaluation of CD107a expression through antibody binding can be used as an indicator of the immune cell effector capacity and has already been shown to be a good marker for NK cell activation and function (Alter *et al*., 2004). Here, CD107a expression was determined after the co-culture for 6 h of the expanded NK cells with the K-562 cell line in the presence of the anti-CD107a antibody and monensin. Due to the low cell numbers obtained, this assay was only performed for cells from donor L at a 1:1, 3:1 and 10:1 effector cell:target cell ratios, and donor J at an effector cell:target cell ratio of 1:1. *Ex vivo* expanded NK cells showed to be capable of degranulating in response to target cell stimulation (**Figure 3.2 A**). Irrespective of the effector cell:target cell ratios, unstimulated NK cells from both donors expressed CD107a, presenting between 0.57 and 3.06% CD107a$^+$ NK cells, revealing that these cells were activated during culture. PMA stimulation only led to a considerable increase in the percentage of CD107a$^+$ NK cells in cells from donor L at a 1:1 (7.99% CD107a$^+$ NK cells) and 10:1 (3.52% CD107a$^+$ NK cells) effector cell:target cell ratios. Upon culture with K-562 cells, the frequency of CD107a$^+$ NK cells increased in cells from both donors, independently of the tested effector cell:target cell ratio, which demonstrate the capacity of the expanded NK cells to respond to target cells. The highest percentage of CD107a$^+$ NK cells was 60.53% and it was observed in cells from donor L at a 1:1 effector cell:target cell ratio. In cells from donor L, the frequency of CD107a$^+$ NK cells appeared to be inversely correlated with the effector cell:target cell ratio once 60.53%, 30.63 % and 13.61% CD107a$^+$ NK cells were obtained at a 1:1, 3:1 and 10:1 effector cell:target cell ratios, respectively. However, it is premature to correlate these two factors, being necessary to analyse more patients.

In addition to NK cell capacity to secrete cytolytic proteins, the release of IFN-γ is also fundamental in anti-tumour directed immune responses since NK cells were shown to infiltrate into tumour-developing lesions at an early stage during cancer progression/malignant transformation and they have been suggested to contribute to the early anti-tumour activity through the production of high

amounts of IFN-γ (Kurosawa *et al*., 1993). Thus, the capacity of the expanded NK cells to produce IFN-γ in response to the MHC-I negative cell line K-562 after a 5-day co-culture at different effector cell:target cell ratios (1:1, 3:1 and/or 10:1) was also investigated. *Ex vivo* expanded NK cells presented the ability to recognize target cells and consequently produce IFN-γ (**Figure 3.2 B**). Irrespective of the effector cell:target cell ratios, unstimulated NK cells from all donors showed some basal production of IFN-γ, ranging between 12.90 and 365.98 pg/mL/5days, which indicates that these cells were activated during culture by the stimuli provided. Although PMA stimulation should induce maximum cell activation, only NK cells from donor J at an effector cell:target cell ratio of 10:1 produced IFN-γ (145.84 pg/mL/5 days) in response to PMA stimulation. The K-562 cell line was recognized and induced NK cells from all donors to produce IFN-γ, however not at all effector cell:target cell ratios. Although NK cells from donor J produced IFN-γ in response to K-562 cells at an effector cell:target cell ratio of 10:1 (63.84 pg/mL/5 days), NK cells from donor K and L produced only IFN-γ at a 1:1 effector cell:target cell ratio (270.17 and 897.02 pg/mL/5 days, respectively).

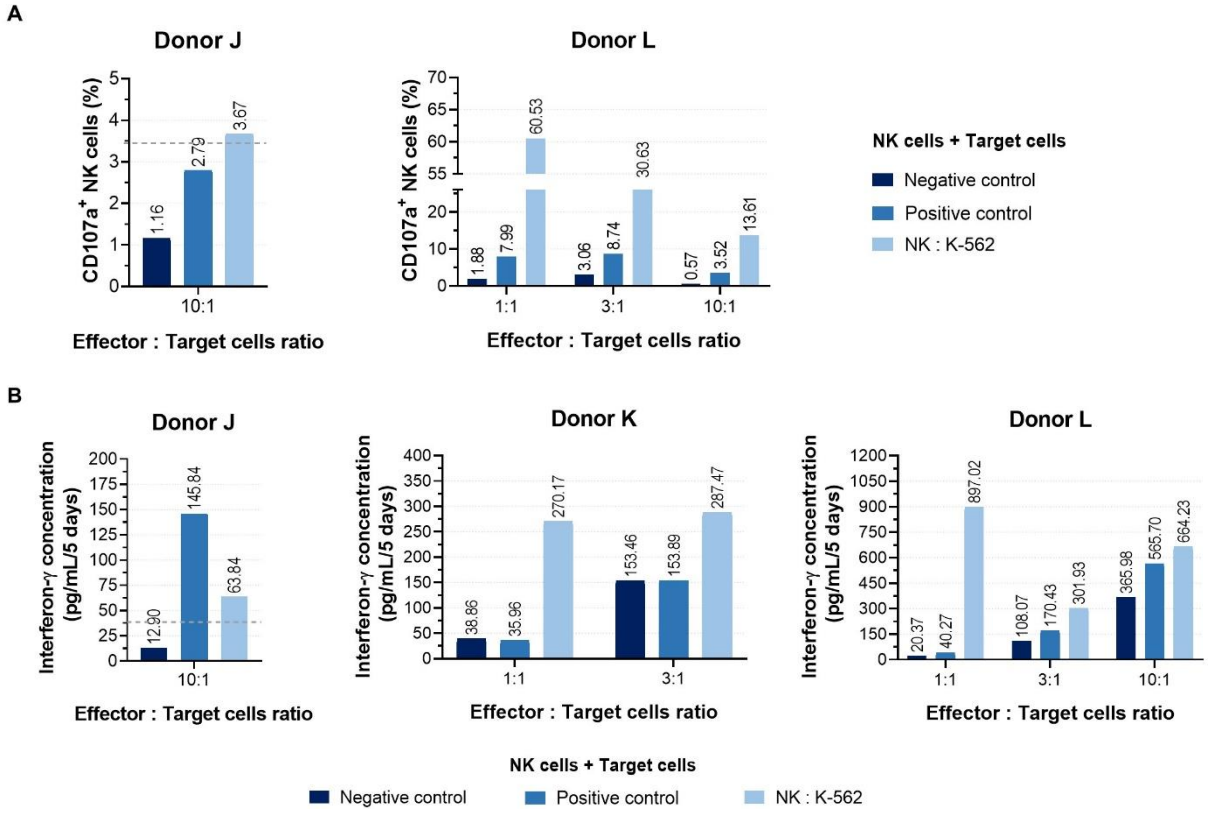


Figure 3.2 - *Ex vivo* expanded NK cells express CD107a and produce IFN-γ in response to the MHC-I negative cell line K-562. NK cells isolated through a positive sorting from PBMCs of donor J, K and L were cultured in the presence of IL-2+IL-15 and stimulated with irradiated K-562 cells at an immune cell:target cell ratio of 10:1. After 14 days in culture, NK cells were harvested, and their functional activity was determined by their ability to degranulate and produce IFN-γ in response to the K-562 cell line. The capacity of NK cells to degranulate was evaluated by the expression of CD107a in $CD3^{-}CD56^{bright/dim}CD16^{+/-}$ cells upon 6 h of co-culture with K-562 cells. Unstimulated NK cells were used as negative control, while NK cells stimulated with PMA for 2 h were used as positive control **(A)**. IFN-γ production was assessed by ELISA after a 5-day co-culture with K-562 cells. Unstimulated NK cells were used as negative control, while NK cells stimulated with PHA were used as positive control **(B)**. To

determine the optimal effector cell:target cell ratios for each assay, different effector cell:target cell ratios (1:1, 3:1 and/or 10:1) were used, when possible. In donor J grahps (A and B), the dashed line represents the threshold at which a response is considered positive (at least three-fold the value obtained in the negative control).

Altogether, these results show that *ex vivo* expanded NK cells maintain their effector capacity, degranulating and producing IFN-γ in response to target cell stimulation.

3.3. NK cells can be isolated directly from patients' tumour tissue

After established the most suitable protocol to grow NK cells *in vitro*, our goal was to ascertain whether NK cells could be isolated and expanded directly from patients' tumour tissue. Patients' tumour tissue was stimulated with irradiated K-562 cells and cultured with IL-2+IL-15. After 10 days, the tumour tissue was removed from culture and the isolated cells were maintained at the same conditions and stimulated more four times with irradiated K-562 cells, completing a total of five stimulations. To evaluate the feasibility of isolating and expanding NK cells from patients' tumour tissue, the total cell number, cell viability and frequency of NK cells and respective subsets were assessed over time (**Figure 3.3**).

Irrespective of patient-associated differences, IL-2+IL-15 combined with the K-562 cell stimulation allowed to isolate and grow NK cells *in vitro*. Only cells from patient D162 exhibited an expansion between the first and last time point assessed, increasing from $3.38x10^6$ to $4.10x10^6$ total cells (**Figure 3.3 A**). Although the total cell number has mostly decreased over time, two of the four patients (patients D155 and D162) showed an increase between the 4^{th} and 5^{th} stimulation, reaching approximately $4.10x10^6$ cells. The highest total cell number ($7.10x10^6$ cells) was obtained after the 2^{nd} stimulation by cells from patient D155. The peak of the total cell number was not consistent between patients since patients D155 and D171 presented it after the 2^{nd} stimulation, whereas patients D157 and D162 exhibited it after the 3^{rd} and 5^{th} stimulation, respectively.

Cell viability initially varied between 48.58 and 78.08% however, like the total cell numbers, this parameter showed a decrease over time, reaching values between 27.66 and 67.21% at the end of the expansion (**Figure 3.3 B**). Similarly, NK cells' frequency has also mostly decreased over time, ranging between 2.43 (patient D155) and 57.54% (patient D171) at the end of the expansion (**Figure 3.3 C**). The maximum NK cells' frequencies were observed after the 3^{rd} stimulation and varied between 25.12 (patient D162) and 76.99% (patient D171). The frequency of each NK cell subset ($CD56^{dim}CD16^-$, $CD56^{dim}CD16^+$, $CD56^{bright}CD16^-$ and $CD56^{bright}CD16^+$) during culture was also determined (**Figure 3.3 D**). Irrespective of the time point, the $CD56^{bright}$ was the main NK cell subset, always comprising more than 95% of the NK cells, except for cells from patient D162 after the 3^{rd} stimulation (90.3%) and patient D155 after the 5^{th} stimulation (83.3%). The $CD56^{dim}$ NK cells always represented a minority of NK cells (<5%). Within each NK cell subset, the $CD16^-$ NK cells ($CD56^{dim}CD16^-$ and $CD56^{bright}CD16^-$) were the most frequent.

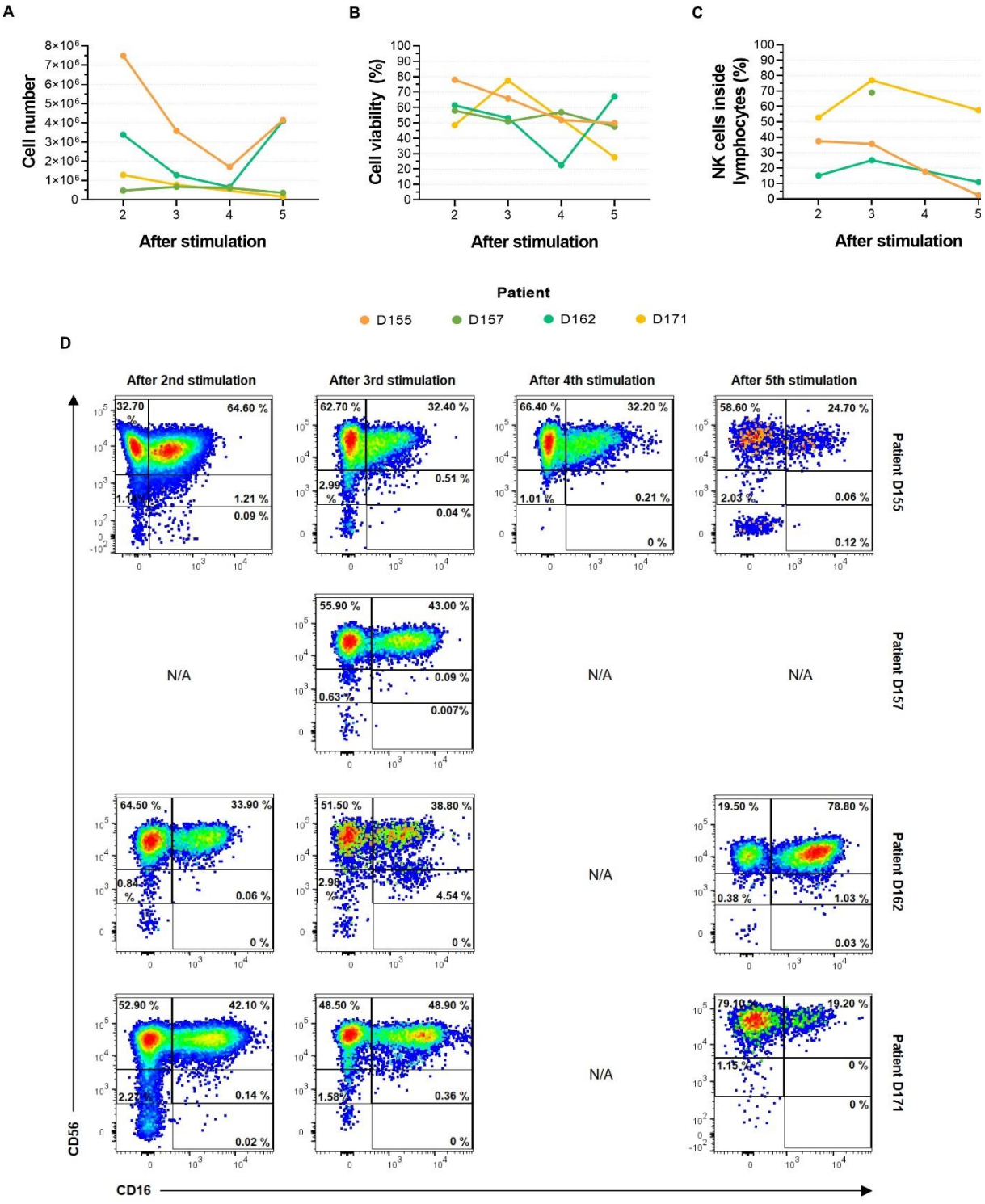
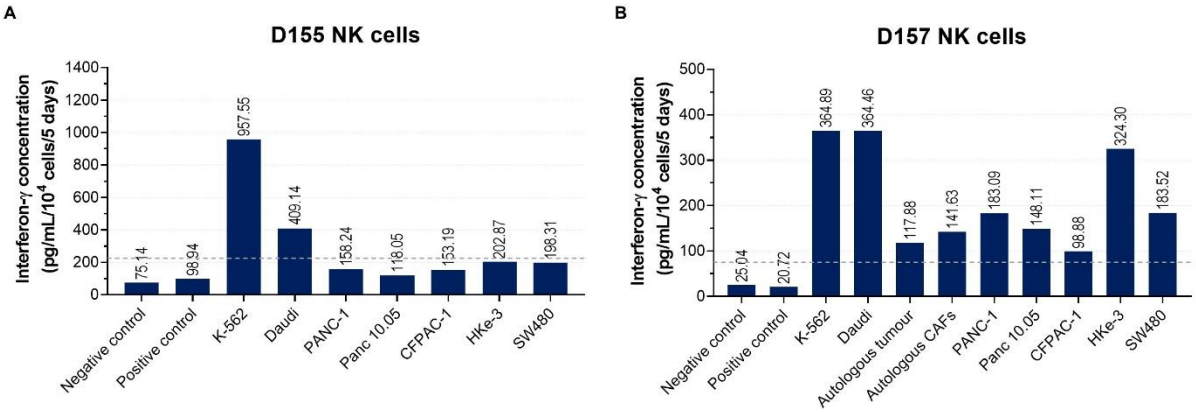


Figure 3.3 – NK cells can be isolated directly from the tumour tissue of patients with cancer and after *in vitro* culture present a CD56^{bright} phenotype. IL-2+IL-15 was used to isolate NK cells directly from the tumour tissue of four patients with cancer: patient D155 (rectal cancer), patient D157 (colon metastasis from ovarian cancer), patient D162 (colon adenocarcinoma) and patient D171 (rectum adenocarcinoma). Cells were stimulated every 10 days with 0.10x10^6 irradiated K-562 cells. A total of five stimulations with K-562 cells was performed and from the 2nd stimulation on, between stimulations, the total cell number **(A)**, cell viability **(B)**, NK cells' frequency within the lymphocyte population **(C)** and NK cell subsets **(D)** were assessed. N/A: not applicable.

3.4. *Ex vivo* expanded NK cells from patients' tumour tissue recognize the autologous tumour and/or allogeneic cancer cell lines and produce IFN-γ

To evaluate the functional capacity of the NK cells expanded from patients' tumour tissue, their ability to produce IFN-γ in response to different target cells was assessed. NK cells from all patients were isolated through a negative sorting procedure and co-cultured for 5 days with three different types of allogeneic tumour cell lines (lymphoblastoid, pancreatic and colorectal cancer cell lines), and when available with autologous cells (tumour cells and/or CAFs). The IFN-γ produced by NK cells in response to the different target cells was evaluated through an ELISA for IFN-γ.

Ex vivo expanded NK cells were capable of producing IFN-γ in response to target cell stimulation (**Figure 3.4**). Independently of patient-associated differences, unstimulated NK cells from all patients, except patient D171, showed a basal production of IFN-γ, ranging between 25.04 and 219.80 pg/mL/10^4 cells/5 days, which suggests that these cells were activated during their expansion. Although PMA stimulation should induce maximum cell activation, no IFN-γ was produced in response to PMA stimulation, which raised some concerns about the functional state of the expanded NK cells, suggesting the entry into anergy or activation-induced cell death. These hypotheses appeared not only to explain the basal production of IFN-γ by cells from patient D162 in all tested conditions (**Figure 3.4 C**), but also the total absence of IFN-γ production by cells from patient D171 in any of the tested conditions (data not shown). Thus, only cells from two of the four patients (patients D155 and D157) responded to target cell stimulation (**Figure 3.4 A and B**), demonstrating to be functionally active. K-562 and Daudi cells were the common targets recognized by NK cells from patients D155 (957.55 and 409.14 IFN-γ pg/mL/10^4 cells/5 days, respectively) and D157 (364.89 and 364.46 IFN-γ pg/mL/10^4 cells/5 days, respectively). NK cells from patient D157 demonstrated to be also capable of producing IFN-γ in response to all the other allogeneic target cells, as well as recognized and responded to the autologous tumour cells (117.88 IFN-γ pg/mL/10^4 cells/5 days) and CAFs (141.63 IFN-γ pg/mL/10^4 cells/5 days).



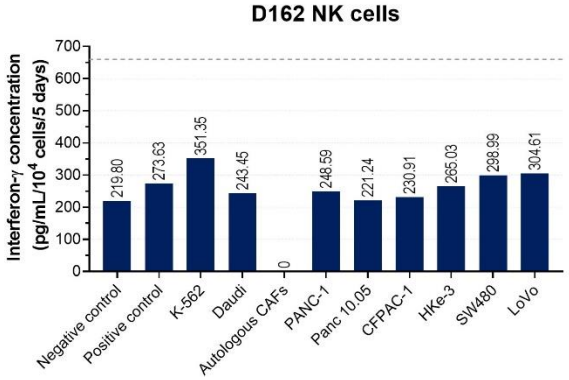


Figure 3.4 – *Ex vivo* expanded NK cells produce IFN-γ in response to autologous and/or allogeneic tumour cell lines. IL-2+IL-15 was used to isolate NK cells from the tumour tissue of four patients with cancer: patient D155 (rectal cancer), patient D157 (colon metastasis from ovarian cancer), patient D162 (colon adenocarcinoma) and patient D171 (rectum adenocarcinoma). Stimulation with $0.10x10^6$ irradiated K-562 cells was performed every 10 days and after five stimulations the expanded NK cells were harvested, negatively sorted and co-cultured for 5 days with autologous cells (tumour cells or CAFs) and lymphoblastoid (K-562 and Daudi), pancreatic (PANC-1, Panc 10.05 and CFPAC-1) and colorectal (HKe-3, SW480 and LoVo) allogeneic cancer cell lines at an effector cell:target cell ratio of 1:1. Graphs show the concentration of IFN-γ (pg/mL/10^4 cells/5 days) produced by NK cells from patients D155 **(A),** D157 **(B)** and D162 **(C)** in response to target cell stimulation. In all graphs, the dashed line represents the threshold at which a response is considered positive (at least three-fold the value obtained in the negative control).

Collectively, these results demonstrate that NK cells expanded directly from patients' tumour tissue are capable of producing IFN-γ in response to target cell stimulation and are able to recognize autologous tumour cells and CAFs. The capacity to recognize and attack MHC-I negative cell lines (K-562 and Daudi), autologous tumour cells and CAFs highlight the contribution that NK cells may have to cancer immunotherapy, complementing αβ T-cells' effector function(s).

3.5. γδ T-cells infiltrate into tumour tissues of different origins

γδ T-cells are found at low frequencies in lymphoid tissues and blood, yet they are abundant in epithelial and mucosal tissues. Since most of the patients involved in this study presented cancer in mucosal tissues, we analysed the infiltration of γδ T-cells in primary or metastatic tumours (**Figure 3.5**). Paraffin sections of patients' tumour tissue(s) were used for immunohistochemistry of the TCR δ chain. Prior to this analysis, the TCR δ chain antibody was titrated and the immunohistochemistry conditions were optimized, namely the pH value for the antigen retrieval and the duration of the incubation with the chromogen (data not shown).

Independently of the tumour type, γδ T-cells were found in most of the available tumour tissues tested, except for three samples: the glioblastoma from patient D82, the peritoneal metastasis from patient D83 and the rectum adenocarcinoma from patient D171 (**Figure 3.5**). Colon and rectal cancer samples presented a higher number of tumour-infiltrating γδ T-cells in comparison with the other tumour types. The colon metastasis from patient D157 was the sample with the highest number of tumour-infiltrating γδ T-cells.

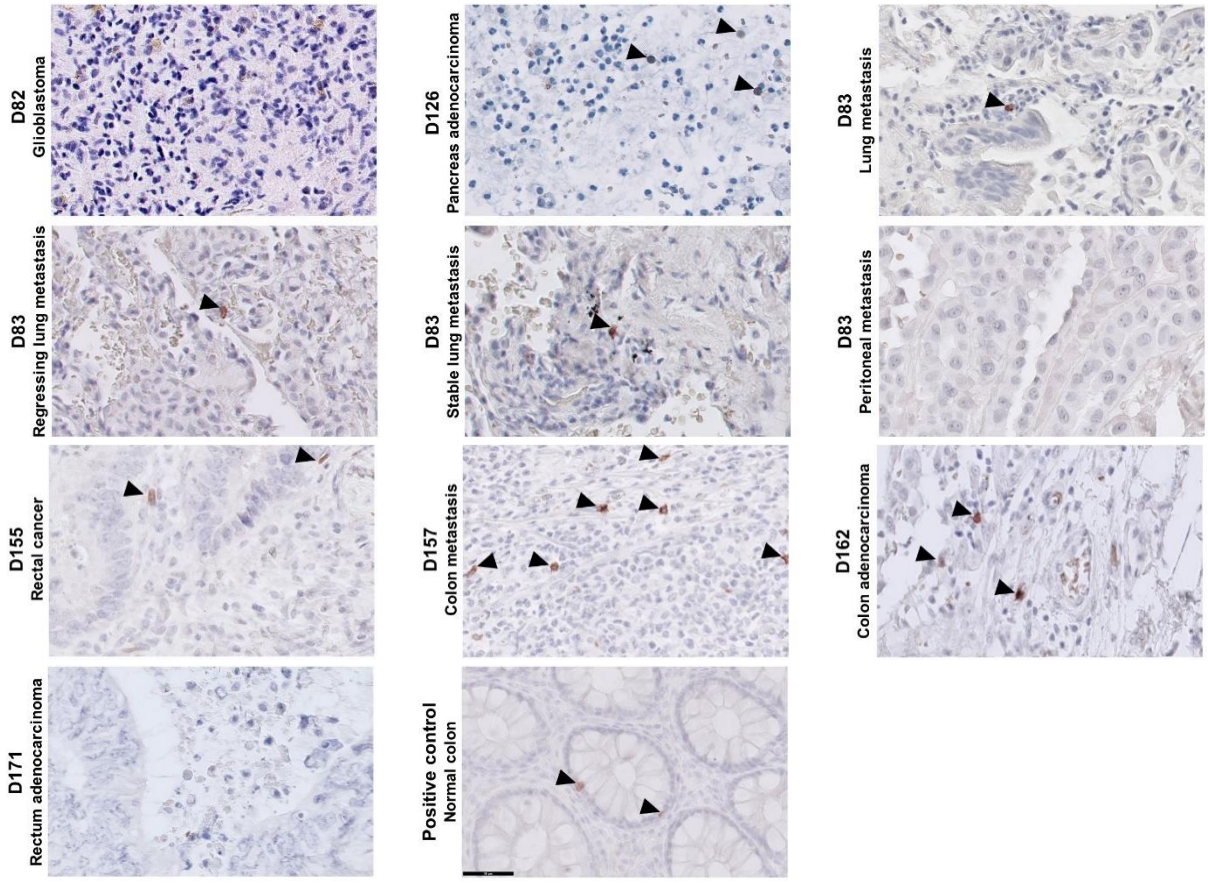


Figure 3.5 – γδ T-cells infiltrate into primary and metastatic lesions of patients with cancer. Immunohistochemical detection of the TCR δ chain (arrows) in paraffin sections of primary or metastatic tumours. Scale bar corresponds to 50 µm.

Collectively, these data confirm γδ T-cell infiltration into tumour tissues of different origins. This is of particular interest since an intra-tumoral γδ T-cell signature is strongly associated with a favourable prognosis (Gentles *et al*., 2015), supporting the use of γδ T-cells in a cellular immunotherapy context.

3.6. γδ T-cells can be isolated directly from patients' tumour tissue

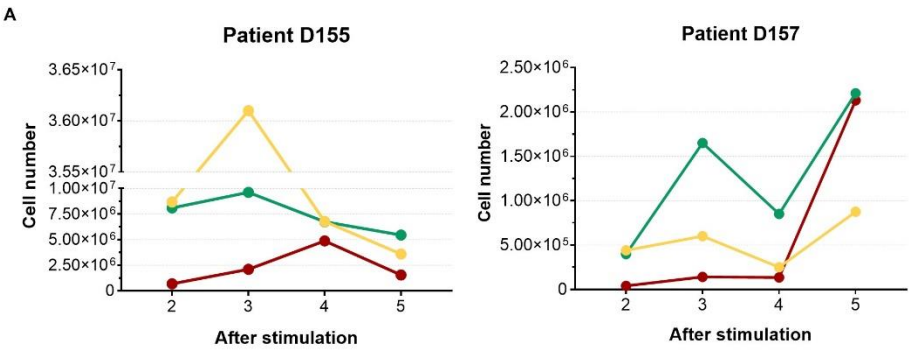
The main goals of the *ex vivo* expansion of γδ T-cells for adoptive cellular immunotherapy are to yield high cell numbers and to obtain a γδ T-cell product characterized by a T helper cells type 1 (Th1) phenotype and an increased cytotoxic capacity that confers high anti-cancer reactivity. During *ex vivo* expansion, γδ T-cells need to be stimulated to proliferate and reach sufficient numbers to be infused into patients. Cytokines are the primary stimulus determining γδ T-cell proliferation, survival, and phenotype. However, γδ T-cells reach higher expansion levels when the use of cytokines is combined with phosphoantigens, *e.g.* IPP, or aminobiphosphonates, such as zoledronic acid (Van Acker et al., 2015). To determine the best stimuli to expand γδ T-cells *ex vivo* directly from patients' tumour tissue, different cytokine combinations were tested, namely a) IL-2+IL-15+IL-1β, b) IL-2+IL-7 and c) IL-7+IL-1β. In addition, γδ T-cells were stimulated five times with irradiated allogeneic PBMCs. In the first two stimulations, PBMCs were pre-treated with zoledronic acid, while the others alternated between addition of PBMCs along with OKT3 (anti-human CD3 antibody) and PBMCs pre-treated with zoledronic acid.

To compare the expansion protocols' efficiency, the total cell number, cell viability and γδ T-cells' frequency in the expanded lymphocytes were assessed between stimulations, whenever it was possible (**Figure 3.6**).

Irrespective of patient-associated differences, IL-2+IL-15+IL-1β and IL-2+IL-7 were the cytokine combinations that led to the highest total cell numbers during culture (**Figure 3.6 A**). Only cells from two of the four patients exhibited an expansion in the total cell number between the first and last time point assessed. Cells from patient D157 demonstrated a total cell number expansion independently of the cytokine combination used, revealing their maximum total cell number-fold increase (53-fold) when cultured with IL-7+IL-1β, increasing from $0.04x10^6$ to $2.13x10^6$ cells between the first and last time point. On the other hand, cells from patient D171 only showed an expansion in the total cell number when cultured with IL-2+IL-7, exhibiting an increase from $1.36x10^6$ to $2.45x10^6$ cells between the first and last time point. Although cells from patient D155 did not expand between the first and last time point assessed, it was possible to observe an increase from $8.68x10^6$ to $36.1x10^6$ cells between the 2nd and 3rd stimulation in the presence of IL-2+IL-15+IL-1β. Due to the high cell number obtained, after the 3rd stimulation, $26x10^6$ cells from patient D155 were frozen and only $9x10^6$ cells were maintained in culture, which explains the high decrease verified in the total cell number between the 3rd and 4th stimulation.

IL-2+IL-15+IL-1β and IL-2+IL-7 were also the cytokine combinations resulting in the highest cell viabilities (**Figure 3.6 B**), except for cells from patients D155 and D157 at the end of the culture, in which IL-7+IL-1β resulted in the highest cell viabilities (54.4 and 83.3%, respectively). The percentage of viable cells decreased over time, never recovering the initial values.

The highest γδ T-cells' frequencies during culture were obtained in the presence of IL-2+IL-15+IL-1β (**Figure 3.6 C**). However, at the end of the culture, the highest γδ T-cells' frequencies were obtained when cells from all patients, except patient D155, were cultured with IL-7+IL-1β. Only cells from two of the four patients (patients D155 and D171) showed an increase in γδ T-cells' frequency between the first and last time point. Cells from patient D155 showed an increase in γδ T-cells' frequency between the first and last time point when cultured with IL-2+IL-15+IL-1β (from 13.00 to 16.30%) or IL-7+IL-1β (from 1.80 to 3.37%). In contrast, cells from patient D171 only demonstrated an increase in γδ T-cells' frequency (from 1.79 to 6.98%) between the first time point and the end of the culture in the presence of IL-2+IL-7. Although the low number of cells in some expansion conditions limited the number of time points assessed, it was possible to observe that in the presence of IL-2+IL-15+IL-1β, γδ T-cells' frequency reached its peak after the 3rd stimulation and then decreased until the end of the culture.



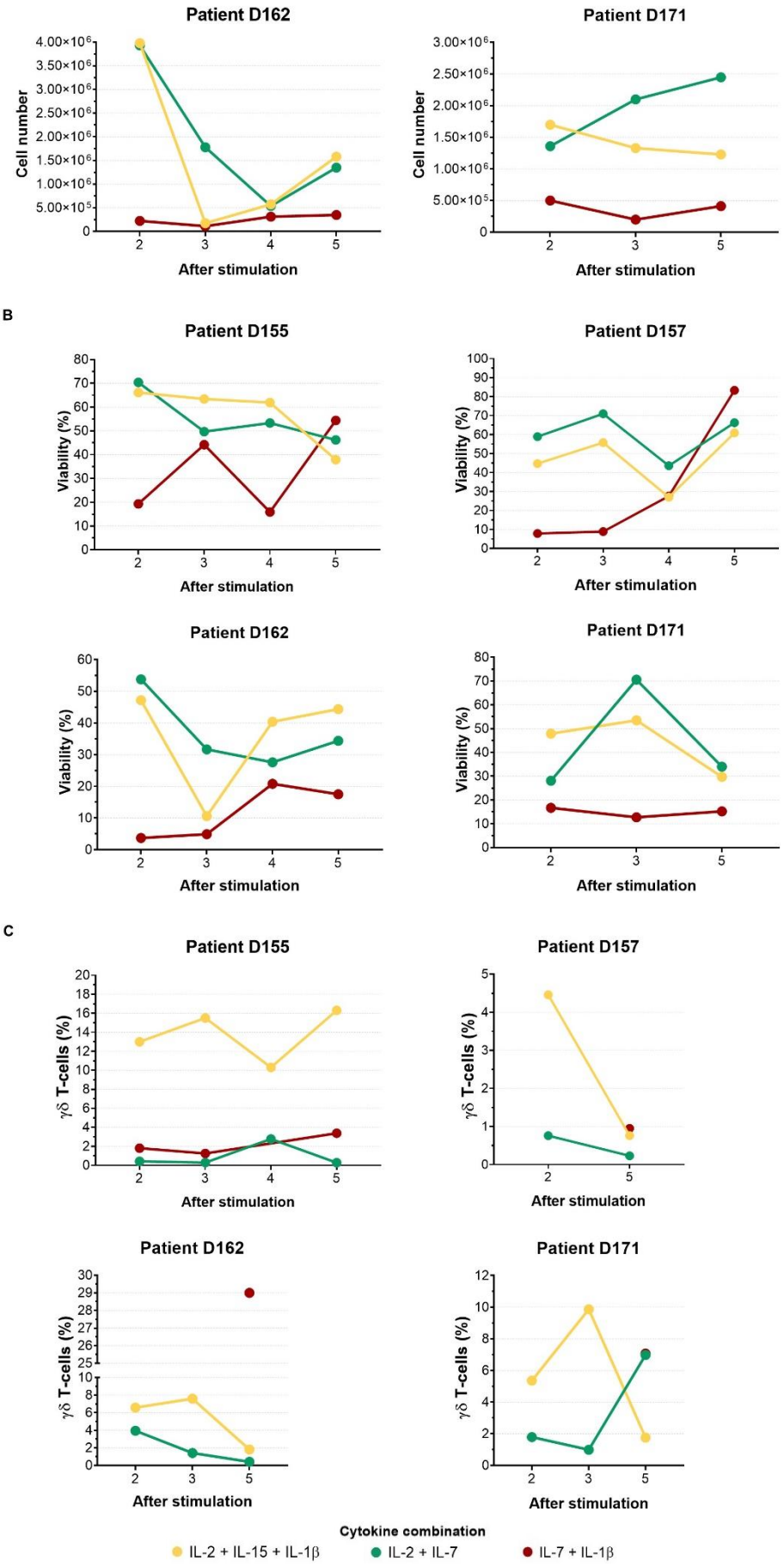

Patient D162
Patient D171
Cell number
After stimulation
B
Patient D155
Patient D157
Viability (%)
Patient D162
Patient D171
C
Patient D155
Patient D157
γδ T-cells (%)
Patient D162
Patient D171
Cytokine combination
IL-2 + IL-15 + IL-1β
IL-2 + IL-7
IL-7 + IL-1β

Figure 3.6 – Three different expansion protocols allow the isolation of γδ T-cells from the tumour tissue of patients with cancer. IL-2+IL-15+IL-1β, IL-2+IL-7 and IL-7+IL-1β were used to expand γδ T-cells from the tumour tissue of four patients with cancer: D155 (rectal cancer), D157 (colon metastasis from ovarian cancer), D162 (colon adenocarcinoma) and D171 (rectum adenocarcinoma). Cells were stimulated five times (every 10 days) with irradiated allogeneic PBMCs pre-treated with zoledronic acid (1st, 2nd and 4th stimulation) or irradiated allogeneic PBMCs along with OKT3 (3rd and 5th stimulation). The total cell number **(A)**, cell viability **(B)** and γδ T-cells' frequency **(C)** were evaluated in all expansion conditions tested after the 2nd, 3rd, 4th and 5th stimulation (when the total cell number was sufficient).

A model was fit to data to determine the impact of the different cytokine combinations in the evaluated parameters, namely total cell number, cell viability and γδ T-cells' frequency. The different cytokine combinations revealed to statistically influence the total cell number and cell viability. IL-7+IL-1β demonstrated to lead to a significant decrease in the total cell number ($p<0.001$) and cell viability ($p<0.001$) over time, while no differences were found between IL-2+IL-15+IL-1β and IL-2+IL-7. In contrast, the γδ T-cells' frequency was not significantly affected by any cytokine combination, however IL-7+IL-1β and IL-2+IL-15+IL-1β were the culture conditions resulting in a higher γδ T-cells' frequencies.

Altogether, these results prove that it is possible to isolate γδ T-cells directly from patients' tumour tissue and expand them *in vitro*. The ideal strategy to expand γδ T-cells should promote the maximum a) total cell number, b) cell viability and c) γδ T-cells' frequency. Despite this was not robustly possible, the best cytokine combination appeared to be IL-2+IL-15+IL-1β, however it needs to be explored in future studies.

3.7. γδ T-cells can be expanded from TIL and PBMCs in the presence of IL-2, IL-15 and IL-1β and with feeder cell stimulation

Once IL-2+IL-15+IL-1β appeared to be the best cytokine combination to expand γδ T-cells from patients' tumour tissue, we investigated the feasibility of using the same expansion conditions to expand γδ T-cells from frozen TIL and PBMCs from patients with cancer. To test our hypothesis, PBMCs from patient D83 and TIL from patients D82 and D126 (**detailed description in section 2.2.2**) were thawed and cultured with medium supplemented with IL-2+IL-15+IL-1β. Cells were stimulated with irradiated allogeneic PBMCs every 10 days. A total of five stimulations were performed, whereas in the first two rounds of stimulation, allogeneic PBMCs were pre-treated with zoledronic acid, in the following stimulations allogeneic PBMCs along with OKT3 (anti-CD3 antibody) were alternated with allogeneic PBMCs pre-treated with zoledronic acid. The total cell number, cell viability and γδ T-cells' frequency were assessed between stimulations, whenever it was possible (**Figure 3.7**).

The combination of IL-2, IL-15 and IL-1β resulted in the expansion of cells from two of the three patients (patients D82 and D83) between the first and last time point. While PBMCs from patient D83 presented an increase from $6.56x10^6$ to $7.55x10^6$ cells between the first and last time point, TIL from patient D82 exhibited the highest total cell number fold-expansion (approximately 3.9-fold), showing an increase from $4.54x10^5$ to $1.75x10^6$ cells (**Figure 3.7 A**). Cells from all patients demonstrated to reach the maximum total cell number after the 4th stimulation, exhibiting $2.25x10^6$ (patient D82), $1.41x10^7$

(patient D83) or 1.05x10^7 total cells (patient D126). Of note, due to the high cell number obtained after the 3rd stimulation (2.99x10^7), 2.10x10^7 TIL from patient D126 were frozen and only 8.90x10^6 cells remained in culture, which explains the high decrease verified in the total cell number between the 3rd and 4th stimulation, and the reason why the total cell number peak in this patient was considered after the 4th stimulation, since in fact the total cell number increased from 8.90x10^6 to 1.05x10^7.

Cell viability showed a decrease over time in cells from all patients. Cells from patients D82, D83 and D126, at the end of the culture, only exhibited 35.90%, 36.00% and 31.50% viability, respectively (**Figure 3.7 B**). In contrast, γδ T-cells' frequency increased over time in cells from all patients, except in cells from patient D126 (**Figure 3.7 C**). PBMCs from patient D83 demonstrated an increase from 0.55 to 1.55% γδ T-cells between the 3rd stimulation and the end of the culture, while TIL from patient D82 exhibited the highest γδ T-cells' frequency of the three patients at the end of the culture, showing an increase from 0.11 to 2.73% γδ T-cells. TIL from patient D126 demonstrated the opposite trend, presenting the highest γδ T-cells' frequency after the 3rd stimulation (4.00%) and then exhibiting a decrease over time, ending with the lowest γδ T-cells' frequency after the 5th stimulation (0.24%).

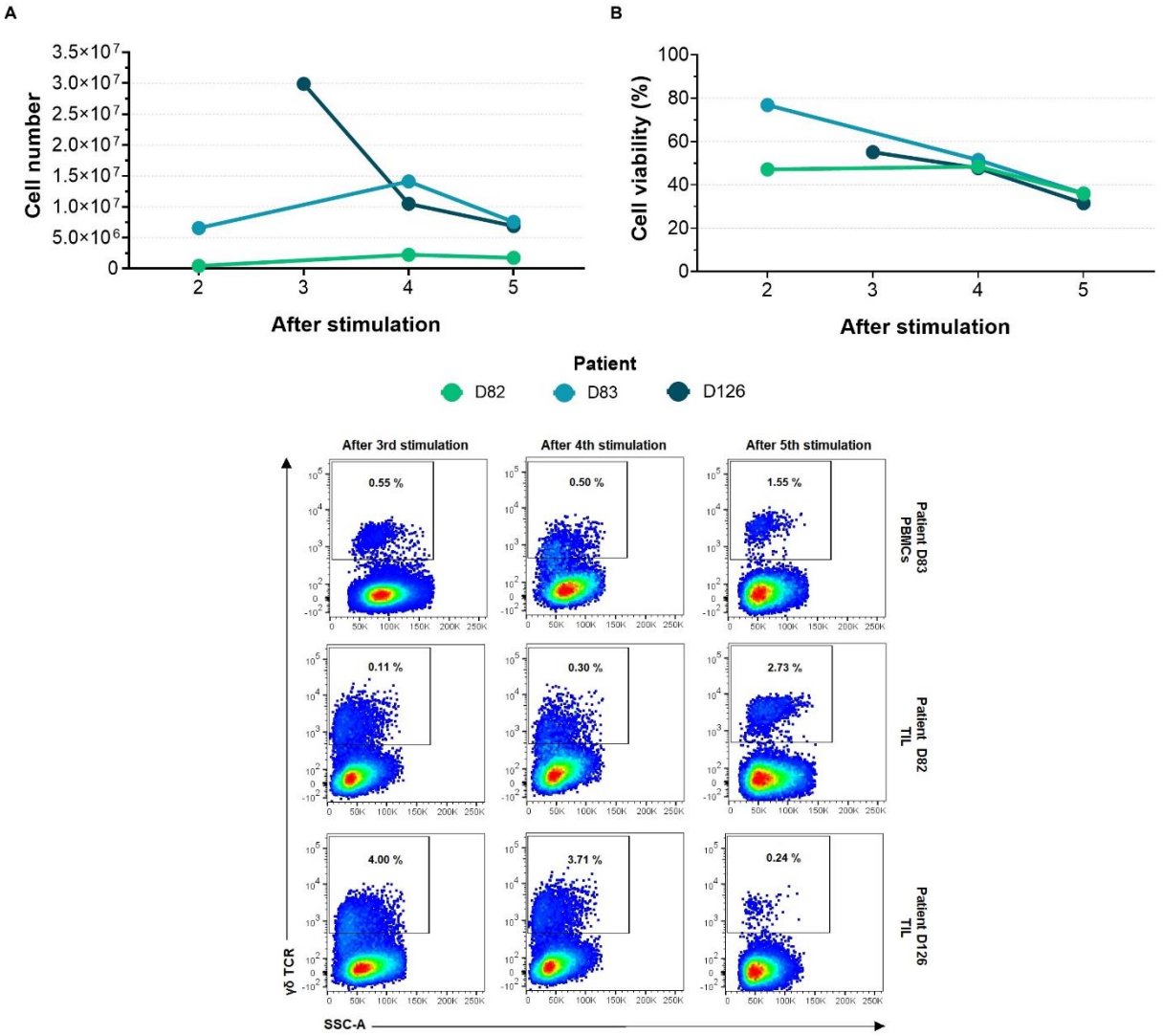


Figure 3.7 – γδ T-cells can be expanded from frozen TIL and PBMCs. IL-2+IL-15+IL-1β was used to expand γδ T-cells *in vitro* from PBMCs of patient D83 (pancreatic cancer) and TIL of patients D82 (glioblastoma) and D126 (PDAC). Cells were stimulated five times (every 10 days) with irradiated allogeneic PBMCs pre-treated with zoledronic acid (1st, 2nd and 4th stimulation) or irradiated allogeneic PBMCs along with OKT3 (3rd and 5th stimulation). The total cell number **(A)**, cell viability **(B)** and γδ T-cells' frequency **(C)** were evaluated after the 2nd, 3rd, 4th and 5th stimulation (when it was possible).
SSC-A: Side scatter area.

In summary, these results show that γδ T-cells can be expanded from frozen TIL and PBMCs using as stimuli IL-2+IL-15+IL-1β and allogeneic PBMCs pre-treated with zoledronic acid or allogeneic PBMCs along with OKT3.

3.8. *In vitro* expanded γδ T-cells produce IFN-γ after recognizing autologous transformed cells and/or allogeneic tumour cell lines

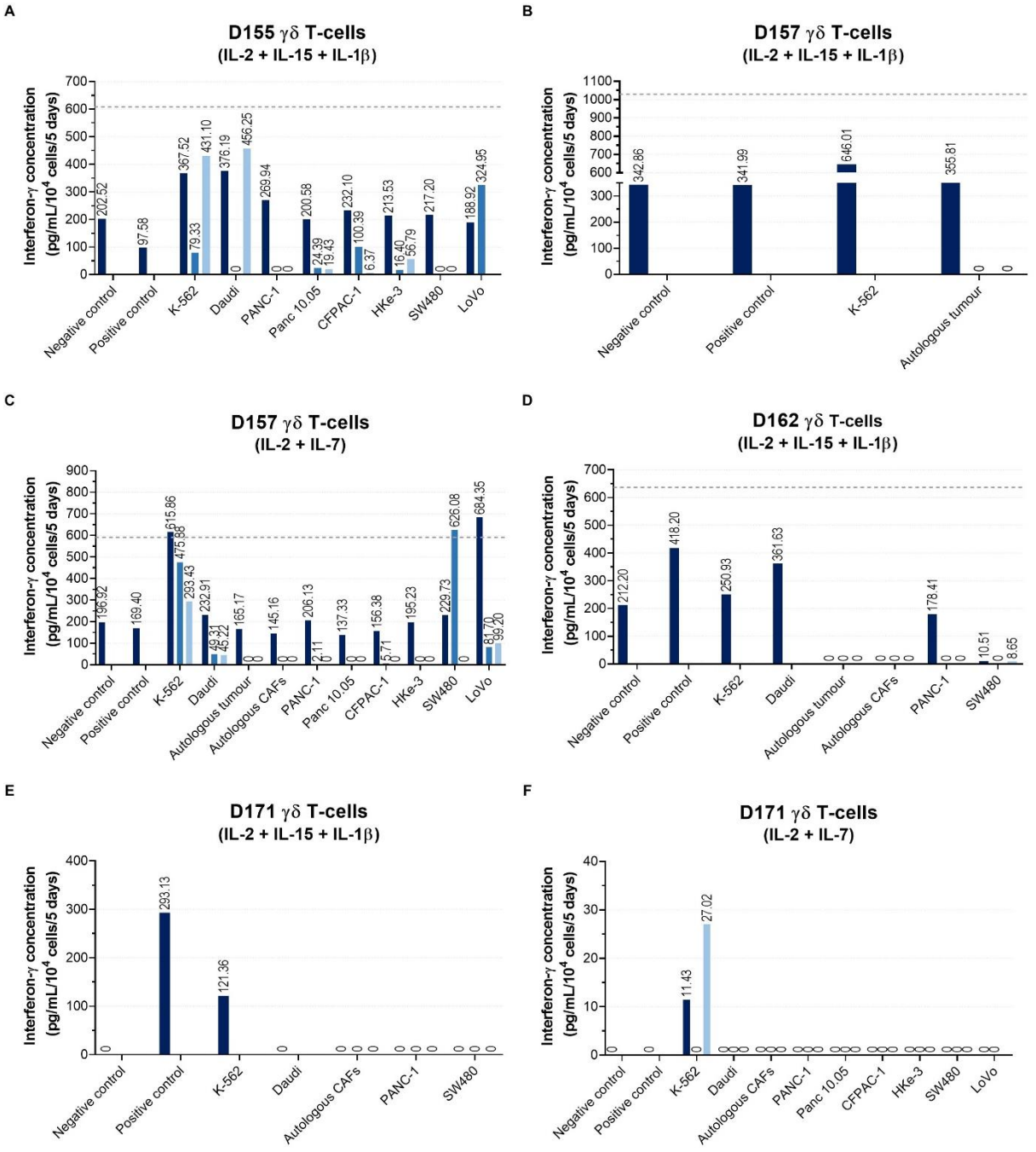
IFN-γ production is one of the most well-analysed mechanism used by γδ T-cells to fight infections and tumours and can be used as an indicator of their effector capacity. Thus, to evaluate the functional capacity of the γδ T-cells expanded from patients' tumour tissue, their IFN-γ production in response to different target cells was assessed. γδ T-cells expanded with IL-2+IL-15+IL-1β were isolated through a negative sorting using an αβ TCR antibody. When the total cell number was sufficient, γδ T-cells were also isolated from the other culture conditions, which was only possible for cells from patients D157 and D171 expanded with IL-2+IL-7. γδ T-cells were co-cultured for 5 days with three different types of allogeneic tumour cell lines (lymphoblastoid, pancreatic and colorectal cell lines), and when available with transformed autologous cells (tumour cells, CAFs or EBV-transformed B-cell lines). The co-culture was also performed in the presence of a γδ TCR blocking antibody or an IgG1 isotype control antibody to determine whether target cell recognition was being driven by the γδ TCR, since γδ T-cells may recognize their targets through the γδ TCR or NK-like receptors, *e.g.* NKG2D. The IFN-γ produced by γδ T-cells in response to the different target cell lines was evaluated through an ELISA for IFN-γ as a way of assessing the effector capacity of the expanded cells.

Ex vivo expanded γδ T-cells in the presence of IL-2+IL-15+IL-1β or IL-2+IL-7 demonstrated to produce IFN-γ in response to target cell stimulation (**Figure 3.8**). Independently of the culture conditions, unstimulated γδ T-cells from all patients, except patient D171 (**Figure 3.8 E and F**), showed some basal production of IFN-γ, ranging from 94.92 to 214.14 pg/mL/10^4 cells/5 days, which indicates that these cells were activated during culture by the stimuli provided. Although PMA stimulation should induce maximum cell activation, only γδ T-cells from patient D171 (IL-2+IL-15+IL-1β) demonstrated to produce IFN-γ (293.13 pg/mL/10^4 cells/5 days) in response to PMA stimulation (**Figure 3.8 E**). These results raised some concerns about the functional state of other patients' γδ T-cells, suggesting the entry into anergy or activation-induced cell death. Irrespective of the culture conditions, γδ T-cells from all patients, except patients D157 (IL-2+IL-7, **Figure 3.8 C**) and D171 (IL-2+IL-15+IL-1β and IL-2+IL-7, **Figure 3.8 E and F**), did not show to produce IFN-γ in response to any of the allogeneic or autologous cells used as targets. γδ T-cells from patient D157 (IL-2+IL-7) revealed to be activated by the K-562 (leukaemia) and LoVo (colorectal) cancer cell lines, demonstrating an IFN-γ production of 615.86 and 684.35 pg/mL/10^4 cells/5 days, respectively (**Figure 3.8 C**). The recognition of the K-562 cell line appeared to

occur in a TCR-independent manner as IFN-γ only decreased from 615.86 to 475.88 pg/mL/10^4 cells/5 days in the presence of the γδ TCR blocking antibody, and with the IgG1 isotype control antibody an IFN-γ production of 293.43 pg/mL/10^4 cells/5 days was observed (**Figure 3.8 C**). Similarly, LoVo cells also appeared to be recognized in a TCR-independent manner since although the IFN-γ production decreased from 684.35 to 81.70 pg/mL/10^4 cells/5 days in the presence of the γδ TCR blocking antibody, a similar amount of IFN-γ (99.20 pg/mL/10^4 cells/5 days) was obtained in the presence of the IgG1 isotype control antibody. γδ T-cells from patient D171, independently of the culture conditions, demonstrated to be also activated by the K-562 cell line, presenting an IFN-γ production of 121.36 pg/mL/10^4 cells/5 days in the presence of IL-2+IL-15+IL-1β (**Figure 3.8 E**) and 11.43 pg/mL/10^4 cells/5 days in the presence of IL-2+IL-7 (**Figure 3.8 F**). γδ T-cells from patient D171 (IL-2+IL-7) appeared to recognize the K-562 cell line through the γδ TCR since the IFN-γ production was impaired in the presence of the γδ TCR blocking antibody (0 pg/mL/10^4 cells/5 days) and recovered in the presence of the IgG1 isotype control antibody (27.02 pg/mL/10^4 cells/5 days; **Figure 3.8 F**), however the IFN-γ production was low for this TIL line.

The effector capacity of γδ T-cells expanded from frozen PBMCs (patient D83) and TIL (patients D82 and D126) was also evaluated through the IFN-γ production assay (**Figure 3.8 G, H and I**). The experimental design was similar to the one used for γδ T-cells expanded from patients' tumour tissue. γδ T-cells from all patients showed a basal production of IFN-γ, suggesting their activation during culture by the stimuli provided. PMA stimulation did not lead any patients' γδ T-cells to produce IFN-γ, despite all cells have demonstrated to be functionally active, recognizing at least one of the target cells used. γδ T-cells from patients D82 and D83 showed to be activated by the K-562 cell line, exhibiting an IFN-γ production of 401.71 (**Figure 3.8 G**) and 761.97 pg/mL/10^4 cells/5 days (**Figure 3.8 H**), respectively. K-562 cells were recognized by γδ T-cells in a TCR-independent manner since the presence of the γδ TCR blocking antibody did not impair the IFN-γ production (**Figure 3.8 H**), and even when it induced a slight decrease, the IFN-γ production was not recovered in the presence of the IgG1 isotype control (**Figure 3.8 G**). Besides K-562 cells, γδ T-cells from patient D82 also recognized the autologous tumour cells, reaching its maximum IFN-γ production (451.59 pg/mL/10^4 cells/5 days, **Figure 3.8 G**). The presence of the γδ TCR blocking antibody led to a decrease in the IFN-γ production (from 451.59 to 92.98 pg/mL/10^4 cells/5 days), which was almost totally recovered in the presence of the IgG1 isotype control antibody (345.86 pg/mL/10^4 cells/5 days), indicating that the recognition of the autologous tumour by γδ T-cells from patient D82 involves the γδ TCR. Although the IFN-γ values have not reached the threshold to be considered a positive response (642.42 pg/mL/10^4 cells/5 days), γδ T-cells from patient D83 also demonstrated to considerably increase their IFN-γ production in the presence of both autologous tumour cell lines PC4 (486.39 pg/mL/10^4 cells/5 days) and PC5 (489.38 pg/mL/10^4 cells/5 days, **Figure 3.8 H**). Moreover, the presence of the γδ TCR blocking antibody resulted in a decrease in the levels of IFN-γ produced (from 486.39 to 102.44 and from 489.38 to 55.02 pg/mL/10^4 cells/5 days, respectively), which were recovered in the presence of the IgG1 isotype control (366.69 and 564.40 pg/mL/10^4 cells/5 days, respectively), indicating that the recognition of both autologous tumour cell lines involves the γδ TCR. γδ T-cells from patient D126 showed to produce IFN-γ only in response to the autologous B-cell line (634.74 pg/mL/10^4 cells/5 days), revealing to recognize it in a TCR-independent

manner since the presence of the γδ TCR blocking antibody did not impair the IFN-γ production (**Figure 3.8 I**).



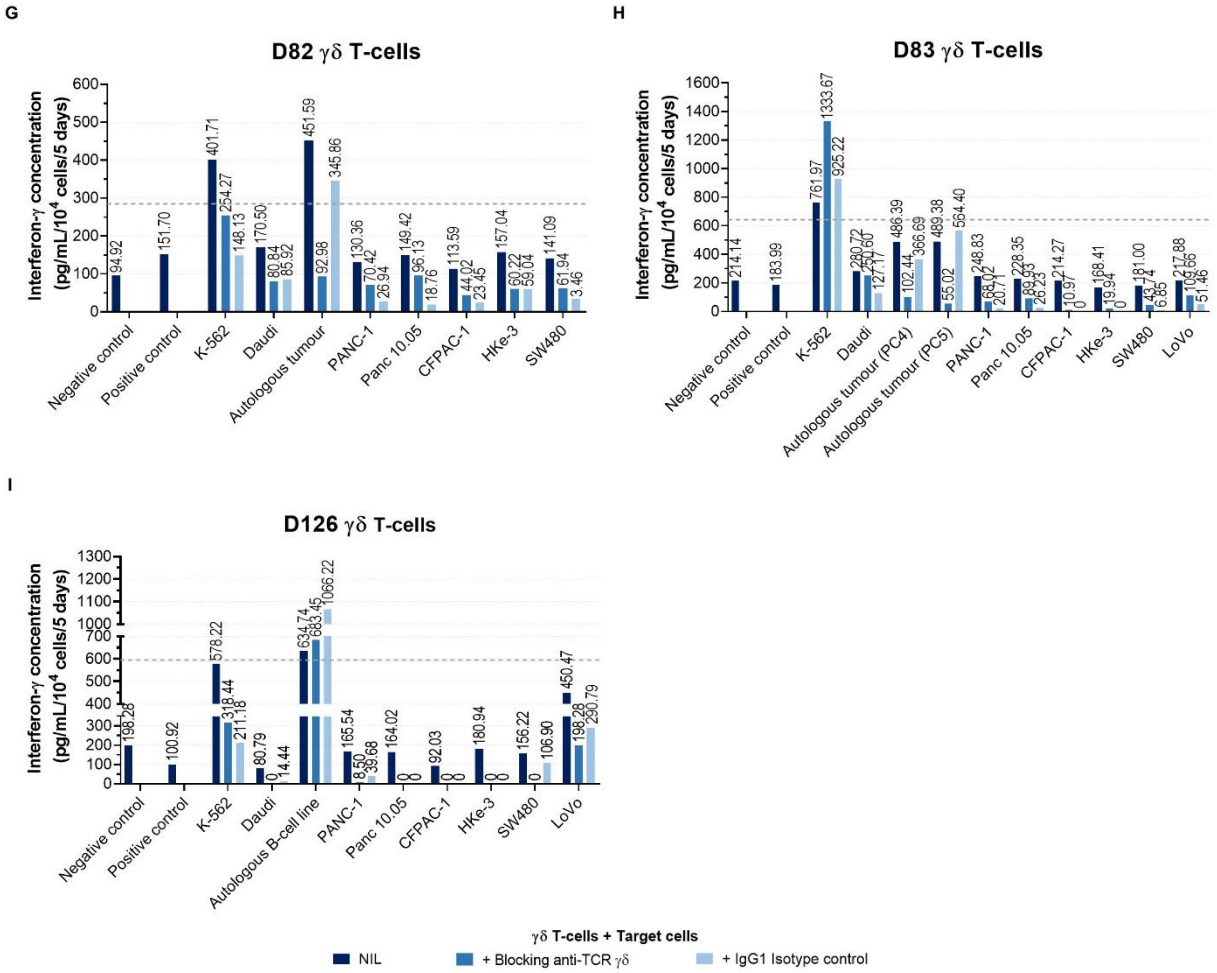


Figure 3.8 - *In vitro* expanded γδ T-cells produce IFN-γ in response to autologous and/or allogeneic tumour cell lines. γδ T-cells were expanded *in vitro* with IL-2+IL-7 and/or IL-2+IL-15+IL-1β from: the tumour tissue from patients D155 (rectal cancer, **A**), D157 (colon metastasis from ovarian cancer, **B and C**), D162 (colon adenocarcinoma, **D**) and D171 (rectum adenocarcinoma, **E and F**); TIL from patients D82 (glioblastoma, **G**) and D126 (PDAC, **I**) and PBMCs from patient D83 (pancreatic cancer, **H**). The expanded γδ T-cells were co-incubated for 5 days at an effector:target cell ratio of 1:1 **(A, B, C, D, E, F, H, I)** or 4:1 **(G)** with transformed autologous cells (tumour cells, CAFs or EBV-transformed B-cell line) and/or lymphoblastoid (K-562 and Daudi), pancreatic (PANC-1, Panc 10.05 and CFPAC-1) and colorectal (HKe-3, SW480 and LoVo) allogeneic cancer cell lines. Graphs show the concentration of IFN-γ (pg/mL/10^4 cells/5 days) produced by γδ T-cells in response to target cell stimulation (NIL) and in the presence of an anti-γδ TCR blocking antibody or an anti-IgG1 isotype control antibody. The dashed line represents the threshold at which a response is considered positive (at least three-fold the value obtained in the negative control).

Altogether, these results show that *ex vivo* expanded γδ T-cells are capable of producing IFN-γ in response to target cell stimulation and recognizing autologous tumour cells in a TCR-dependent manner. Furthermore, these results highlight the contribution that γδ T-cells may have to cancer immunotherapy due to their capacity to recognize and attack autologous tumour cells that may not be detected by αβ T-cells.

4. Discussion

αβ T-cell-based ACT have been showing promising results, however, they only target MHC-I positive cancer cells, which implies that tumour cell clones that downregulate or lose MHC-I expression remain in the body and may lead to cancer recurrence. There is therefore an urgent need to complement αβ T-cell-based ACT through the development of new therapies that target MHC-I negative tumour cells. NK cells or γδ T-cells would fill that gap since they may be able to recognize MHC-I or -II negative tumour cells. Due to their target cell recognition characteristics and more innate role in tumour immune surveillance, NK cells and γδ T-cells started to receive an increased attention and several NK cell and γδ T-cell-based immunotherapies have been developed during the last years. Both types of therapies (NK or γδ T-cells) demonstrated to be successful in targeting haematological malignancies (**Table 9.1** and **Table 9.2** in appendix; (Bachanova *et al*., 2014; Bjorklund *et al*., 2018). In contrast, this was not the case for solid cancers. Besides negative factors, such as the presence of inhibitory signals within the TME and altered immunogenicity of tumour cells, the lack of access of immune cells to tumour lesions is considered one of the major factors that contribute to the failure of ACT for solid tumours. The interaction between chemokine receptors and ligands is responsible for cell infiltration into tissues. NK cell and γδ T-cell subsets express distinct chemokine receptor repertoires (Berahovich *et al*., 2006; Campbell *et al*., 2001; Glatzel *et al*., 2002; Lança *et al*., 2013; Wendel *et al*., 2008; Ye *et al*., 2013) and are differently distributed in blood and tissues. Most NK cell or γδ T-cell therapies often use PBMCs to obtain the effector cells, which in fact may result in a cell product with low anti-tumour reactivity and/or homing capacity due to the expression of a chemokine receptor repertoire that does not correspond to the ligands expressed by tumour cells. Based on the assumption that tumour-infiltrating NK cells and γδ T-cells are tumour-reactive and may indeed exhibit homing receptors that enable them to access more easily into tumour lesions, the isolation and expansion of these cells from the patients' tumour tissue for ACT may be a good alternative. Thus, the main goals of this work were: a) explore the feasibility of isolating and expanding NK cells and γδ T-cells directly from the patients' tumour tissue (yet the homing markers of the NK cells or γδ T-cells populations examined in this master thesis have not been tested) and b) determine whether these immune cells exhibited reactivity against autologous tumour cells.

4.1. NK cells

Tumours presenting NK cell infiltration and high expression of genes encoding NK cell stimuli, such as the cytokine IL-15, were found to be associated with improved survival in patients with metastatic cutaneous melanoma (Cursons *et al*., 2019). More recently, in several types of solid tumours, high levels of NK cell markers, *e.g.* CD56, CD57, NKp30 and NKp46, were correlated with a better prognosis in patients with solid malignancies (Zhang *et al*., 2020). Thus, NK cell-based immunotherapies receive now more attention, despite their exploration in the cancer immunotherapy field had started some time ago when their involvement in tumour immunosurveillance, especially through the recognition and killing of MHC-I negative tumour cells, was discovered. Although NK cell expansion and activation may be promoted *in vivo* through cytokine infusion or the use of monoclonal antibodies, most NK cell-

based therapies expand and activate autologous or allogeneic NK cells *ex vivo* and infuse them into the patient.

Several protocols to culture and expand NK cells have been tested using different cytokines (*e.g.* IL-2, IL-12, IL-15, IL-18 and IL-21) and co-stimulation with feeder cells of different origins (target cell lines, autologous or allogeneic PBMCs) (Granzin *et al*., 2017), however none is currently considered the "standard protocol". Therefore, before determining the feasibility of isolating and expanding NK cells directly from patients' tumour tissue, different expansion protocols were tested. Four different cytokine combinations were analysed (IL-2+IL-15+IL-21; IL-2; IL-2+IL-15 and IL-15) in the presence or absence of K-562 cell stimulation. Different time points to sort NK cells (at day 0, after a 7- or 14-day expansion of the $CD3^-$ population) were also evaluated for each condition. PBMCs from healthy donors (donor G, H and I) were used and a 14-day expansion was performed, during which the total cell number, cell viability and NK cells' frequency were assessed to evaluate the effectiveness of the different expansion protocols. None of the expansion protocols showed to promote simultaneously an expansion in the total cell number, NK cells' frequency and at the same time to maintain cell viability. However, the isolation of NK cells at day 0 and subsequent culture with IL-2+IL-15 and K-562 cell stimulation appeared to result in the best trade-off between total cell number, cell viability and NK cells' frequency. It is not surprising that IL-2+IL-15 led to the most promising results since several studies have previously reported that the combination of these two cytokines promotes NK cell expansion from PBMCs of healthy donors or patients with cancer, showing NK cell expansion-folds ranging between 15.7 and 993 (Peragine et al., 2015; Siegler et al., 2010; Torelli et al., 2015). Peragine and colleagues demonstrated that the stimulation of NK cells with IL-2+IL-15 or only with autologous feeder cells results in lower fold-expansions than the combination of both (Peragine et al., 2015). This observation is in line with our results, despite we used the K-562 cell line as feeder or 'stimulator' cells, and also with the necessity of cell-to-cell contact beyond humoral signals to maintain NK cell proliferation (Miller *et al*., 1992; Robertson *et al*., 1997). However, while Peragine *et al.* obtained $24.4x10^6$ $CD3^-CD56^+$ cells (Peragine et al., 2015), we only obtained approximately $0.182x10^6$ $CD3^-CD56^+CD16^{-/+}$ cells. Thus, due to the low cell numbers obtained, the influence of different initial cell concentrations was ascertained by co-culturing for 14 days in the presence of IL-2+IL-15 NK cells isolated from PBMCs of three healthy donors (donor J, K and L) with irradiated K-562 cells at different cell densities ($0.5x10^6$, $1x10^6$ and $2x10^6$ cells/mL). Cell number and cell viability were assessed at various time points and although, as expected, higher initial cell densities result in higher cell numbers at the end of the expansion, the initial cell concentration did not show to be a determinant factor in the fold-expansion reached by NK cells. As Peragine and colleagues suggested, the variability in the NK cell expansion may be ascribed to the effectiveness of the protocol used in supporting NK cell expansion and activation, the source of feeder cells used, the use of animal-derived reagents and grading of culture components, *e.g.* culture media, growth factors and serum components (Peragine et al., 2015).

In addition to their use to assess the effect of different initial cell densities on the expansion of NK cells, cells from donors J, K and L were also used to evaluate the NK cell subsets obtained with our expansion protocol and the effector potential of the expanded cells. The frequency of each NK cell subset was determined at the beginning and end of the experiment. Except for one donor in which most

NK cells presented a $CD56^{bright}$ phenotype, $CD56^{dim}$ was the main NK cell subset detected in NK cells at day 0, which is in line with the fact that this is the dominant population in peripheral blood (Ferlazzo *et al*., 2004). Most NK cells from both subsets expressed CD16, so $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{+}$ were the most frequent phenotypes. The CD56 expression increased during culture and at day 14 $CD56^{bright}$ was the main NK cell subset in cells from all donors as it has already been shown by Torelli and colleagues (Torelli *et al*., 2015) and Nhung *et al.* (Nhung *et al*., 2018). Although $CD56^{bright}CD16^{-}$ NK cells were observed, $CD56^{bright}CD16^{+}$ represented the most common phenotype among the expanded NK cells, which is consistent with the results obtained by Peragine and colleagues (Peragine et al., 2015). Moreover, Takahashi *et al.* also demonstrated that stimulation of $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{-}$ NK cells with cytokines promotes a $CD56^{bright}CD16^{+}$ phenotype (Takahashi *et al*., 2007). In contrast, Luhm *et al.* (Luhm *et al*., 2002) and Klingemann and Martinson (Klingemann and Martinson, 2004) reported that expanding $CD56^{+}$ NK cells leads primarily to a high expansion of the $CD16^{-}$ population. However, our results showed that the only donor presenting $CD56^{bright}$ NK cells at the beginning of the culture, after expansion presented both populations $CD16^{+}$ and $CD16^{-}$, revealing inclusive a higher frequency of $CD16^{+}$ NK cells.

$CD56^{bright}$ and $CD56^{dim}$ NK cell subsets differ not only in their CD56 expression but also in their effector functions: while $CD56^{dim}$ are more effective promoting cytolysis due to the high amounts of perforin and granzyme A that are contained in their cytolytic granules, $CD56^{bright}$ NK cells are more efficient producing pro-inflammatory cytokines, such as IFN-γ and TNF-α (Jacobs *et al*., 2001). Accordingly, since $CD56^{bright}$ was the main NK cell subset in cells from all donors, it would be expected that these cells, upon target cell recognition, would respond through the production of pro-inflammatory cytokines. However, Poznanski *et al.* recently demonstrated that degranulation, cytotoxicity and IFN-γ production increase with the increase of CD56 brightness, revealing that $CD56^{bright}$ NK cells may be cytotoxic or regulatory cells (Poznanski *et al*., 2018). Moreover, $CD56^{dim}$ NK cells showed also to be an important source of cytokines and chemokines upon recognition of aberrant cells (Fauriat *et al*., 2010), for example through the release of high amounts of IFN-γ very early after activation (De Maria *et al*., 2011). Thus, CD56 expression does not distinguish regulatory from cytotoxic NK cells (Poznanski *et al*., 2018).

To determine the functional capacity of the expanded NK cells, they were co-cultured with the MHC-I negative cell line K-562 at different effector:target cells ratios (1:1, 3:1 and 10:1) - when possible - and CD107a expression as well as IFN-γ production were assessed. The analysis of the expanded NK cells revealed their ability to respond to target cell stimulation after 14 days of *in vitro* expansion. Irrespective of the effector cell:target cell ratio, NK cells from both donors J and L showed to degranulate in response to K-562 cell stimulation, revealing to maintain their degranulation ability after *ex vivo* expansion as it has been already reported in previous studies (Alter *et al*., 2004; Knorr *et al*., 2013; Spanholtz *et al*., 2011; Torelli *et al*., 2015). However, at the same effector cell:target cell ratio, the frequency of $CD107a^{+}$ NK cells obtained was not consistent between donors. These donor-associated differences may occur because although the K-562 cell line is a “gold standard” target for NK cells, the capacity of a certain stimulus to induce NK cell priming is donor-dependent (Hood *et al*., 2019). For example, Hood *et al.* induced NK cell priming with two different stimuli (IL-2 or CTV-1 cell line) and

observed that only NK cells from approximately 50% of the patients responded to each stimulus (Hood *et al*., 2019). Moreover, the same activation stimulus only resulted in certain subpopulations of NK cells from an individual to respond since different NK cell subpopulations express different combinations of activating and inhibitory receptors (Horowitz et al., 2013). Therefore, NK cell function is determined by a) which subpopulations of resting NK cells are present and their respective phenotype; b) the type of stimulus used to activate NK cells and c) the effect that this stimulus is going to have on the NK cells, which ultimately determines the ability of these cells to lyse or not their targets. The nature of the stimuli to which NK cells were previously exposed in each donor, may also condition their response since memory-like NK cells may have been generated. Memory-like NK cells are characterized by a long-lasting and specific memory, presenting a more robust response after restimulation. These cells were reported to be generated upon stimulation with haptens (O'Leary *et al*., 2006), viral pathogens (Sun *et al*., 2009) or cytokines (Romee *et al*., 2012, 2016), and to be characterized by the lack of KIRs and CD57, and an increased expression of CD94, NKG2A, NKp46 and CD69 (Romee *et al*., 2012). Thus, future experiments should include the analysis of these cell markers.

CD107a expression occurs in concert with target cell lysis, but also with cytokine secretion, being significantly associated with the production of IFN-γ and TNF-α (Alter *et al*., 2004). In agreement with this, our results demonstrated that NK cells from donor J (effector cell:target cell ratio of 10:1) and L (effector cell:target cell ratio of 1:1), besides expressing CD107a in response to K-562 cell stimulation, secreted IFN-γ. Thus, *ex vivo* expanded NK cells revealed to maintain also their capacity to produce IFN-γ in response to target cells, as it was shown in previous studies (Lee *et al*., 2017; Masuyama *et al*., 2016). Although NK cells from all donors demonstrated to be functionally active through the production of a considerable amount of IFN-γ in response to K-562 cell stimulation, this was not verified in all effector cell:target cell ratios tested. Lower effector cell:target cell ratios appeared to be associated with a higher production of IFN-γ, which may probably be explained due to a faster recognition of the target cells at higher effector cell:target cell ratios and consequently an earlier release of IFN-γ that suppress K-562 cell proliferation and induce apoptosis by increasing the expression of Fas and FasL (Xia *et al*., 2017).

IL-2+IL-15 combined with K-562 cell stimulation was the protocol leading to the most promising results in the *in vitro* expansion of NK cells. Thus, this protocol was used to ascertain the feasibility of isolating and expanding NK cells directly from patients' tumour tissue. Due to the use of patients' tumour tissue instead of PBMCs as cell source, two main alterations in the protocol were performed, namely NK cells were not sorted before expansion and the culture lasted approximately 50 days instead of 14, which resulted in a total of five stimulations with K-562 cells instead of one. The feasibility of isolating and expanding NK cells from patients' tumour tissue was assessed by evaluating over time the total cell number, cell viability and the frequency of NK cells and respective subsets. The combination of IL-2+IL-15 and K-562 cell stimulation allowed to isolate and grow NK cells *in vitro*. Cells from all patients demonstrated a decrease in their total cell numbers over time. At the end of the expansion, only cells from one of the four patients exhibited an expansion in the total cell number. The maximum total cell number obtained was observed after the 2^{nd} stimulation and corresponded to $7.49x10^6$ cells. In comparison with the results from other studies, this value is very low for the total cell number since

usually values in the order of 10^9 NK cells are obtained (Granzin *et al*., 2015; Masuyama *et al*., 2016), however using PBMCs of healthy donors. NK cells expanded from PBMCs of healthy donors have demonstrated higher fold-expansions than NK cells expanded from PBMCs of patients with cancer (Kim *et al*., 2013). However, Nhung *et al.* expanded NK cells from PBMCs of patients with cancer using a 21-day expansion method involving simulation with IL-2 (700 IU/mL) and Picibanil (a lyophilized mixture of group A *Streptococcus pyogenes* treated with Benzylpenicillin) in culture flasks-coated with an anti-CD16 monoclonal antibody and also obtained values in the order of 10^9 NK cells, namely $3.5874x10^9$ NK cells in a total of $4.1372x10^9$ cells (Nhung *et al*., 2018). In contrast, Peragine *et al.* expanded NK cells from PBMCs of patients with cancer using a 14-day expansion protocol combining IL-2+IL-15 with autologous feeder cell stimulation and obtained $18.6x10^6$ NK cells (Peragine et al., 2015). This value is much lower than the one reported by Nhung and colleagues (Nhung *et al*., 2018), however it is still very high in comparison with our results, especially because we are considering the total cell number and not the number of NK cells obtained. Some factors that may have contributed to the low total cell numbers obtained and that must be considered and investigated in future experiments are: the duration of the culture, the size of the tumour sample used and the initial number of NK cells infiltrated in the tumour, which is very challenging to determine. Although larger tumour samples may provide a much higher cell number, the fact that cells from all patients showed their highest total cell numbers after the 2nd stimulation suggests that the long-term culture may also be a main factor contributing to the low total cell numbers obtained.

IL-2 and IL-15 have a very important role in NK cell activation and survival. IL-2 promotes NK cell survival by inhibiting mitochondrial-mediated apoptosis through the induction of Bcl-2 expression (Armant *et al*., 1995), whereas IL-15 limits Bim expression through extracellular-signal-regulated kinase 1 (Erk1) and 2 (Erk2), and promotes Mcl-1 expression, which is necessary and sufficient for NK cell survival (Huntington *et al*., 2007). Although the expansion protocol used include both cytokines, the viability from cells of all patients at the end of the expansion varied between 27.66 to 67.21% compared to >90% usually reported in other studies (Nhung et al., 2018; Peragine et al., 2015). Similarly, at the end of the expansion, the NK cells' frequencies obtained were also low, varying between 2.43 and 57.54%. The highest NK cells' frequencies were detected after the 3rd stimulation and ranged between 25.12 and 76.99%. Although these values are still low, they are more similar to the ones reported for example by Nhung *et al.* that after a 21-day expansion from PBMCs of patients with cancer obtained between 54 to 96.7% NK cells (Nhung *et al*., 2018). In general, the low NK cells' frequency was not that surprising since the expansion carried out in this study was performed in a long-term culture, which has shown to have a direct impact in the NK cell expansion and viability. While Berg *et al.* demonstrated that the viability and expansion rates of NK cells reach their peak 9 to 15 days after the beginning of the culture and start to decline after 21 days (Berg *et al*., 2009), Fujisaki and colleagues showed that NK cells after 8 to 15 weeks of continuous proliferation enter into a senescent state (Fujisaki *et al*., 2009). Besides the long-term culture, the constant stimulation with IL-2 and IL-15 for a long period may also have negatively affected NK cell expansion and survival. The consecutive stimulation of NK cells with IL-15 has been shown to induce a functional NK cell change consistent with exhaustion, with these cells presenting an increased expression of cell cycle checkpoint inhibitors and arrest genes, and becoming

more susceptible to cell death (Felices *et al*., 2018). This susceptibility to cell death may have been more potentiated since IL-2 was used in combination with IL-15, and NK cells expanded and activated by IL-2 for more than 2 days have shown to upregulate Fas and other death receptors, resulting in a higher susceptibility to apoptosis mediated through these receptors (Betters *et al*., 2010). In addition, since IL-2 is known to be the "gold standard" cytokine used to expand T-cells, these cells may have also been stimulated to proliferate. Immunosuppressive Treg cells are comprised in the TME of several tumours, such as gastric (Mao *et al*., 2017) or breast cancer (Khaja et al., 2017), and their possible proliferation may have been a limiting factor in NK cell expansion since Treg cells are known to inhibit NK cell proliferation and cytotoxicity (Ghiringhelli *et al*., 2005). Thus, in future experiments the presence of Treg cells should be investigated. Moreover, a growth curve considering earlier time points should be performed to optimize the duration of the culture guaranteeing that NK cells are harvested in the peak of the exponential phase of growth and do not enter in senescence.

The frequency of NK cells in TIL varies according to the tumour histology. For example, NK cells are frequently found among TIL in bladder cancer (Mukherjee *et al*., 2018), but are scarce in the tumour infiltrate of endometrial cancer (Degos *et al*., 2019). Similarly, $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets are found in different frequencies among intra-tumoral lymphocytes. Apart from some exceptions, such as late stages of bladder cancer (Mukherjee *et al*., 2018), a higher prevalence of $CD56^{bright}$ NK cells is found in TIL from solid malignancies, *e.g.* melanoma, breast and colon cancers (Levi *et al*., 2015), NSCLC (Bruno *et al*., 2013), gastrointestinal sarcoma (Delahaye *et al*., 2011), endometrial cancer (Degos *et al*., 2019) and RCC (Schleypen *et al*., 2003, 2006). In agreement with these findings, the main NK cell subset detected in the first time point assessed in cells from all patients was the $CD56^{bright}$. Independently of the time point, this NK cell subset was always the most frequent, comprising more than 95% of all NK cells. Most NK cells were $CD16^{-}$ ($CD56^{dim}CD16^{-}$ and $CD56^{bright}CD16^{-}$) and during culture the $CD56^{bright}CD16^{-}$ NK cell subset was the most well-represented. Although the assessment of the NK cell subsets was performed for the first time after approximately 20 days of the beginning of the *in vitro* culture and not right after the tumour biopsy collection, these results are consistent with the prevalence of $CD56^{bright}CD16^{-}$ NK cells in solid malignancies, such as melanoma, breast and colon cancers (Levi *et al*., 2015), NSCLC (Bruno *et al*., 2013) and gastrointestinal sarcoma (Delahaye *et al*., 2011). However, since *in vitro* culture may have led to the preferential expansion of the $CD56^{bright}CD16^{-}$ NK cell subset, an immunofluorescence for CD3 and CD56 could be performed in paraffin sections of patients' tumour tissue to offer an overview of the frequency of $CD3^{-}CD56^{+}$ NK cells infiltrated into the tumour tissue prior to the *ex vivo* expansion.

NK cells expanded and activated with IL-2 alone or combined with IL-15 have demonstrated the capacity to kill cancer stem cells from breast cancer (Ames *et al*., 2015; Yin *et al*., 2016), PDAC, sarcoma (Ames *et al*., 2015), CRC (Tallerico *et al*., 2013) and glioblastoma (Castriconi *et al*., 2009). NK cells contribute to the killing of tumour cells through different mechanisms, however, the production of IFN-γ is one of the most important since these cells are an early source of this cytokine in tumour-developing lesions, contributing to an early anti-tumour response (Kurosawa *et al*., 1993). Thus, the functional capacity of the expanded NK cells was evaluated by their ability to produce IFN-γ in response to target cell stimulation. This analysis showed the capacity of NK cells to maintain their effector phenotype

almost after three months of *in vitro* culture. Only cells from two of the four patients (patients D155 and D157) responded to target cell stimulation, both recognizing the cell lines K-562 and Daudi. The activation of NK cells comprises two stages: the priming and the triggering (North *et al*., 2007). The priming phase is characterized by the activation of NK cells by a non-specific signal, such as an activating cytokine (*e.g.* IL-2 or IFN-γ) or a target cell expressing the proper intensity and combination of ligands for the activating receptors (North *et al*., 2007; Sabry and Lowdell, 2013). The triggering phase requires the co-engagement of at least one additional NK cell activating receptor, which should be specific to the recognition of stressed cells in order to prevent autoreactivity (North *et al*., 2007; Sabry and Lowdell, 2013). Tumour cells usually evade NK cell-mediated cytotoxicity through the lack of a priming or triggering signal (North *et al*., 2007). An example is the human leukemic cell line CTV-1 that avoids its lysis by NK cells by priming but not triggering them (North *et al*., 2007). Thus, the targeting of K-562 cells by the expanded NK cells was not surprising since according to their ligand profile, these cells can stimulate NK cells through different activating receptors, *e.g.* NKG2D, NKp30, NKp44 and DNAM1 (Tremblay-Mclean *et al*., 2019). In contrast, Daudi cells express fewer ligands for the NK cell activating receptors (Sabry *et al*., 2019) and despite they present low to no MHC-I expression, they fail to prime NK cells, being resistant to NK cell activity (North *et al*., 2007). However, when NK cells are prior primed with an activating cytokine or an appropriate target cell, such as the CTV-1 cell line, Daudi cells become susceptible to NK cell lysis (North *et al*., 2007). Therefore, the unexpected targeting of Daudi cells may have resulted from the priming of NK cells with IL-2 and IL-15 during their *in vitro* culture.

Besides K-562 and Daudi cell lines, NK cells from patient D157 produced IFN-γ not only in response to all the other allogeneic target cells but also to the autologous tumour cells and CAFs. The new observation in these results was the recognition of the autologous tumour by NK cells expanded *ex vivo* directly from the patient's tumour tissue and not from the PBMCs of patients with cancer as it has been already reported in previous studies (Poznanski *et al*., 2018; Turin *et al*., 2018; Voskens *et al*., 2010). Besides reporting the recognition of an autologous tumour with the same histology (ovarian cancer) than the one that we report here, Poznanski *et al.* described a new NK cell subset, the $CD56^{superbright}CD16^{+}$ (Poznanski *et al*., 2018). These NK cells demonstrated an enhanced activation state and anti-tumour function compared with IL-2 activated NK cells, suggesting that these capacities increase with the increase of CD56 expression. Although it was not possible to evaluate the NK cell subsets in cells of patient D157 before the IFN-γ assay, considering the results from the last time point available and the gating strategy used by Poznanski *et al.* (Poznanski *et al*., 2018), approximately half of the $CD56^{bright}$ NK cells from patient D157 were $CD56^{superbright}$. This fact may explain the capacity of NK cells from patient D157 to target the autologous tumour and CAFs, but also their ability to produce IFN-γ in response to all the tumour cell lines tested. On the other hand, this high effector capacity may suggest that our protocol promoted the differentiation of lymphokine-activated (LAK) cells, which are characterized by a potent *in vitro* cytotoxicity against syngeneic and autologous tumours and NK-resistant cells (Grimm *et al*., 1982; Lotze *et al*., 1981; Yron *et al*., 1980). LAK cells are a population of killer cells generated through the incubation of lymphocytes with high-dose IL-2 (800-1000 U/mL) (Grimm *et al*., 1982; Lotze *et al*., 1981; Yron *et al*., 1980). This cell population present MHC non-restricted anti-tumour activity (Chadwick and Miller, 1991; Phillips and Lanier, 1986) and is mainly

composed of $CD3^{-}CD56^{+}$ (NK cells), $CD3^{+}CD56^{+}$ (NKT cells) and $CD3^{+}CD56^{-}$ cells (T-cells) (West *et al*., 2011). The markers of LAK cells are similar to the ones exhibited on T-cells, despite LAK progenitor cells lack both T-cell and NK cell markers (Grimm et al., 1983; Rosenstein et al., 1984). After IL-2 activation, all cells included in the LAK cell population increase the expression of the adhesion molecule LFA-1 and the activation markers CD69, NKG2D and DNAM1. NK cells show also an increase in the expression of CD16 and their NK-specific activation markers, namely NKp30, NKp44 and NKp46, whereas T-cells present an increase in CCR7 expression (West *et al*., 2011). Due to their potent cytotoxic capacity, LAK cells together with high-dose administration of IL-2 were explored in the cancer immunotherapy field, first revealing some promising results in patients with renal cancer and melanoma (Rosenberg *et al*., 1985). However, Law *et al.* demonstrated that the therapeutic benefit achieved with LAK cells and high-dose administration of IL-2 could be obtained with the administration of IL-2 alone (Law *et al*., 1995). In future experiments, a more extensive analysis of cell surface markers must be performed to determine the type of cells that are being expanded with our protocol. Moreover, a tumour piece may be cultured using IL-2 to promote the differentiation of LAK cells and then compare their effector capacity with the one presented by the cells expanded with IL-2+IL15.

In contrast with cells from patients D155 and D157, NK cells from patients D162 and D171 did not respond to any target cell stimulation. However, while NK cells from patient D162 showed a basal production of IFN-γ not only when unstimulated but also in the presence of any target cell line, NK cells from patient D171 did not produce IFN-γ in any of the conditions tested. The basal production of IFN-γ by NK cells from patient D162 indicated that these cells were activated during their *in vitro* culture, despite they were unresponsive to target cell stimulation. NK cell activation is regulated through a balance between activating and inhibitory receptors. These two types of receptors are expressed in NK cells in different combinations, resulting in distinct NK cell populations according to the receptors expressed (Horowitz et al., 2013). Therefore, target cells may have expressed activating ligands for which the expanded NK cells lacked the corresponding activating receptors. However, at least K-562 cells should have induced NK cells to produce IFN-γ and although it cannot be considered a positive response, a considerable increase in the IFN-γ production relatively to the negative control was observed. This fact suggests that during expansion NK cells may have downregulated their activating receptors, which would make them less responsive or even unresponsive to any target cell stimulation. This is commonly observed in the dysfunctional NK cells isolated from patients with cancer (Costello *et al*., 2002; Healy *et al*., 1998; Konjević *et al*., 2007; Kono *et al*., 1996). The unresponsiveness may have also been enhanced by the constant stimulation of NK cells with K-562 cells during culture since it has been shown to lead NK cells less responsive to further stimulation (Cavalcanti et al., 1999; Jewett and Bonavida, 1995, 1996). Another hypothesis that cannot be excluded is the expression of any type of inhibitory ligands (**summarized in Table 9.5 in Appendix**) in target cells, which would impair NK cell activation.

In contrast, none of the previously mentioned hypothesis consistently explains the unresponsiveness of NK cells from patient D171. The total absence of IFN-γ production may indicate that NK cells were producing other cytokines instead of IFN-γ. Generically, activation responses result in the expression of IFN-γ, TNF-α, MIP-1α and MIP-1β (Brady *et al*., 2010; Fauriat *et al*., 2010). However,

the cytokine secreting profile presented by NK cells is strongly influenced by the surrounding cytokine milieu. Some reports have demonstrated that solid tumours induce an immunoregulatory phenotype in NK cells, which impairs their cytotoxic ability and consequently leads to the lack of therapeutic efficacy observed in NK-cell based immunotherapies for solid malignancies (Belisle *et al*., 2007; Krneta *et al*., 2016; Parkhurst *et al*., 2011). For example, $CD56^{bright}CD16^{-}$ NK cells within different solid tumours, such as melanoma, breast and colon cancers (Levi *et al*., 2015) and NSCLC (Bruno *et al*., 2013), present a pro-angiogenic phenotype, expressing *e.g.* VEGF, PIGF and IL-8 (Bruno *et al*., 2013), and contributing to tumour progression. According to their cytokine secreting profile, NK cells are commonly described as NK1 or NK2 due to their similarities to Th1 and Th2. The NK1 phenotype is characterized by the production of IFN-γ and IL-10 and may be induced by stimulation with IL-12, whereas the NK2 phenotype is known for the secretion of IL-5 and IL-13 and may be induced by stimulation with IL-4 (Deniz *et al*., 2002; Peritt *et al*., 1998). NK cells may also present a third phenotype known as NK17/NK1 and characterized by the production of IL-17 in concert with IFN-γ upon IL-2 activation (Pandya *et al*., 2011). Thus, although the absence of IFN-γ production by the NK cells from patient D171 may be explained by a different cytokine secreting profile, it does not exclude the possible entry into a state of functional anergy. However, it is important to consider that NK cell activation requires the correct stimulus to achieve a functional response, so the lack of response to one stimulus or target cell does not imply the failure to respond to another (Hood *et al*., 2019). Therefore, it is premature to conclude that the expanded NK cells had impaired cytotoxicity without performing more functional tests, such as the CD107a assay and evaluate the production of other cytokines. Of note that the production of angiogenic factors should also be assessed to confirm whether the expansion protocol used was inducing pro-tumoral NK cells. The results could also be complemented by analysing the NK cell ligands and receptors expressed in target and NK cells, respectively.

NK cells isolated from patients with cancer are usually reported to be dysfunctional (Costello *et al*., 2002; Healy *et al*., 1998; Konjević *et al*., 2007; Kono *et al*., 1996). Platonova *et al.* have inclusive demonstrated that intra-tumoral NK cells have an impaired cytotoxicity, not being able to degranulate or produce IFN-γ in response to K-562 cell stimulation (Platonova *et al*., 2011). However, although NK cell functionality was not evaluated before *in vitro* culture, our results show that after *in vitro* activation intra-tumoral NK cells are functionally active, producing IFN-γ in response to target cell stimulation. Moreover, the capacity to recognize and attack MHC-I negative cell lines (K-562 and Daudi), autologous tumour cells and CAFs highlight the contribution that NK cells may have to cancer immunotherapy, complementing αβ T-cells' effector functions.

4.2. γδ T-cells

γδ T-cells can be found in peripheral blood or as tissue-resident cells, being particularly abundant in the epithelium and mucosa (Bonneville *et al*., 2010). Tumours located in epithelial or mucosal tissues are found to be more frequently infiltrated by γδ T-cells, which may contribute to tumour eradication. In fact, γδ T-cell infiltration into tumours was shown to be the most favourable prognostic marker in many cancers (Gentles *et al*., 2015). To evaluate γδ T-cell infiltration in primary or metastatic lesions from all patients involved in this study, immunohistochemistry for the TCR δ chain was performed

in the respective paraffin tumour sections. Independently of the tumour histology, γδ T-cells were found in most analysed tumours, except for three samples: glioblastoma from patient D82, peritoneal metastasis from patient D83 and rectum adenocarcinoma from patient D171. The absence of γδ T-cells in a tumour section of a patient does not imply that this tumour does not feature γδ T-cells. Tumours have a high heterogeneity, so despite the stained section may not show γδ T-cells, it does not invalidate that these cells may have infiltrated other areas of the tumour. This was the case of the glioblastoma from patient D82 and the rectum adenocarcinoma from patient D171, since although γδ T-cells were hardly or not detectable in these patients' tumour sections, it was possible to expand them from patients' TIL or tumour tissue, respectively. The γδ T-cell ability to infiltrate into tumour tissues of different origins combined with the fact that an intra-tumoral γδ T-cell signature is the most positive prognostic marker in many cancers (Gentles *et al*., 2015) supports the use of γδ T-cells in cellular immunotherapy.

γδ T-cell-based immunotherapies, either promoting activation and expansion of γδ T-cells *in vivo* or *ex vivo*, have been shown to be feasible and safe (Fournié *et al*., 2013). Most clinical protocols expand γδ T-cells with IL-2 and zoledronic acid (Hoeres *et al*., 2018), resulting in the expansion of the γδ T-cell subset $Vγ9^{+}Vδ2^{+}$ (Meraviglia et al., 2010). Several other protocols, mainly for $Vδ2^{+}$ γδ T-cell expansion, have already been tested. Besides the combination of IL-2 and zoledronic acid (Kondo *et al*., 2008, 2011) or BrHPP (Bennouna *et al*., 2008; Kobayashi *et al*., 2011), some other protocols explored the use of a) IL-2+IL-18 (Domae *et al*., 2017), b) IL-2+IL-15 (Klimpel *et al*., 2003), c) IL-2+IL-4 (Dokouhaki *et al*., 2010) or d) IL-7+IL-1β (Maeurer *et al*., 1996b). However, a 21-day two-step protocol using IL-1β, IL-4, IL-21 and IFN-γ with anti-CD3 stimulation in the first 14 days, and IL-15 and IFN-γ with anti-CD3 stimulation in the last 7 days, was shown to be the most successful using PBMCs of patients with CLL as starting point. This protocol promoted the expansion of $Vδ1^{+}$ γδ T-cells with high cytotoxic activity against CLL cells, as well as reported the highest expansion until now (in plastic culture plates) of $Vδ1^{+}$ γδ T-cells from PBMCs of patients with cancer (Almeida *et al*., 2016). Despite the progress made in the development of protocols with some success in the expansion of γδ T-cells, currently none is widely accepted. Therefore, in the present study, to isolate γδ T-cells directly from patients' tumour tissue, we tested three expansion protocols differing in the cytokine combinations used: a) IL-2+IL-15+IL-1β, b) IL-2+IL-7 or c) IL-7+IL-1β. Moreover, irradiated allogeneic PBMCs pre-treated with zoledronic acid were used as a second stimulus since zoledronic acid demonstrated to successfully promote γδ T-cell expansion in previous studies (Kondo *et al*., 2008, 2011). Cells were stimulated five times with irradiated allogeneic PBMCs pre-treated with zoledronic acid (1^{st}, 2^{nd} and 4^{th} stimulation) or irradiated allogeneic PBMCs along with OKT3 (3^{rd} and 5^{th} stimulation). To compare the effectiveness of the different expansion protocols tested, the total cell number, cell viability and γδ T-cells' frequency were evaluated at different time points.

Our study reports for the first time, to our best knowledge, the isolation of γδ T-cells directly from patients' tumour tissue. The ideal expansion protocol should lead simultaneously to high cell numbers, cell viabilities and γδ T-cell percentages. None of the cytokine combinations tested demonstrated the capacity of providing these three requirements simultaneously. IL-2+IL-15+IL-1β and IL-2+IL-7 were the cytokine combinations that resulted in the highest total cell numbers. Higher cell numbers in the presence of IL-2 are not surprising since IL-2 is known to be the "gold standard" cytokine used to expand

T-cells. However, this raises the possibility that αβ T-cells have also been stimulated to proliferate. Although one could hypothesize that this may have resulted in competition between αβ and γδ T-cells, it may have also contributed to γδ T-cell expansion since Siegers *et al.* demonstrated that γδ T-cell expansion is higher in the presence of αβ T-cells as compared to αβ T-cells deprived cultures (Siegers *et al*., 2013). Only cells from two of the four patients (patients D157 and D171) showed an expansion in the total cell number between the first and last time point assessed, both reaching the highest total cell numbers ($2.21x10^6$ and $2.45x10^6$ cells, respectively) in the presence of IL-2+IL-7. However, the highest total cell number ($36.1x10^6$ cells) was obtained in TIL from patient D155 after the 3rd stimulation in the presence of IL-2+IL-15+IL-1β. Other studies usually present values in the order of the $36x10^6$ cells for the total number of γδ T-cells. For example, Kondo *et al.* obtained a total of $44.8x10^6$ γδ T-cells from PBMCs after a 14-day expansion using IL-2 and zoledronic acid (Kondo *et al*., 2008). This reveals that the total cell numbers and consequently the final number of γδ T-cells obtained with our expansion protocols were low. Some factors that may have contributed to these results and that must be considered and investigated in future experiments are: the duration of the culture, the size of the tumour sample used and the initial number of tumour-infiltrating γδ T-cells.

IL-2+IL-15+IL-1β and IL-2+IL-7 were the cytokine combinations leading to the highest cell viabilities during culture. The viability of the expanded cells decreased over time and at the end of the culture, cells in the presence of IL-2+IL-15+IL-1β, IL-2+IL-7 and IL-7+IL-1β presented values varying from 29.7 to 60.9%, 34.0 to 66.3% and 15.2 to 83.3%, respectively. The long-term culture and persistent exposure of γδ T-cells to their antigens have been shown to lead to a downregulation of the nuclear factor-κB (NF-κB) level in γδ T-cells, resulting in a higher sensitivity to activation-induced cell death (Ferrarini *et al*., 2008). This may explain the low cell viabilities obtained with any of the expansion protocols. It may also justify the low total cell numbers observed since long-term culture and persistent exposure of γδ T-cells to their antigens was also associated with lower cell proliferation (Ferrarini *et al*., 2008). Another hypothesis is that the expansion protocols tested, as most of the available protocols, have preferentially expanded γδ T-cells with a terminally differentiated phenotype ($CD27^-CD45RO^+$), which are characterized by low proliferative capacity and reduced survival (Dieli *et al*., 2003; Salot *et al*., 2007).

IL-2+IL-15+IL-1β led to the highest γδ T-cell percentages during culture, which presented their maximum value after the 3rd stimulation (ranging between 7.59 and 15.50%). At the end of the culture, it was the combination of IL-7 and IL-1β that resulted in the highest γδ T-cell percentages in cells from all patients, except patient D155. Although IL-7+IL-1β has shown to significantly contribute for the decrease of the total cell number and cell viability, it appears to promote the highest γδ T-cells' frequency after long-term culture. This is supported by a study that used a 3-month expansion protocol using IL-7+IL-1β to expand γδ T-cells from TIL, revealing a γδ T-cells' frequency of 96 to 98% after 2 months of culture (Maeurer *et al*., 1996b). In addition, IL-7 was already shown to be essential to prolong the life span of mature γδ T-cells, despite it is not required for their proliferation (Laky *et al*., 2003). At the end of the culture, the highest γδ T-cells' frequencies were 16.3 (patient D155 IL-2+IL-15+IL-1β) and 29% (patient D162 IL-7+IL-1β) compared to more than 60% obtained by Almeida and colleagues (Almeida *et al*., 2016), who showed to achieve the highest expansion of $Vδ1^+$ γδ T-cells recorded *in vitro* using a

two-step protocol to expand γδ T-cells from PBMCs of patients with CLL. Besides the low values observed between the first and last time point performed, only cells from two of the four patients (patients D155 and D171) demonstrated an increase in γδ T-cells' frequency, namely in the presence of IL-2+IL-15+IL-1β and IL-2+IL-7, respectively. Together these results may be explained by the fact that γδ T-cells of some patients are refractory to zoledronic acid stimulation, which was shown to be directly associated with the frequency and number of γδ T-cells present at culture initiation (Kondo *et al*., 2008). Another possible explanation is that Treg cells have co-expanded with γδ T-cells and limit their expansion since Treg cells have been shown to inhibit γδ T-cell *in vitro* proliferation (Kunzmann et al., 2008). Although none of the tested protocols allowed a robust γδ T-cell expansion, our results are the first proof of concept that γδ T-cells can be directly isolated from patients' tumour tissue and that the best cytokine combination among those tested appears to be IL-2+IL-15+IL-1β.

γδ T-cells are divided into different subsets according to the Vδ chain expressed (mainly $Vδ1^+$ or $Vδ2^+$) and their effector capacity (naïve, $CD45RA^+CD27^+$; memory, $CD45RA^-CD27^+$ or effector cells, $CD45RA^-CD27^-$). Most of the clinical protocols developed until now are based in the use of stimulants, *e.g.* aminobiphosphonates, which promote the preferential expansion of $Vδ2^+$ γδ T-cells. Zoledronic acid is an example of a commonly used aminobiphosphonate that is a strong activator of $Vγ9^+Vδ2^+$ γδ T-cells (Gober *et al*., 2003). The *in vivo* use of zoledronic acid showed to promote a decrease in the proliferative capacity of γδ T-cells, characteristic of naïve and memory cells, while it led to an increase in the IFN-γ production (Dieli *et al*., 2003). Although it was not possible to determine the Vδ chain expressed in the expanded cells, we can assume that independently of the cytokine combination used, our protocols promoted the preferential expansion of effector $Vδ2^+$ γδ T-cells, possibly $Vγ9^+Vδ2^+$, since all protocols used zoledronic acid as a stimulus. This may explain the low γδ T-cells' frequency obtained since although effector γδ T-cells are excellent IFN-γ-producers, they present a low proliferative capacity. On the other hand, the low γδ T-cells' frequency may also be explained by the fact that in comparison to $Vδ1^+$ γδ T-cells, $Vδ2^+$ γδ T-cells are usually found in lower levels in the tissue (Bonneville *et al*., 2010), despite in patients with melanoma, $Vδ2^+$ γδ T-cells were shown to be recruited to the tumour tissue and found at the same level than $Vδ1^+$ γδ T-cells (Cordova *et al*., 2012).

γδ T-cells have the capacity to kill tumour cells of different origins, namely hematopoietic tumour cell lines (Ferrarini *et al*., 1995; Fisch *et al*., 1997; Gober *et al*., 2003; Selin *et al*., 1992), colon carcinoma cells (Corvaisier *et al*., 2005), breast cancer cells (Wu *et al*., 2019), prostate tumour cells (Liu *et al*., 2005) or glioma cells (Nakazawa *et al*., 2014). γδ T-cells participate in the anti-tumour immune response through the production of IFN-γ, constituting an important early source of this cytokine (Gao *et al*., 2003). Thus, the effector capacity of the expanded γδ T-cells was evaluated by assessing their ability to produce IFN-γ in response to target cell stimulation. The analysis of the expanded γδ T-cells revealed their capacity to maintain the functional effector phenotype almost after three months of *in vitro* culture. Both IL-2+IL-15+IL-1β and IL-2+IL-7 were shown to induce the differentiation of IFN-γ-producing γδ T-cells. However, only cells from two of the four patients (patient D157 IL-2+IL-7 and patient D171 IL-2+IL-15+IL-1β and IL-2+IL-7) produced IFN-γ in response to target cell stimulation. The K-562 cell line was recognized by γδ T-cells from both patients, despite the dependence on the TCR to trigger γδ T-cells was unclear. K-562 cells are known to be lysed by γδ T-cells due to the triggering of the γδ TCR (Di

Fabrizio *et al*., 1991), which is consistent with the recognition pattern of TIL obtained from patient D171 (IL-2+IL-7), despite the low levels of IFN-γ production observed. Besides K-562 cells, the CRC cell line LoVo was the only target recognized, namely by γδ T-cells from patient D157 (IL-2+IL-7). Although LoVo cells appeared to be recognized in a TCR-independent manner since the impairment of IFN-γ production was not recovered in the presence of the IgG1 isotype control, it is well known that CRC cell lines are recognized by $Vγ9^{+}Vδ2^{+}$ γδ T-cells through their TCR (Corvaisier *et al*., 2005). NKG2D had also been reported to participate in the targeting of CRC cell lines by γδ T-cells, namely as a co-stimulatory molecule (Corvaisier *et al*., 2005). Consistent with this, CRC cell lines present high expression levels of NKG2D ligands, *e.g.* MICA (Corvaisier *et al*., 2005; Viey *et al*., 2005), MICB (Viey *et al*., 2005), ULBP1 (Corvaisier *et al*., 2005), ULBP2 (Corvaisier *et al*., 2005) and ULBP3 (Corvaisier *et al*., 2005). NKG2D was also shown to be involved in the targeting of metastatic RCC by γδ T-cells (Viey *et al*., 2005).

Most target cells did not induce γδ T-cells to produce IFN-γ, which may be explained through different factors. One hypothesis is that tumour cells impaired γδ T-cell effector function, leading to a downmodulation or even abolishment of cytokine production as Schilbach *et al.* demonstrated after challenged $Vδ2^{+}$ γδ T-cells with neuroblastoma cells (Schilbach *et al*., 2008). Although the results may also suggest that cells entered anergy or underwent activation-induced cell death due to the long-term culture, they may also indicate that the expanded γδ T-cells were producing other cytokines instead of IFN-γ. For example, in patients with SCC, tumour-infiltrating $Vδ1^{+}$ and $Vδ2^{+}$ γδ T-cells showed to preferentially produce IL-17 but not IFN-γ (Lo Presti *et al*., 2017). The cytokine production pattern is usually associated with the effector phenotype presented by γδ T-cells, namely Th1, Th2, Th17 or Treg. γδ T-cells default toward a Th1 phenotype, which is characterized by IFN-γ production upon activation. However, the polarization into other cytokine-producing subsets may occur, according to the stimuli that γδ T-cells receive from the environment. The cytokine milieu imprints on the effector phenotype fate. For example, IL-7 fails to induce Th1 γδ T-cells, yet was found to be important for the induction of a Th17 phenotype (Michel *et al*., 2012). The combination of IL-1β, IL-6, IL-23 and TGF-β together with TCR triggering lead to the expression of the transcription factor RORC, resulting in the polarization to IL-17-producing γδ T-cells (Caccamo *et al*., 2011). Although these cells have shown some anti-tumour properties (Ma *et al*., 2011), more recently they have been associated with the promotion of tumour cell proliferation and metastasis (Coffelt *et al*., 2015; Rei *et al*., 2014; Wu *et al*., 2014). In contrast, a Th1 response has been shown to be essential for tumour regression (Dai *et al*., 2018). IL-15 and IL-2 induce the polarization of γδ T-cells into a Th1 phenotype (Ribot *et al*., 2014). However, IL-2 also promotes Th17 γδ T-cell proliferation even though it does not support their survival since it induces the downregulation of the IL-7 receptor (Rei *et al*., 2014). The cytokine release pattern is also correlated with the disease clinical stage. IL-17-producing γδ T-cells showed to be more abundant in patients with advanced SCC (stage III and IV), whereas IFN-γ-producing γδ T-cells were mainly found in patients with early stage disease (stage I and II) (Lo Presti *et al*., 2017). In human CRC, $Vδ1^{+}$ γδ T-cells producing IL-17A showed to be positively correlated with a more advanced tumour stage, which has been attributed to an inflammatory regulatory axis established between dendritic cells, IL-17-producing γδ T-cells and myeloid derived stem cells (Wu *et al*., 2014). Thus, for immunotherapeutic purposes, IFN-γ and TNF-α-producing γδ T-cells are preferable, which results in the necessity of determining the cytokine

release pattern of the *ex vivo* expanded γδ T-cells before their infusion into the patient. This is an important missing piece in our work since it was only possible to evaluate the IFN-γ production. The evaluation of IL-17 production by the expanded γδ T-cells in response to target cell stimulation would have been extremely helpful to determine whether the tested expansion protocols were inducing a Th17 phenotype.

IL-2+IL-15+IL-1β was the cytokine combination leading to the most promising results in the isolation of γδ T-cells from patients' tumour tissue. Thus, the feasibility of using this protocol to expand γδ T-cells from frozen TIL and PBMCs of patients with cancer was also investigated. The total cell number, cell viability and γδ T-cells' frequency were evaluated to address the effectiveness of the expansion protocol, whereas the IFN-γ production assay was performed to determine the effector potential of the expanded cells. Cells from two of the three patients (patients D82 and D83) demonstrated an expansion in their total cell numbers between the first and last time point assessed. At the end of the culture, the total cell numbers obtained ranged between $1.75x10^6$ to $7.55x10^6$ cells. However, the total cell number peak was observed after the 4^{th} stimulation ($2.25x10^6$, $1.41x10^7$ and $1.05x10^7$ total cells) and the maximum total cell number obtained was detected after the 3^{rd} stimulation and corresponded to $2.99x10^7$ cells. These values are similar to those recorded in the expansion of γδ T-cells from patients' tumour tissue, which as previously mentioned, are very low, especially for an expansion using as starting material PBMCs and TIL. Values in the order of the ones we report are usually obtained for the total number of γδ T-cells in other studies. Numerous studies have already demonstrated to grow 10^9 γδ T-cells from PBMCs of patients with cancer, despite the level of expansion has shown to be influenced by the disease stage and/or the γδ T-cells' frequency (Bennouna et al., 2008; Bouet-Toussaint et al., 2008; Burjanadzé et al., 2007; Cabillic et al., 2010; Khan et al., 2014; Kobayashi et al., 2007, 2011; Kondo et al., 2008; Kunzmann et al., 2008; Nicol et al., 2011; Salot et al., 2007; Sugie et al., 2013; Yamasaki et al., 2011).

Cells of the three patients showed a decrease over time in cell viability, exhibiting at the end of the culture values ranging between 31.50 and 36.00%. In contrast, in cells from two of the three patients (patients D82 and D83), the γδ T-cells' frequency increased over time. At the end of the culture, the γδ T-cells' frequency varied between 0.24 and 2.73%, which is very low in comparison with several previous studies that independently of the protocol used demonstrated always to reach between 29 and approximately 100% γδ T-cells after expansion (Bouet-Toussaint et al., 2008; Burjanadzé et al., 2007; Khan et al., 2014; Kunzmann et al., 2008; Nicol et al., 2011; Salot et al., 2007; Sugie et al., 2013; Yamasaki et al., 2011). The only exceptions were found in cells from patients that did not respond to zoledronic acid stimulation. For example, while an expansion of γδ T-cells from cells of a non-responder patient only resulted in 1.1% γδ T-cells, an expansion from cells of a responder patient resulted in 49% γδ T-cells (Kunzmann et al., 2008). As previously explored, the initial γδ T-cells' frequency may also be an obstacle to their expansion. An example was the poor expansion detected in cells from patients with breast cancer that initially only exhibited 1% or less $V\delta2^+$ γδ T-cells (Sugie *et al*., 2013). Some other probable causes for the low total cell numbers, cell viabilities and γδ T-cells' frequency were already explored in the discussion of the γδ T-cell expansion from patients' tumour tissue and may reflect the a) long-term culture, b) persistent exposure of γδ T-cells to their antigens, c) promotion of a terminally

differentiated phenotype ($CD27^{-}CD45RO^{+}$) and/or d) inhibition of γδ T-cell proliferation by Treg cells. Another important consideration is that the PBMCs and TIL used were not fresh, but thawed, and this may have influenced cell expansion. Siegers *et al.* demonstrated that the expansion of $Vδ2^{+}$ γδ T-cells from frozen PBMCs was lower than that from fresh PBMCs (Siegers *et al*., 2013).

As for γδ T-cells expanded from patients' tumour tissue, certainly the use of zoledronic acid promoted the preferential expansion of the $Vδ2^{+}$ γδ T-cell subset. Although the protocol does not promote $Vδ1^{+}$ γδ T-cells' growth, it would be also unlikely to expand them because they appear to not recover from freezing and thawing since Siegers *et al.* showed that was only possible to expand $Vδ2^{+}$ γδ T-cells from frozen PBMCs even when these presented a $Vδ1^{+}$ γδ T-cell predominance (Siegers *et al*., 2013).

The effector capacity of the γδ T-cells expanded from frozen PBMCs or TIL of patients with cancer was also evaluated through their ability to produce IFN-γ in response to target cell stimulation. This analysis revealed that these cells maintained their effector capacity almost after three months of *in vitro* culture and were capable of targeting transformed autologous cells. Among all allogeneic target cells tested (lymphoblastoid, pancreatic and colorectal cancer cell lines), only K-562 cells were recognized, namely by γδ T-cells from two of the three patients (patients D82 and D83). The γδ T-cell triggering appeared to occur in a TCR-independent manner, however it is well described that the K-562 cell line is lysed by γδ T-cells due to the activation of the TCR (Di Fabrizio *et al*., 1991). The transformed autologous cells used as targets included the autologous tumour cells from patients D82 (glioblastoma cells) and D83 (pancreatic tumour cells), and the EBV-transformed B-cell line from patient D126. γδ T-cells from patient D126 produced IFN-γ only in response to the autologous EBV-transformed B-cell line. Although γδ T-cells are capable to detect virus-infected cells through their TCR, for example, due to the recognition of viral glycoproteins (Sciammas *et al*., 1994), γδ T-cells from patient D126 showed to be activated in a TCR-independent manner by the autologous B-cell line. In addition, the absence of recognition of Daudi cells, also an EBV-transformed cell line, suggests that the activation by the autologous transformed B-cells did not occur due to the recognition of EBV-encoded components, implying a non-specific recognition mechanism as it was also described in a previous study *(Orsini et al.*, 1993). Thus, the recognition of the autologous EBV-transformed B-cell line by γδ T-cells from patient D126 possibly involved a Toll-like receptor or an NK receptor. NKG2D is an example of an NK receptor that has been already reported to be involved in the recognition of virus-infected cells by human $Vγ9^{+}Vδ2^{+}$ γδ T-cells (Qin *et al*., 2009).

Although the level of IFN-γ produced did not reach the threshold to be considered a positive response, γδ T-cells from patient D83 exhibited an increase in the IFN-γ production in response to the autologous pancreatic tumour cell lines PC4 and PC5. The ability of e*x vivo* expanded γδ T-cells to recognize autologous pancreatic cancer cells was already demonstrated in a previous study, which showed that γδ T-cells present a stronger lytic activity than $CD8^{+}$ αβ T-cells (Kitayama *et al*., 1993). γδ T-cells target pancreatic carcinoma cells through two different mechanisms depending on whether γδ T-cells were or not previously stimulated by phosphoantigens: phosphoantigen-activated γδ T-cells present a TCR-dependent recognition mechanism of pancreatic tumour cells (Kabelitz *et al*., 2004), while γδ T-cells not stimulated by phosphoantigens depend almost completely on the NKG2D to target

pancreatic tumour cells (Märten *et al*., 2006). γδ T-cells from patient D83 recognized both autologous tumour cell lines through a TCR-dependent mechanism, despite they have not been stimulated with phosphoantigens. Therefore, the alternated stimulation with allogeneic PBMCs pre-treated with zoledronic acid and allogeneic PBMCs along with OKT3 also appears to influence the mechanism used by γδ T-cells to target tumour cells, in this case by favouring the expansion of γδ T-cells that recognize pancreatic tumour cells via the γδ TCR.

The autologous glioblastoma cells activated patient D82 γδ T-cells in a TCR-dependent manner and resulted in the production of a higher amount of IFN-γ than that obtained in response to the K-562 cell line. Previous studies had already demonstrated the ability of *ex vivo* expanded γδ T-cells to recognize autologous glioblastoma cells *in vitro* (Fujimiya *et al*., 1997; Yamaguchi *et al*., 1997). In comparison with the previously mentioned studies, our major experimental achievement was the expansion from TIL instead from PBMCs of γδ T-cells capable to recognize autologous glioblastoma cells. In addition to their capacity of being activated by glioblastoma cells in a TCR-dependent manner and to produce IFN-γ, previous studies showed that γδ T-cells can eradicate glioblastoma cells through the granzyme/perforin axis (Cimini *et al*., 2011; Joalland *et al*., 2018). Their activation occurs not only through the TCR but also involves the NKG2D (Chauvin *et al*., 2019; Cimini *et al*., 2011). Besides the numerous studies revealing the capacity of γδ T-cells to recognize glioblastoma cells (Bryant *et al*., 2009, 2011; Chauvin *et al*., 2019; Cimini *et al*., 2011; Joalland *et al*., 2018; Nakazawa *et al*., 2014), the efficacy of γδ T-cell-based immunotherapy was already shown in preclinical models (Bryant *et al*., 2011; Chauvin *et al*., 2019; Jarry *et al*., 2016) and is also being addressed in a phase I clinical trial (NCT04165941). However, here we raise another hypothesis for the future of cellular immunotherapy in cancer treatment rather than a γδ T-cell-based immunotherapy. The fact that γδ T-cells from patient D82 were expanded from the TIL infused in the patient as part of his cellular immunotherapy and their capacity to recognize the autologous tumour cells suggests that TIL-based immunotherapies may contain low numbers of γδ T-cells that cooperatively help αβ T-cells fighting the tumour. This encourages the application of γδ and αβ T-cells as a combined therapy for the cellular treatment of patients with solid cancers.

5. Conclusion

Our results prove the feasibility of isolating and expanding tumour-infiltrating NK cells and γδ T-cells from patients with solid cancers. The protocol used for the isolation of tumour-infiltrating γδ T-cells (IL-2+IL-15+IL-1β and alternated stimulation with allogeneic PBMCs pre-treated with zoledronic acid and allogeneic PBMCs along with OKT3) also revealed to be effective in the isolation of γδ T-cells from frozen TIL and PBMCs. Therefore, our data highlight the potential of using tumour tissues of patients with solid cancers as a new source of NK cells and γδ T-cells for ACT-based cancer immunotherapies. This new platform for ACT will allow to offer a personalized therapy with tumour-reactive NK cells or γδ T-cells that will have a set of characteristics that facilitate their infiltration into tumours. However, expansion protocols require improvement since ACT requires a large number of cells.

Our data also suggest that NK cells and γδ T-cells isolated from patients' tumour tissue, after *in vitro* culture, present a functional effector phenotype, producing IFN-γ in response to autologous transformed cells and/or allogeneic cancer cell lines of different origins (*e.g.* lymphoblastoid, pancreatic and colorectal cancer cell lines). Moreover, although the γδ T-cell receptors and tumour antigens interactions are not completely defined, our results show that tumour-infiltrating γδ T-cells use their TCR to target autologous tumour cells. Altogether, these data point out the biological relevance of both NK cells and γδ T-cells in cancer treatment and encourage their use not only as single therapies but combined with other cancer immunotherapies, especially with αβ T-cell-based ACT. A therapy combining αβ T-cells with NK cells or γδ T-cells may lead to better clinical responses in patients with cancer since it will allow to target both MHC positive and negative tumour cells, resulting in the complete eradication of the tumours. However, *in vitro* and preclinical studies are needed to evaluate in detail the synergistic action of NK cells and γδ T-cells with αβ T-cells.

Finally, although tumour-infiltrating NK cells and γδ T-cells were explored in early clinical trials to treat patients with primary solid tumours, they may also represent a very promising treatment for patients with an advanced clinical condition characterized by the presence of multiple tumours (primary and/or metastatic). Since metastases are the major cause of death in patients with solid tumours, cancer immunotherapies based on tumour-infiltrating NK cells and γδ T-cells may offer a glimpse of hope for a better clinical outcome. Thus, this study provides the first proof of concept necessary to start exploring tumour-infiltrating NK cells or γδ T-cells ACT in cancer immunotherapy.

6. Future perspectives

Although our work opens a new chapter in the ACT-based cancer immunotherapies, there is still much more to be explored. Some of the investigational lines to follow as a complement of this work are:

1) Analysing the TCR in tumour-infiltrating γδ T-cells

Although the use of zoledronic acid in our expansion protocols may lead us to assume that the obtained γδ T-cell product is enriched in Vδ2$^+$ γδ T-cells, the TCR analysis will allow to confirm this hypothesis, as well as to determine whether the obtained cell product corresponds to a monoclonal or polyclonal γδ T-cell population. Since γδ T-cells from some patients recognized the autologous tumour cells, would also be interesting to determine the Vγ and Vδ repertoires present in the PBMCs to ascertain whether tumour-specific γδ T-cells were only located at the tumour site or also in circulation. This can be done using deep TCR γδ sequencing.

2) Improving expansion protocols

After successfully isolating NK cells and γδ T-cells directly from patients' tumour tissue, the ultimate goal is to improve the expansion protocols to yield higher cell numbers. Tumour tissues from patients with solid cancers will be cultured with the cytokine combination reported here as the best to expand NK cells and γδ T-cells. With less cytokine combinations being tested, larger tumour tissue pieces will be used to isolate and expand each cell type, which will certainly allow to yield higher cell numbers. In addition, the tumour tissue sample will be removed from culture at day 3 instead of day 10. This will allow NK cells and γδ T-cells to expand without the presence of the TME, which may contribute to higher yields since TME is usually immunosuppressive and thus its presence may impair NK cell and γδ T-cell proliferation. Moreover, the duration of the *in vitro* culture will also be reduced to 14-21 days since most *ex vivo* expansion protocols for both cell types range this duration and at least the NK cells' growth peak was shown to occur during this period.

3) Exploring protocols to expand Vδ1$^+$ instead of Vδ2$^+$ γδ T-cells

Our data provide the first proof of concept that γδ T-cells can be directly isolated from patients' tumour tissue. After demonstrating this by using a protocol that is commonly employed to promote the preferential expansion of Vδ2$^+$ γδ T-cells, due to the use of feeder cells pre-treated with zoledronic acid as stimulus, our ultimate goal is to develop a protocol that promotes the expansion of tumour-infiltrating Vδ1$^+$ γδ T-cells. In comparison with Vδ2$^+$ γδ T-cells, Vδ1$^+$ γδ T-cells present certain characteristics that make them much more attractive for cancer immunotherapies, namely: a) exhibit a broader expression of adhesion molecules, which confers them a greater potential to migrate and be retained in the tumours, as it was demonstrated in SCC (Thomas *et al*., 2001); b) present CCL2-mediated chemotaxis toward tumours (Lança *et al*., 2013) and c) are less susceptible to activation-induced cell death (Siegers *et al*., 2011). Vδ1$^+$ γδ T-cells were shown to directly interact with lipids presented by CD1d, such as α-galactosylceramide (Uldrich *et al*., 2013). Since cardiolipin binds to human CD1d (Cox *et al*.,

2009) and it was already reported in murine to activate γδ T-cells (Dieudé *et al*., 2011), we plan to isolate and expand Vδ1⁺ γδ T-cells from patients' tumour tissue by using the best cytokine combination reported here (IL-2+IL-15+IL-1β) but instead of feeder cells treated with zoledronic acid, we would like to use cardiolipin.

4) Determine chemokine receptors and perform migration assays

The interaction between chemokine receptors and their ligands is a critical factor for the proper homing of immune cells into tumour sites, a point that has been one of the main challenges of the ACT-based cancer immunotherapies for solid tumours. Although theoretically our work provides a promising alternative, it is still necessary to perform some chemotaxis assays that confirm the migration capacity of the expanded tumour-infiltrating NK cells and γδ T-cells into the tumour. Firstly, a transwell migration assay should be performed where the chemoattractant gradient can be established by the autologous tumour cells, or when these are not available, allogeneic tumour cell lines with similar histology to the patient's tumour can be used. Besides the staining and further counting of the migratory cells, a live imaging video could also be performed to capture the migratory process. Further this first validation, an *in vivo* chemotaxis assay should also be carried out. To complement these *in vitro* and *in vivo* assays, the chemokines released by the autologous tumour could be determined through an ELISA, allowing to further confirm through RNA-sequencing or flow cytometry whether the respective chemokine receptors are expressed by the expanded tumour-infiltrating NK cells and γδ T-cells. Perhaps a more biologically relevant, but also more technically challenging way to study immune cell homing and tissue interaction is to use the so-called 'thick tumour section', which would allow to keep the TME intact and study the direct interaction of *ex vivo* added autologous immune cells with the tumour tissue.

5) Study the combined action of NK cells and γδ T-cells with αβ T-cells

This work aimed to explore a new cell source for NK cells and γδ T-cells-based cancer immunotherapies that allows the expansion of tumour-reactive cells with a higher tumour-homing capacity. The improvement of these ACT as single therapies will allow their future combination with αβ T-cells in a unique cell-based cancer immunotherapy, which will offer the simultaneously targeting of MHC positive and negative tumour cells, resulting in tumour eradication. Thus, it would be interesting to first demonstrate *in vitro* the synergistic action of NK cells and γδ T-cells with αβ T-cells. For this, the three cell types should be isolated and expanded directly from patients' tumour tissue and cultured with autologous tumour cells individually or combined (NK cells and αβ T-cells or γδ and αβ T-cells). Then, besides evaluating the IFN-γ production, IL-17 production, CD107a expression and tumour cell death could also be determined. Moreover, it would also be interesting to observe the live interactions between tumour cells and the different immune cell types, individually or combined, since γδ T-cells have been suggested to take up killed tumour cells and to cross-present them to αβ T-cells (Brandes *et al*., 2009).

7. References

Abdolvahab, M.H., Darvishi, B., Zarei, M., Majidzadeh-A, K., and Farahmand, L. (2020). Interferons: Role in cancer therapy. Immunotherapy *12*, 833–855.

Abe, Y., Muto, M., Nieda, M., Nakagawa, Y., Nicol, A., Kaneko, T., Goto, S., Yokokawa, K., and Suzuki, K. (2009). Clinical and immunological evaluation of zoledronate-activated Vγ9γδ T-cell-based immunotherapy for patients with multiple myeloma. Exp. Hematol. *37*, 956–968.

Van Acker, H.H., Anguille, S., Willemen, Y., Smits, E.L., and Van Tendeloo, V.F. (2015). Bisphosphonates for cancer treatment: Mechanisms of action and lessons from clinical trials. Pharmacol. Ther. *158*, 24–40.

Alexander, A.A.Z., Maniar, A., Cummings, J.-S., Hebbeler, A.M., Schulze, D.H., Gastman, B.R., Pauza, C.D., Strome, S.E., and Chapoval, A.I. (2008). Isopentenyl pyrophosphate-activated CD56+ γδ T lymphocytes display potent antitumor activity toward human squamous cell carcinoma. Clin. Cancer Res. *14*, 4232–4240.

Almeida, A.R., Correia, D. V., Fernandes-Platzgummer, A., Da Silva, C.L., Da Silva, M.G., Anjos, D.R., and Silva-Santos, B. (2016). Delta one T cells for immunotherapy of chronic lymphocytic leukemia: Clinical-grade expansion/differentiation and preclinical proof of concept. Clin. Cancer Res. *22*, 5795–5804.

Alnaggar, M., Xu, Y., Li, J., He, J., Chen, J., Li, M., Wu, Q., Lin, L., Liang, Y., Wang, X., et al. (2019). Allogenic Vγ9Vδ2 T cell as new potential immunotherapy drug for solid tumor: A case study for cholangiocarcinoma. J. Immunother. Cancer *7*, 1–7.

Alter, G., Malenfant, J.M., and Altfeld, M. (2004). CD107a as a functional marker for the identification of natural killer cell activity. J. Immunol. Methods *294*, 15–22.

Ames, E., Canter, R.J., Grossenbacher, S.K., Mac, S., Chen, M., Smith, R.C., Hagino, T., Perez-Cunningham, J., Sckisel, G.D., Urayama, S., et al. (2015). NK Cells Preferentially Target Tumor Cells with a Cancer Stem Cell Phenotype. J Immunol. *195*, 4010–4019.

Aoki, T., Matsushita, H., Hoshikawa, M., Hasegawa, K., Kokudo, N., and Kakimi, K. (2017). Adjuvant combination therapy with gemcitabine and autologous γδ T-cell transfer in patients with curatively resected pancreatic cancer. Cytotherapy *19*, 473–485.

Armant, M., Delespesse, G., and Sarfati, M. (1995). IL-2 and IL-7 but not IL-12 protect natural killer cells from death by apoptosis and up-regulate bcl-2 expression. Immunology *85*, 331–337.

Bachanova, V., Cooley, S., Defor, T.E., Verneris, M.R., Zhang, B., McKenna, D.H., Curtsinger, J., Panoskaltsis-Mortari, A., Lewis, D., Hippen, K., et al. (2014). Clearance of acute myeloid leukemia by haploidentical natural killer cells is improved using IL-2 diphtheria toxin fusion protein. Blood *123*, 3855–3863.

Bauer, S., Groh, V., Wu, J., Steinle, A., Phillips, J.H., Lanier, L.L., and Spies, T. (1999). Activation of NK cells and T cells by NKG2D, a receptor for stress- inducible MICA. Science. *285*, 727–729.

Belisle, J.A., Gubbels, J.A.A., Raphael, C.A., Migneault, M., Rancourt, C., Connor, J.P., and Patankar, M.S. (2007). Peritoneal natural killer cells from epithelial ovarian cancer patients show an altered phenotype and bind to the tumour marker MUC16 (CA125). Immunology *122*, 418–429.

Bennouna, J., Bompas, E., Neidhardt, E.M., Rolland, F., Philip, I., Galéa, C., Salot, S., Saiagh, S., Audrain, M., Rimbert, M., et al. (2008). Phase-I study of Innacell γδ^{TM}, an autologous cell-therapy product highly enriched in γ9δ2 T lymphocytes, in combination with IL-2, in patients with metastatic renal cell carcinoma. Cancer Immunol. Immunother. *57*, 1599–1609.

Bennouna, J., Levy, V., Sicard, H., Senellart, H., Audrain, M., Hiret, S., Rolland, F., Bruzzoni-Giovanelli, H., Rimbert, M., Galéa, C., et al. (2010). Phase i study of bromohydrin pyrophosphate (BrHPP, IPH 1101), a Vγ9Vδ2 T lymphocyte agonist in patients with solid tumors. Cancer Immunol. Immunother. *59*, 1521–1530.

Benyamine, A., Le Roy, A., Mamessier, E., Gertner-Dardenne, J., Castanier, C., Orlanducci, F., Pouyet, L., Goubard, A., Collette, Y., Vey, N., et al. (2016). BTN3A molecules considerably improve Vγ9Vδ2T cells-based immunotherapy in acute myeloid leukemia. Oncoimmunology *5*, e1146843.

Berahovich, R.D., Lai, N.L., Wei, Z., Lanier, L.L., and Schall, T.J. (2006). Evidence for NK Cell Subsets Based on Chemokine Receptor Expression. J. Immunol. *177*, 7833–7840.

Berg, M., Lundqvist, A., McCoy Jr, P., Samsel, L., Fan, Y., Tawab, A., and Childs, R. (2009). Clinical-grade ex vivo-expanded human natural killer cells up-regulate activating receptors and death receptor ligands and have enhanced cytolytic activity against tumor cells. Cytotherapy *11*, 341–355.

Betters, D.M., Smith, A.L., Berg, M., Lundqvist, A., and Childs, R.W. (2010). In vitro expanded natural killer (NK) cells are more susceptible to Fas-mediated apoptosis compared to fresh and overnight IL-2 activated NK cells. In Cancer Research, (American Association for Cancer Research (AACR)), p. Abstract 1271.

Bhatia, A., and Kumar, Y. (2014). Cellular and molecular mechanisms in cancer immune escape: A comprehensive review. Expert Rev. Clin. Immunol. *10*, 41–62.

Bjorklund, A.T., Carlsten, M., Sohlberg, E., Liu, L.L., Clancy, T., Karimi, M., Cooley, S., Miller, J.S., Klimkowska, M., Schaffer, M., et al. (2018). Complete remission with reduction of high-risk clones following haploidentical NK-cell therapy against MDS and AML. Clin. Cancer Res. *24*, 1834–1844.

Bonneville, M., O'Brien, R.L., and Born, W.K. (2010). γδ T cell effector functions: A blend of innate programming and acquired plasticity. Nat. Rev. Immunol. *10*, 467–478.

Bottino, C., Falco, M., Parolini, S., Marcenaro, E., Augugliaro, R., Sivori, S., Landi, E., Biassoni, R., Notarangelo, L.D., Moretta, L., et al. (2001). NTB-A, a novel SH2D1A-associated surface molecule contributing to the inability of natural killer cells to kill Epstein-Barr virus-infected B cells in X-linked lymphoproliferative disease. J. Exp. Med. *194*, 235–246.

Bouet-Toussaint, F., Cabillic, F., Toutirais, O., Le Gallo, M., Thomas De La Pintière, C., Daniel, P., Genetet, N., Meunier, B., Dupont-Bierre, E., Boudjema, K., et al. (2008). Vγ9Vδ2 T cell-mediated recognition of human solid tumors. Potential for immunotherapy of hepatocellular and colorectal carcinomas. Cancer Immunol. Immunother. *57*, 531–539.

Brady, J., Carotta, S., Thong, R.P.L., Chan, C.J., Hayakawa, Y., Smyth, M.J., and Nutt, S.L. (2010). The Interactions of Multiple Cytokines Control NK Cell Maturation. J. Immunol. *185*, 6679–6688.

Brandes, M., Willimann, K., and Moser, B. (2005). Professional antigen-presentation function by human γδ cells. Science. *309*, 264–268.

Brandes, M., Willimann, K., Bioley, G., Lévy, N., Eberl, M., Luo, M., Tampé, R., Lévy, F., Romero,

P., and Moser, B. (2009). Cross-presenting human γδ T cells induce robust CD8+ αβ T cell responses. Proc. Natl. Acad. Sci. U. S. A. *106*, 2307–2312.

Brandt, C.S., Baratin, M., Yi, E.C., Kennedy, J., Gao, Z., Fox, B., Haldeman, B., Ostrander, C.D., Kaifu, T., Chabannon, C., et al. (2009). The B7 family member B7-H6 is a tumor cell ligand for the activating natural killer cell receptor NKp30 in humans. J. Exp. Med. *206*, 1495–1503.

Braud, V.M., Allan, D.S.J., O'Callaghan, C.A., Soderstrom, K., D'Andrea, A., Ogg, G.S., Lazetic, S., Young, N.T., Bell, J.I., Phillips, J.H., et al. (1998). HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. Nature *391*, 795–799.

Brooks, M.E., Kristensen, K., van Benthem, K.J., Magnusson, A., Berg, C.W., Nielsen, A., Skaug, H.J., Mächler, M., and Bolker, B.M. (2017). glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. R J. *9*, 378–400.

Bruno, A., Focaccetti, C., Pagani, A., Imperatori, A.S., Spagnoletti, M., Rotolo, N., Cantelmo, A.R., Franzi, F., Capella, C., Ferlazzo, G., et al. (2013). The proangiogenic phenotype of natural killer cells in patients with non-small cell lung cancer. Neoplasia *15*, 133–142.

Bryant, N.L., Suarez-Cuervo, C., Gillespie, G.Y., Markert, J.M., Nabors, L.B., Meleth, S., Lopez, R.D., and Lamb, L.S. (2009). Characterization and immunotherapeutic potential of γδ T-cells in patients with glioblastoma. Neuro. Oncol. *11*, 357–367.

Bryant, N.L., Gillespie, G.Y., Lopez, R.D., Markert, J.M., Cloud, G.A., Langford, C.P., Arnouk, H., Su, Y., Haines, H.L., Suarez-Cuervo, C., et al. (2011). Preclinical evaluation of ex vivo expanded/activated γδ T cells for immunotherapy of glioblastoma multiforme. J. Neurooncol. *101*, 179–188.

Burjanadzé, M., Condomines, M., Reme, T., Quittet, P., Latry, P., Lugagne, C., Romagne, F., Morel, Y., Rossi, J.F., Klein, B., et al. (2007). In vitro expansion of gamma delta T cells with anti-myeloma cell activity by Phosphostim and IL-2 in patients with multiple myeloma. Br. J. Haematol. *139*, 206–216.

Burshtyn, D.N., Scharenberg, A.M., Wagtmann, N., Rajagopalan, S., Berrada, K., Y, T., Kinet, J.-P., and Long, E.O. (1996). Recruitment of Tyrosine Phosphatase HCP (SHP-1) by the Killer Cell Inhibitory Receptor. Immunity *4*, 77–85.

Cabillic, F., Toutirais, O., Lavoué, V., De La Pintière, C.T., Daniel, P., Rioux-Leclerc, N., Turlin, B., Mönkkönen, H., Mönkkönen, J., Boudjema, K., et al. (2010). Aminobisphosphonate-pretreated dendritic cells trigger successful Vγ9Vδ2 T cell amplification for immunotherapy in advanced cancer patients. Cancer Immunol. Immunother. *59*, 1611–1619.

Cabrera, T., Fernandez, M.A., Sierra, A., Garrido, A., Herruzo, A., Escobedo, A., Fabra, A., and Garrido, F. (1996). High frequency of altered HLA class I phenotypes in invasive breast carcinomas. Hum. Immunol. *50*, 127–134.

Cabrera, T., Collado, A., Fernandez, M.A., Ferron, A., Sancho, J., Ruiz-Cabello, F., and Garrido, F. (1998). High frequency of altered HLA class I phenotypes in invasive colorectal carcinomas. Tissue Antigens *52*, 114–123.

Caccamo, N., La Mendola, C., Orlando, V., Meraviglia, S., Todaro, M., Stassi, G., Sireci, G., Fournié, J.J., and Dieli, F. (2011). Differentiation, phenotype, and function of interleukin-17-producing human vγ9vδ2 T cells. Blood *118*, 129–138.

Campbell, J.J., Qin, S., Unutmaz, D., Soler, D., Murphy, K.E., Hodge, M.R., Wu, L., and Butcher,

E.C. (2001). Unique Subpopulations of CD56 + NK and NK-T Peripheral Blood Lymphocytes Identified by Chemokine Receptor Expression Repertoire . J. Immunol. *166*, 6477–6482.

Campbell, K.S., Dessing, M., Lopez-Botet, M., Cella, M., and Colonna, M. (1996). Tyrosine phosphorylation of a human killer inhibitory receptor recruits protein tyrosine phosphatase 1C. J. Exp. Med. *184*, 93–100.

Castriconi, R., Daga, A., Dondero, A., Zona, G., Poliani, P.L., Melotti, A., Griffero, F., Marubbi, D., Spaziante, R., Bellora, F., et al. (2009). NK Cells Recognize and Kill Human Glioblastoma Cells with Stem Cell-Like Properties. J. Immunol. *182*, 3530–3539.

Cavalcanti, M., Jewett, A., and Bonavida, B. (1999). Irreversible cancer cell-induced functional anergy and apoptosis in resting and activated NK cells. Int. J. Oncol. *14*, 361–366.

Chadwick, B.S., and Miller, R.G. (1991). Heterogeneity of the lymphokine-activated killer cell phenotype. Cell. Immunol. *132*, 168–176.

Chauvin, C., Joalland, N., Perroteau, J., Jarry, U., Lafrance, L., Willem, C., Retiere, C., Oliver, L., Gratas, C., Gautreau-Rolland, L., et al. (2019). NKG2D controls natural reactivity of Vg9Vd2 T lymphocytes against mesenchymal glioblastoma cells. Clin. Cancer Res. *25*, 7218–7228.

Chen, D.S., and Mellman, I. (2013). Oncology meets immunology: The cancer-immunity cycle. Immunity *39*, 1–10.

Chen, G., Huang, A.C., Zhang, W., Zhang, G., Wu, M., Xu, W., Yu, Z., Yang, J., Wang, B., Sun, H., et al. (2018). Exosomal PD-L1 Contributes to Immunosuppression and is Associated with anti-PD-1 Response. Nature *560*, 382–386.

Chen, M.L., Pittet, M.J., Gorelik, L., Flavell, R.A., Weissleder, R., Von Boehmer, H., and Khazaie, K. (2005). Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-β signals in vivo. Proc. Natl. Acad. Sci. U. S. A. *102*, 419–424.

Choi, A.H., O'Leary, M.P., Fong, Y., and Chen, N.G. (2016). From benchtop to bedside: A review of oncolytic virotherapy. Biomedicines *4*, 1–20.

Choudhry, H., Helmi, N., Abdulaal, W.H., Zeyadi, M., Zamzami, M.A., Wu, W., Mahmoud, M.M., Warsi, M.K., Rasool, M., and Jamal, M.S. (2018). Prospects of IL-2 in Cancer Immunotherapy. Biomed Res. Int. *2018*, 9056173.

Christmas, S.E., and Meager, A. (1990). Production of interferon-gamma and tumour necrosis factor-alpha by human T-cell clones expressing different forms of the gamma delta receptor. Immunology *71*, 486–492.

Christofi, T., Baritaki, S., Falzone, L., Libra, M., and Zaravinos, A. (2019). Current perspectives in cancer immunotherapy. Cancers (Basel). *11*, 1472.

Cimini, E., Piacentini, P., Sacchi, A., Gioia, C., Leone, S., Lauro, G.M., Martini, F., and Agrati, C. (2011). Zoledronic acid enhances Vδ2 T-lymphocyte antitumor response to human glioma cell lines. Int. J. Immunopathol. Pharmacol. *24*, 139–148.

ClinicalTrials.gov. Nk cells Completed, Terminated Studies - List Results. Retrieved 30th November 2020, from https://clinicaltrials.gov/ct2/results?term=nk+cells&Search=Apply&recrs=h&recrs=e&age_v=&gndr=&type=&rslt=.

ClinicalTrials.gov. Nk cells Recruiting, Not yet recruiting, Active, not recruiting, Enrolling by invitation Studies - List Results. Retrieved 30th November 2020, from https://clinicaltrials.gov/ct2/results?term=Nk+cells&Search=Apply&recrs=b&recrs=a&recrs=f&recrs=d&age_v=&gndr=&type=&rslt=.

Coffelt, S.B., Kersten, K., Doornebal, C.W., Weiden, J., Vrijland, K., Hau, C.-S., Verstegen, N.J., Ciampricotti, M., JAC Hawinkels, L., Jonkers, J., et al. (2015). IL17-producing γδ T cells and neutrophils conspire to promote breast cancer metastasis. Nature *522*, 345–348.

Cordova, A., Toia, F., la Mendola, C., Orlando, V., Meraviglia, S., Rinaldi, G., Todaro, M., Cicero, G., Zichichi, L., Donni, P.L., et al. (2012). Characterization of Human γδ T Lymphocytes Infiltrating Primary Malignant Melanomas. PLoS One *7*, e49878.

Correia, D. V., Fogli, M., Hudspeth, K., Gomes Da Silva, M., Mavilio, D., and Silva-Santos, B. (2011). Differentiation of human peripheral blood Vδ1+ T cells expressing the natural cytotoxicity receptor NKp30 for recognition of lymphoid leukemia cells. Blood *118*, 992–1001.

Corvaisier, M., Moreau-Aubry, A., Diez, E., Bennouna, J., Mosnier, J.-F., Scotet, E., Bonneville, M., and Jotereau, F. (2005). Vγ9Vδ2 T Cell Response to Colon Carcinoma Cells. J. Immunol. *175*, 5481–5488.

Cosman, D., Müllberg, J., Sutherland, C.L., Chin, W., Armitage, R., Fanslow, W., Kubin, M., and Chalupny, N.J. (2001). ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. Immunity *14*, 123–133.

Costello, R.T., Sivori, S., Marcenaro, E., Lafage-Pochitaloff, M., Mozziconacci, M.J., Reviron, D., Gastaut, J.A., Pende, D., Olive, D., and Moretta, A. (2002). Defective expression and function of natural killer cell-triggering receptors in patients with acute myeloid leukemia. Blood *99*, 3661–3667.

Couzi, L., Pitard, V., Sicard, X., Garrigue, I., Hawchar, O., Merville, P., Moreau, J.F., and Déchanet-Merville, J. (2012). Antibody-dependent anti-cytomegalovirus activity of human γδ T cells expressing CD16 (FcγRIIIa). Blood *119*, 1418–1427.

Cox, D., Fox, L., Tian, R., Bardet, W., Skaley, M., Mojsilovic, D., Gumperz, J., and Hildebrand, W. (2009). Determination of Cellular Lipids Bound to Human CD1d Molecules. PLoS One *4*, e5325.

Cursons, J., Souza-Fonseca-Guimaraes, F., Foroutan, M., Anderson, A., Hollande, F., Hediyeh-Zadeh, S., Behren, A., Huntington, N.D., and Davis, M.J. (2019). A gene signature predicting natural killer cell infiltration and improved survival in melanoma patients. Cancer Immunol. Res. *7*, 1162–1174.

D'Asaro, M., La Mendola, C., Di Liberto, D., Orlando, V., Todaro, M., Spina, M., Guggino, G., Meraviglia, S., Caccamo, N., Messina, A., et al. (2010). Vγ9Vδ2 T Lymphocytes Efficiently Recognize and Kill Zoledronate-Sensitized, Imatinib-Sensitive, and Imatinib-Resistant Chronic Myelogenous Leukemia Cells. J. Immunol. *184*, 3260–3268.

Dai, M., Hellstrom, I., Yip, Y.Y., Sjögren, H.O., and Hellstrom, K.E. (2018). Tumor Regression and Cure Depends on Sustained Th1 Responses. J. Immunother. *41*, 369–378.

Das, H., Groh, V., Kuijl, C., Sugita, M., Morita, C.T., Spies, T., and Bukowski, J.F. (2001). MICA engagement by human Vγ2Vδ2 T cells enhances their antigen-dependent effector function. Immunity *15*, 83–93.

Degos, C., Heinemann, M., Barrou, J., Boucherit, N., Lambaudie, E., Savina, A., Gorvel, L., and

Olive, D. (2019). Endometrial tumor microenvironment alters human NK cell recruitment, and resident NK cell phenotype and function. Front. Immunol. *10*, 877.

Delahaye, N.F., Rusakiewicz, S., Martins, I., Ménard, C., Roux, S., Lyonnet, L., Paul, P., Sarabi, M., Chaput, N., Semeraro, M., et al. (2011). Alternatively spliced NKp30 isoforms affect the prognosis of gastrointestinal stromal tumors. Nat. Med. *17*, 700–707.

Deniz, G., Akdis, M., Aktas, E., Blaser, K., and Akdis, C.A. (2002). Human NK1 and NK2 subsets determined by purification of IFN-γ-secreting and IFN-γ-nonsecreting NK cells. Eur. J. Immunol. *32*, 879–884.

Dieli, F., Gebbia, N., Poccia, F., Caccamo, N., Montesano, C., Fulfaro, F., Arcara, C., Valerio, M.R., Meraviglia, S., Di Sano, C., et al. (2003). Induction of γδ T-lymphocyte effector functions by bisphosphonate zoledronic acid in cancer patients in vivo. Blood *102*, 2310–2311.

Dieli, F., Vermijlen, D., Fulfaro, F., Caccamo, N., Meraviglia, S., Cicero, G., Roberts, A., Buccheri, S., D'Asaro, M., Gebbia, N., et al. (2007). Targeting human γδ T cells with zoledronate and interleukin-2 for immunotherapy of hormone-refractory prostate cancer. Cancer Res. *67*, 7450–7457.

Dieudé, M., Striegl, H., Tyznik, A.J., Wang, J., Behar, S.M., Piccirillo, C.A., Levine, J.S., Zajonc, D.M., and Rauch, J. (2011). Cardiolipin Binds to CD1d and Stimulates CD1d-Restricted γδ T Cells in the Normal Murine Repertoire. J. Immunol. *186*, 4771–4781.

Dokouhaki, P., Han, M., Joe, B., Li, M., Johnston, M.R., Tsao, M.S., and Zhang, L. (2010). Adoptive immunotherapy of cancer using ex vivo expanded human γδ T cells: A new approach. Cancer Lett. *297*, 126–136.

Dokouhaki, P., Schuh, N.W., Joe, B., Allen, C.A.D., Der, S.D., Tsao, M.S., and Zhang, L. (2013). NKG2D regulates production of soluble TRAIL by ex vivo expanded human γδ T cells. Eur. J. Immunol. *43*, 3175–3182.

Dolstra, H., Fredrix, H., Van Der Meer, A., De Witte, T., Figdor, C., and Van De Wiel-Van Kemenade, E. (2001). TCR γδ cytotoxic T lymphocytes expressing the killer cell-inhibitory receptor p58.2 (CD158b) selectively lyse acute myeloid leukemia cells. Bone Marrow Transplant. *27*, 1087–1093.

Domae, E., Hirai, Y., Ikeo, T., Goda, S., and Shimizu, Y. (2017). Cytokine-mediated activation of human ex vivo-expanded Vγ9Vδ2 T cells. Oncotarget *8*, 45928–45942.

Van Driel, W.J., Tjiong, M.Y., Hilders, C.G.J.M., Trimbos, B.J., and Fleuren, G.J. (1996). Association of allele-specific HLA expression and histopathologic progression of cervical carcinoma. Gynecol. Oncol. *62*, 33–41.

Eberl, M., Hintz, M., Reichenberg, A., Kollas, A.K., Wiesner, J., and Jomaa, H. (2003). Microbial isoprenoid biosynthesis and human γδ T cell activation. FEBS Lett. *544*, 4–10.

Di Fabrizio, L., Kimura, Y., Ware, R., Rogozinski, L., and Chess, L. (1991). Specific triggering of gamma, delta T cells by K562 activates the gamma, delta T cell receptor and may regulate natural killer-like function. J. Immunol. *146*, 2495–2503.

Fauriat, C., Long, E.O., Ljunggren, H.-G., and Bryceson, Y.T. (2010). Regulation of human NK-cell cytokine and chemokine production by target cell recognition. Blood *115*, 2167–2176.

Felices, M., Lenvik, A.J., McElmurry, R., Chu, S., Hinderlie, P., Bendzick, L., Geller, M.A., Tolar, J., Blazar, B.R., and Miller, J.S. (2018). Continuous treatment with IL-15 exhausts human NK cells via a

metabolic defect. JCI Insight *3*, e96219.

Ferlazzo, G., Thomas, D., Lin, S.-L., Goodman, K., Morandi, B., Muller, W.A., Moretta, A., and Münz, C. (2004). The Abundant NK Cells in Human Secondary Lymphoid Tissues Require Activation to Express Killer Cell Ig-Like Receptors and Become Cytolytic. J. Immunol. *172*, 1455–1462.

Ferrarini, M., Heltai, S., Toninelli, E., Sabbadini, M.G., Pellicciari, C., and Manfredi, A.A. (1995). Daudi lymphoma killing triggers the programmed death of cytotoxic V gamma 9/V delta 2 T lymphocytes. J. Immunol. *154*, 3704–3712.

Ferrarini, M., Delfanti, F., Gianolini, M., Rizzi, C., Alfano, M., Lazzarin, A., and Biswas, P. (2008). NF-κB Modulates Sensitivity to Apoptosis, Proinflammatory and Migratory Potential in Short- versus Long-Term Cultured Human γδ Lymphocytes. J. Immunol. *181*, 5857–5864.

Fisch, P., Meuer, E., Pende, D., Rothenfußer, S., Viale, O., Kock, S., Ferrone, S., Fradelizi, D., Klein, G., Moretta, L., et al. (1997). Control of a B cell lymphoma recognition via natural killer inhibitory receptors implies a role for human Vγ9/Vδ2 T cells in tumor immunity. Eur. J. Immunol. *27*, 3368–3379.

Fournié, J.J., Sicard, H., Poupot, M., Bezombes, C., Blanc, A., Romagné, F., Ysebaert, L., and Laurent, G. (2013). What lessons can be learned from γδ T cell-based cancer immunotherapy trials? Cell. Mol. Immunol. *10*, 35–41.

Fujimiya, Y., Suzuki, Y., Katakura, R., Miyagi, T., Yamaguchi, T., Yoshimoto, T., and Ebina, T. (1997). In Vitro Interleukin 12 Activation of Peripheral Blood CD3+ CD56+ and CD3+ CD56- γδ T cells from glioblastoma patients. Clin. Cancer Res. *3*, 633–643.

Fujisaki, H., Kakuda, H., Imai, C., Mullighan, C.G., and Campana, D. (2009). Replicative Potential of Human Natural Killer Cells. Br. J. Haematol. *145*, 606–613.

Gao, Y., Yang, W., Pan, M., Scully, E., Girardi, M., Augenlicht, L.H., Craft, J., and Yin, Z. (2003). γδ T Cells Provide an Early Source of Interferon γ in Tumor Immunity. J. Exp. Med. *198*, 433–442.

Gentles, A.J., Newman, A.M., Liu, C.L., Bratman, S. V., Feng, W., Kim, D., Nair, V.S., Xu, Y., Khuong, A., Hoang, C.D., et al. (2015). The prognostic landscape of genes and infiltrating immune cells across human cancers. Nat. Med. *21*, 938–945.

Ghiringhelli, F., Ménard, C., Terme, M., Flament, C., Taieb, J., Chaput, N., Puig, P.E., Novault, S., Escudier, B., Vivier, E., et al. (2005). CD4+CD25+ regulatory T cells inhibit natural killer cell functions in a transforming growth factor-β-dependent manner. J. Exp. Med. *202*, 1075–1085.

Girardi, M., Oppenheim, D.E., Steele, C.R., Lewis, J.M., Glusac, E., Filler, R., Hobby, P., Sutton, B., Tigelaar, R.E., and Hayday, A.C. (2001). Regulation of cutaneous malignancy by γδ T cells. Science. *294*, 605–609.

Glatzel, A., Wesch, D., Schiemann, F., Brandt, E., Janssen, O., and Kabelitz, D. (2002). Patterns of Chemokine Receptor Expression on Peripheral Blood γδ T Lymphocytes: Strong Expression of CCR5 Is a Selective Feature of Vδ2/Vγ9 γδ T Cells. J. Immunol. *168*, 4920–4929.

Gober, H.-J., Kistowska, M., Angman, L., Jenö, P., Mori, L., and De Libero, G. (2003). Human T cell receptor γδ cells recognize endogenous mevalonate metabolites in tumor cells. J. Exp. Med. *197*, 163–168.

Godder, K., Henslee-Downey, P., Mehta, J., Park, B., Chiang, K.-Y., Abhyankar, S., and Lamb, L. (2007). Long term disease-free survival in acute leukemia patients recovering with increased γδ T cells

after partially mismatched related donor bone marrow transplantation. Bone Marrow Transplant. *39*, 751–757.

Granzin, M., Soltenborn, S., Müller, S., Kollet, J., Berg, M., Cerwenka, A., Childs, R.W., and Huppert, V. (2015). Fully automated expansion and activation of clinical-grade natural killer cells for adoptive immunotherapy. Cytotherapy *17*, 621–632.

Granzin, M., Wagner, J., Köhl, U., Cerwenka, A., Huppert, V., and Ullrich, E. (2017). Shaping of natural killer cell antitumor activity by ex vivo cultivation. Front. Immunol. *8*, 458.

Griffioen, A.W., Damen, C.A., Martinotti, S., Blijham, G.H., and Groenewegen, G. (1996). Endothelial intercellular adhesion molecule-1 expression is suppressed in human malignancies: The role of angiogenic factors. Cancer Res. *56*, 1111–1117.

Grimm, E.A., Mazumder, A., Zhang, H.Z., and Rosenberg, S.A. (1982). Lymphokine-activated killer cell phenomenon. Lysis of natural killer-resistant fresh solid tumor cells by interleukin 2-activated autologous human peripheral blood lymphocytes. J. Exp. Med. *155*, 1823–1841.

Grimm, E.A., Ramsey, K.M., Mazumder, A., Wilson, D.J., Djeu, J.Y., and Rosenberg, S.A. (1983). Lymphokine-activated Killer Cell Phenomenon II. Precursor Phenotype Is Serologically Distinct from Peripheral T Lymphocytes, Memory Cytotoxic Thymus-derived Lymphocytes, and Natural Killer Cells. J. Exp. Med. *157*, 884–897.

Gross, G., Waks, T., and Eshhar, Z. (1989). Expression of immunoglobulin-T-cell receptor chimeric molecules as functional receptors with antibody-type specificity. Proc. Natl. Acad. Sci. U. S. A. *86*, 10024–10028.

Gu, S., Borowska, M.T., Boughter, C.T., and Adams, E.J. (2018). Butyrophilin3A proteins and Vγ9Vδ2 T cell activation. Stem Cell Dev Biol. *84*, 65–74.

Guerra, N., Tan, Y.X., Joncker, N.T., Choy, A., Gallardo, F., Xiong, N., Knoblaugh, S., Cado, D., Greenberg, N.R., and Raulet, D.H. (2008). NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. Immunity *28*, 571–580.

Halary, F., Peyrat, M.-A., Champagne, E., Lopez-Botet, M., Moretta, A., Moretta, L., Vié, H., Fournié, J.J., and Bonneville, M. (1997). Control of self-reactive cytotoxic T lymphocytes expressing γδ T cell receptors by natural killer inhibitory receptors. Eur. J. Immunol. *27*, 2812–2821.

Halfteck, G.G., Elboim, M., Gur, C., Achdout, H., Ghadially, H., and Mandelboim, O. (2009). Enhanced In Vivo Growth of Lymphoma Tumors in the Absence of the NK-Activating Receptor NKp46/NCR1. J. Immunol. *182*, 2221–2230.

Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of cancer: The next generation. Cell *144*, 646–674.

Hansasuta, P., Dong, T., Thananchai, H., Weekes, M., Willberg, C., Aldemir, H., Rowland-Jones, S., and Braud, V.M. (2004). Recognition of HLA-A3 and HLA-A11 by KIR3DL2 is peptide-specific. Eur. J. Immunol. *34*, 1673–1679.

Harrer, D.C., Simon, B., Fujii, S., Shimizu, K., Uslu, U., Schuler, G., Gerer, K.F., Hoyer, S., Dörrie, J., and Schaft, N. (2017). RNA-transfection of γ/δ T cells with a chimeric antigen receptor or an α/β T-cell receptor: A safer alternative to genetically engineered α/β T cells for the immunotherapy of melanoma. BMC Cancer *17*, 551.

Healy, C.G., Simons, J.W., Carducci, M.A., DeWeese, T.L., Bartkowski, M., Tong, K.P., and Bolton, W.E. (1998). Impaired expression and function of signal-transducing zeta chains in peripheral T cells and natural killer cells in patients with prostate cancer. Cytometry *32*, 109–119.

Hoeres, T., Smetak, M., Pretscher, D., and Wilhelm, M. (2018). Improving the efficiency of Vγ9Vδ2 T-cell immunotherapy in cancer. Front. Immunol. *9*, 800.

Hood, S.P., Foulds, G.A., Imrie, H., Reeder, S., McArdle, S.E.B., Khan, M., and Pockley, A.G. (2019). Phenotype and function of activated natural killer cells from patients with prostate cancer: Patient-dependent responses to priming and IL-2 activation. Front. Immunol. *9*, 3169.

Horowitz, A., Strauss-albee, D.M., Leipold, M., Kubo, J., Nemat-Gorgani, N., Dogan, O.C., Dekker, C.L., Mackey, S., Maecker, H., Swan, G.E., et al. (2013). Genetic and environmental determinants of human NK cell diversity revealed by mass cytometry. Sci. Transl. Med. *5*, 208ra145.

Huntington, N.D., Puthalakath, H., Gunn, P., Naik, E., Michalak, E.M., Smyth, M.J., Tabarias, H., Degli-Esposti, M.A., Dewson, G., Willis, S.N., et al. (2007). Interleukin 15-mediated survival of natural killer cells is determined by interactions among Bim, Noxa and Mcl-1. Nat. Immunol. *8*, 856–863.

Igarashi, T., Wynberg, J., Srinivasan, R., Becknell, B., McCoy, J.P., Takahashi, Y., Suffredini, D.A., Linehan, W.M., Caligiuri, M.A., and Childs, R.W. (2004). Enhanced cytotoxicity of allogeneic NK cells with killer immunoglobulin-like receptor ligand incompatibility against melanoma and renal cell carcinoma cells. Blood *104*, 170–177.

Izumi, T., Kondo, M., Takahashi, T., Fujieda, N., Kondo, A., Tamura, N., Murakawa, T., Nakajima, J., Matsushita, H., and Kakimi, K. (2013). Ex vivo characterization of γδ T-cell repertoire in patients after adoptive transfer of Vγ9Vδ2 T cells expressing the interleukin-2 receptor β-chain and the common γ-chain. Cytotherapy *15*, 481–491.

Jacobs, R., Hintzen, G., Kemper, A., Beul, K., Kempf, S., Behrens, G., Sykora, K.-W., and Schmidt, R.E. (2001). CD56bright cells differ in their KIR repertoire and cytotoxic features from CD56dim NK cells. Eur. J. Immunol. *31*, 3121–3126.

Jarry, U., Chauvin, C., Joalland, N., Léger, A., Minault, S., Robard, M., Bonneville, M., Oliver, L., Vallette, F.M., Vié, H., et al. (2016). Stereotaxic administrations of allogeneic human Vγ9Vδ2 T cells efficiently control the development of human glioblastoma brain tumors. Oncoimmunology *5*, e1168554.

Jewett, A., and Bonavida, B. (1995). Target-induced anergy of natural killer cytotoxic function is restricted to the NK-target conjugate subset. Cell. Immunol. *160*, 91–97.

Jewett, A., and Bonavida, B. (1996). Target-induced inactivation and cell death by apoptosis in a subset of human NK cells. J. Immunol. *156*, 907–915.

Jinushi, M., Takehara, T., Tatsumi, T., Kanto, T., Groh, V., Spies, T., Kimura, R., Miyagi, T., Mochizuki, K., Sasaki, Y., et al. (2003). Expression and role of MICA and MICB in human hepatocellular carcinomas and their regulation by retinoic acid. Int. J. Cancer *104*, 354–361.

Joalland, N., Chauvin, C., Oliver, L., Vallette, F.M., Pecqueur, C., Jarry, U., and Scotet, E. (2018). IL-21 Increases the Reactivity of Allogeneic Human Vγ9Vδ2 T Cells Against Primary Glioblastoma Tumors. J. Immunother. *41*, 224–231.

Kabelitz, D., Wesch, D., Pitters, E., and Zöller, M. (2004). Characterization of Tumor Reactivity of Human Vγ9Vδ2 γδ T Cells In Vitro and in SCID Mice In Vivo. J. Immunol. *173*, 6767–6776.

Kärre, K., Ljunggren, H.G., Piontek, G., and Kiessling, R. (1986). Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. Nature *319*, 675–678.

Kerkar, S.P., and Restifo, N.P. (2012). Cellular constituents of immune escape within the tumor microenvironment. Cancer Res. *72*, 3125–3130.

Khaja, A.S.S., Toor, S.M., Salhat, H. El, Faour, I., Haq, N.U., Ali, B.R., and Elkord, E. (2017). Preferential accumulation of regulatory T cells with highly immunosuppressive characteristics in breast tumor microenvironment. Oncotarget *8*, 33159–33171.

Khan, M.W.A., Curbishley, S.M., Chen, H.-C., Thomas, A.D., Pircher, H., Mavilio, D., Steven, N.M., Eberl, M., and Moser, B. (2014). Expanded human blood-derived γδT cells display potent antigen-presentation functions. Front. Immunol. *5*, 344.

Kim, E.-K., Ahn, Y.-O., Kim, S., Kim, T.M., Keam, B., and Heo, D.S. (2013). Ex vivo activation and expansion of natural killer cells from patients with advanced cancer with feeder cells from healthy volunteers. Cytotherapy *15*, 231–241.

Kitayama, J., Atomi, Y., Nagawa, H., Kuroda, A., Mutoh, T., Minami, M., and Juji, T. (1993). Functional analysis of TCR γδ+ T cells in tumour-infiltrating lymphocytes (TIL) of human pancreatic cancer. Clin. Exp. Immunol. *93*, 442–447.

Klein, E., Klein, G., Nadkarni, J.S., Nadkarni, J.J., Wigzell, H., and Clifford, P. (1968). Surface IgM-kappa Specificity on a Burkitt Lymphoma Cell In Vivo and in Derived Culture Lines. Cancer Res. *28*, 1300–1310.

Klimpel, G.R., Matthias, M.A., and Vinetz, J.M. (2003). Leptospira interrogans Activation of Human Peripheral Blood Mononuclear Cells: Preferential Expansion of TCRγδ + T Cells vs TCRαβ + T Cells . J. Immunol. *171*, 1447–1455.

Klingemann, H.-G., and Martinson, J. (2004). Ex vivo expansion of natural killer cells for clinical applications. Cytotherapy *6*, 15–22.

Knorr, D.A., Ni, Z., Hermanson, D., Hexum, M.K., Bendzick, L., Cooper, L.J.N., Lee, D.A., and Kaufman, D.S. (2013). Clinical-Scale Derivation of Natural Killer Cells From Human Pluripotent Stem Cells for Cancer Therapy. Stem Cells Transl. Med. *2*, 274–283.

Kobayashi, H., Tanaka, Y., Yagi, J., Osaka, Y., Nakazawa, H., Uchiyama, T., Minato, N., and Toma, H. (2007). Safety profile and anti-tumor effects of adoptive immunotherapy using gamma-delta T cells against advanced renal cell carcinoma: A pilot study. Cancer Immunol. Immunother. *56*, 469–476.

Kobayashi, H., Tanaka, Y., Yagi, J., Minato, N., and Tanabe, K. (2011). Phase I/II study of adoptive transfer of γδ T cells in combination with zoledronic acid and IL-2 to patients with advanced renal cell carcinoma. Cancer Immunol. Immunother. *60*, 1075–1084.

Kondo, M., Sakuta, K., Noguchi, A., Ariyoshi, N., Sato, K., Sato, S., Sato, K., Hosoi, A., Nakajima, J., Yoshida, Y., et al. (2008). Zoledronate facilitates large-scale ex vivo expansion of functional γδ T cells from cancer patients for use in adoptive immunotherapy. Cytotherapy *10*, 842–856.

Kondo, M., Izumi, T., Fujieda, N., Kondo, A., Morishita, T., Matsushita, H., and Kakimi, K. (2011). Expansion of human peripheral blood γδ T cells using zoledronate. J. Vis. Exp. *55*, e3182.

Kong, Y., Cao, W., Xi, X., Ma, C., Cui, L., and He, W. (2009). The NKG2D ligand ULBP4 binds to TCRγ9/δ2 and induces cytotoxicity to tumor cells through both TCRγδ and NKG2D. Blood *114*, 310–

317.

Konjević, G., Mirjačić Martinović, K., Vuletić, A., Jović, V., Jurisić, V., Babović, N., and Spužić, I. (2007). Low expression of CD161 and NKG2D activating NK receptor is associated with impaired NK cell cytotoxicity in metastatic melanoma patients. Clin. Exp. Metastasis *24*, 1–11.

Kono, K., Ressing, M.E., Brandt, R.M.P., Melief, C.J.M., Potkul, R.K., Andersson, B., Petersson, M., Kast, W.M., and Kiessling, R. (1996). Decreased expression of signal-transducing ζ chain in peripheral T cells and natural killer cells in patients with cervical cancer. Clin. Cancer Res. *2*, 1825–1828.

Kress, E., Hedges, J.F., and Jutila, M.A. (2006). Distinct gene expression in human Vδ1 and Vδ2 γδ T cells following non-TCR agonist stimulation. Mol. Immunol. *43*, 2002–2011.

Krneta, T., Gillgrass, A., Chew, M., and Ashkar, A.A. (2016). The breast tumor microenvironment alters the phenotype and function of natural killer cells. Cell. Mol. Immunol. *13*, 628–639.

Kunzmann, V., Kimmel, B., Herrmann, T., Einsele, H., and Wilhelm, M. (2008). Inhibition of phosphoantigen-mediated γδ T-cell proliferation by CD4+ CD25+ FoxP3+ regulatory T cells. Immunology *126*, 256–267.

Kunzmann, V., Smetak, M., Kimmel, B., Weigang-Koehler, K., Goebeler, M., Birkmann, J., Becker, J., Schmidt-Wolf, I.G.H., Einsele, H., and Wilhelm, M. (2012). Tumor-promoting versus tumor-antagonizing roles of γδ T cells in cancer immunotherapy: Results from a prospective phase I/II trial. J. Immunother. *35*, 205–213.

Kurosawa, S., Matsuzaki, G., Harada, M., Ando, T., and Nomoto, K. (1993). Early appearance and activation of natural killer cells in tumor-infiltrating lymphoid cells during tumor development. Eur. J. Immunol. *23*, 1029–1033.

Kuwana, Y., Asakura, Y., Utsunomiya, N., Nakanishi, M., Arata, Y., Itoh, S., Nagase, F., and Kurosawa, Y. (1987). Expression of chimeric receptor composed of immunoglobulin-derived V regions and T-cell receptor-derived C regions. Biochem. Biophys. Res. Commun. *149*, 960–968.

Laad, A.D., Thomas, M.L., Fakih, A.R., and Chiplunkar, S. V. (1999). Human gamma delta T cells recognize heat shock protein-60 on oral tumor cells. Int. J. Cancer *80*, 709–714.

Laky, K., Lewis, J.M., Tigelaar, R.E., and Puddington, L. (2003). Distinct Requirements for IL-7 in Development of TCR γδ Cells During Fetal and Adult Life. J. Immunol. *170*, 4087–4094.

Lança, T., Correia, D. V., Moita, C.F., Raquel, H., Neves-Costa, A., Ferreira, C., Ramalho, J.S., Barata, J.T., Moita, L.F., Gomes, A.Q., et al. (2010). The MHC class Ib protein ULBP1 is a nonredundant determinant of leukemia/lymphoma susceptibility to γδ T-cell cytotoxicity. Blood *115*, 2407–2411.

Lança, T., Costa, M.F., Gonçalves-Sousa, N., Rei, M., Grosso, A.R., Penido, C., and Silva-Santos, B. (2013). Protective Role of the Inflammatory CCR2/CCL2 Chemokine Pathway through Recruitment of Type 1 Cytotoxic γδ T Lymphocytes to Tumor Beds. J. Immunol. *190*, 6673–6680.

Lang, J.M., Kaikobad, M.R., Wallace, M., Staab, M.J., Horvath, D.L., Wilding, G., Liu, G., Eickhoff, J.C., McNeel, D.G., and Malkovsky, M. (2011). Pilot trial of interleukin-2 and zoledronic acid to augment γδ T cells as treatment for patients with refractory renal cell carcinoma. Cancer Immunol. Immunother. *60*, 1447–1460.

Law, T.M., Motzer, R.J., Mazumdar, M., Sell, K.W., Walther, P., O'Connell, M., Khan, A., Vlamis, V., Vogelzang, N.J., and Bajorin, D.F. (1995). Phase III randomized trial of interleukin-2 with or without

lymphokine-activated killer cells in the treatment of patients with advanced renal cell carcinoma. Cancer *76*, 824–832.

Lee, H.R., Son, C.H., Koh, E.K., Bae, J.H., Kang, C.D., Yang, K., and Park, Y.S. (2017). Expansion of cytotoxic natural killer cells using irradiated autologous peripheral blood mononuclear cells and anti-CD16 antibody. Sci. Rep. *7*, 11075.

Lelièvre, L., Clézardin, P., Magaud, L., Roche, L., Tubiana-Mathieu, N., Tigaud, J.D., Topart, D., Raban, N., Mouret-Reynier, M.A., and Mathevet, P. (2018). Comparative Study of Neoadjuvant Chemotherapy With and Without Zometa for Management of Locally Advanced Breast Cancer With Serum VEGF as Primary Endpoint: The NEOZOL Study. Clin. Breast Cancer *18*, e1311–e1321.

Levi, I., Amsalem, H., Nissan, A., Darash-Yahana, M., Peretz, T., Mandelboim, O., and Rachmilewitz, J. (2015). Characterization of tumor infiltrating Natural Killer cell subset. Oncotarget *6*, 13835–13843.

Li, Z., Xu, Q., Peng, H., Cheng, R., Sun, Z., and Ye, Z. (2011). IFN-γ enhances HOS and U2OS cell lines susceptibility to γδ T cell-mediated killing through the Fas/Fas ligand pathway. Int. Immunopharmacol. *11*, 496–503.

Liu, E., Marin, D., Banerjee, P., Macapinlac, H.A., Thompson, P., Basar, R., Kerbauy, L.N., Overman, B., Thall, P., Kaplan, M., et al. (2020). Use of CAR-transduced natural killer cells in CD19-positive lymphoid tumors. N. Engl. J. Med. *382*, 545–553.

Liu, Z., Guo, B.L., Gehrs, B.C., Nan, L., and Lopez, R.D. (2005). Ex vivo expanded human Vγ9Vδ2+ γδ-T cells mediate innate antitumor activity against human prostate cancer cells in vitro. J. Urol. *173*, 1552–1556.

Ljunggren, H.-G., and Kärre, K. (1985). Host Resistance Directed Selectively Against H-2-Deficient Lymphoma Variants Analysis of the Mechanism. J. Exp. Med. *162*, 1745–1759.

Ljunggren, H.-G., and Kärre, K. (1990). In search of the “missing self”: MHC molecules and NK cell recognition. Immunol. Today *11*, 237–244.

Lotze, M.T., Grimm, E.A., Mazumder, A., Strausser, J.L., and Rosenberg, S.A. (1981). Lysis of fresh and cultured autologous tumor by human lymphocytes cultured in T-cell growth factor. Cancer Res. *41*, 4420–4425.

Lozzio, C.B., and Lozzio, B.B. (1975). Human With Chronic Myelogenous Leukemia Cell-Line With Positive Philadelphia Chromosome. Blood *45*, 321–334.

Luhm, J., Brand, J.M., Koritke, P., Höppner, M., Kirchner, H., and Frohn, C. (2002). Large-scale generation of natural killer lymphocytes for clinical application. J. Hematotherapy Stem Cell Res. *11*, 651–657.

Luoma, A.M., Castro, C.D., Mayassi, T., Bembinster, L.A., Bai, L., Picard, D., Anderson, B., Scharf, L., Kung, J.E., Sibener, L. V., et al. (2013). Crystal Structure of Vδ1 T Cell Receptor in Complex with CD1d-Sulfatide Shows MHC-like Recognition of a Self-Lipid by Human γδ T Cells. Immunity *39*, 1032–1042.

Ma, Y., Aymeric, L., Locher, C., Mattarollo, S.R., Delahaye, N.F., Pereira, P., Boucontet, L., Apetoh, L., Ghiringhelli, F., Casares, N., et al. (2011). Contribution of IL-17-producing γδ T cells to the efficacy of anticancer chemotherapy. J. Exp. Med. *208*, 491–503.

Maeurer, M.J., Gollin, S.M., Martin, D., Swaney, W., Bryant, J., Castelli, C., Robbins, P., Parmiani,

G., Storkus, W.J., and Lotze, M.T. (1996a). Tumor escape from immune recognition: Lethal recurrent melanoma in a patient associated with downregulation of the peptide transporter protein TAP-1 and loss of expression of the immunodominant MART-1/Melan-A antigen. J. Clin. Invest. *98*, 1633–1641.

Maeurer, M.J., Martin, D., Walter, W., Liu, K., Zitvogel, L., Halusczcak, K., Rabinowich, H., Duquesnoy, R., Storkus, W., and Lotzer, M.T. (1996b). Human Intestinal Vδ1+ T Lymphocytes Recognize Tumor Cells of Epithelial Origin. J. Exp. Med. *183*, 1681–1696.

Maniar, A., Zhang, X., Lin, W., Gastman, B.R., Pauza, C.D., Strome, S.E., and Chapoval, A.I. (2010). Human γδ T lymphocytes induce robust NK cell-mediated antitumor cytotoxicity through CD137 engagement. Blood *116*, 1726–1733.

Mao, F.Y., Kong, H., Zhao, Y.L., Peng, L.S., Chen, W., Zhang, J.Y., Cheng, P., Wang, T.T., Lv, Y.P., Teng, Y.S., et al. (2017). Increased tumor-infiltrating CD45RA-CCR7- regulatory T-cell subset with immunosuppressive properties foster gastric cancer progress. Cell Death Dis. *8*, e3002.

Marcenaro, E., Augugliaro, R., Falco, M., Castriconi, R., Parolini, S., Sivori, S., Romeo, E., Millo, R., Moretta, L., Bottino, C., et al. (2003). CD59 is physically and functionally associated with natural cytotoxicity receptors and activates human NK cell-mediated cytotoxicity. Eur. J. Immunol. *33*, 3367–3376.

De Maria, A., Bozzano, F., Cantoni, C., and Moretta, L. (2011). Revisiting human natural killer cell subset function revealed cytolytic CD56dimCD16+ NK cells as rapid producers of abundant IFN-γ on activation. Proc. Natl. Acad. Sci. U. S. A. *108*, 728–732.

Marlin, R., Pappalardo, A., Kaminski, H., Willcox, C.R., Pitard, V., Netzer, S., Khairallah, C., Lomenech, A.M., Harly, C., Bonneville, M., et al. (2017). Sensing of cell stress by human γδ TCR-dependent recognition of annexin A2. Proc. Natl. Acad. Sci. U. S. A. *114*, 3163–3168.

Märten, A., Von Lilienfeld-Toal, M., Büchler, M.W., and Schmidt, J. (2006). Soluble MIC is elevated in the serum of patients with pancreatic carcinoma diminishing γδ T cell cytotoxicity. Int. J. Cancer *119*, 2359–2365.

Masuyama, J. ichi, Murakami, T., Iwamoto, S., and Fujita, S. (2016). Ex vivo expansion of natural killer cells from human peripheral blood mononuclear cells co-stimulated with anti-CD3 and anti-CD52 monoclonal antibodies. Cytotherapy *18*, 80–90.

Melandri, D., Zlatareva, I., Chaleil, R.A.G., Dart, R.J., Chancellor, A., Nussbaumer, O., Polyakova, O., Roberts, N.A., Wesch, D., Kabelitz, D., et al. (2018). The γδTCR combines innate immunity with adaptive immunity by utilizing spatially distinct regions for agonist selection and antigen responsiveness. Nat. Immunol. *19*, 1352–1365.

Meraviglia, S., Eberl, M., Vermijlen, D., Todaro, M., Buccheri, S., Cicero, G., La Mendola, C., Guggino, G., D'Asaro, M., Orlando, V., et al. (2010). In vivo manipulation of Vγ9Vδ2 T cells with zoledronate and low-dose interleukin-2 for immunotherapy of advanced breast cancer patients. Clin. Exp. Immunol. *161*, 290–297.

Met, Ö., Jensen, K.M., Chamberlain, C.A., Donia, M., and Svane, I.M. (2019). Principles of adoptive T cell therapy in cancer. Semin. Immunopathol. *41*, 49–58.

Meza Guzman, L.G., Keating, N., and Nicholson, S.E. (2020). Natural killer cells: Tumor surveillance and signaling. Cancers (Basel). *12*, 952.

Michel, M.L., Pang, D.J., Haque, S.F.Y., Potocnik, A.J., Pennington, D.J., and Hayday, A.C. (2012). Interleukin 7 (IL-7) selectively promotes mouse and human IL-17-producing γδ cells. Proc. Natl. Acad. Sci. U. S. A. *109*, 17549–17554.

Mikulak, J., Oriolo, F., Bruni, E., Roberto, A., Colombo, F.S., Villa, A., Bosticardo, M., Bortolomai, I., Presti, E. Lo, Meraviglia, S., et al. (2019). NKp46-expressing human gut-resident intraepithelial Vδ1 T cell subpopulation exhibits high antitumor activity against colorectal cancer. JCI Insight *4*, 1–19.

Miller, J.S., Oelkers, S., Verfaillie, C., and McGlave, P. (1992). Role of monocytes in the expansion of human activated natural killer cells. Blood *80*, 2221–2229.

Moulin, M., Alguacil, J., Gu, S., Mehtougui, A., Adams, E.J., Peyrottes, S., and Champagne, E. (2017). Vγ9Vδ2 T cell activation by strongly agonistic nucleotidic phosphoantigens. Cell. Mol. Life Sci. *74*, 4353–4367.

Mukherjee, N., Ji, N., Hurez, V., Curiel, T.J., Montgomery, M.O., Braun, A.J., Nicolas, M., Aguilera, M., Kaushik, D., Liu, Q., et al. (2018). Intratumoral CD56bright natural killer cells are associated with improved survival in bladder cancer. Oncotarget *9*, 36492–36502.

Muto, M., Baghdadi, M., Maekawa, R., Wada, H., and Seino, K.I. (2015). Myeloid molecular characteristics of human γδ T cells support their acquisition of tumor antigen-presenting capacity. Cancer Immunol. Immunother. *64*, 941–949.

Nakajima, J., Murakawa, T., Fukami, T., Goto, S., Kaneko, T., Yoshida, Y., Takamoto, S., and Kakimi, K. (2010). A phase I study of adoptive immunotherapy for recurrent non-small-cell lung cancer patients with autologous γδ T cells. Eur. J. Cardio-Thoracic Surg. *37*, 1191–1197.

Nakazawa, T., Nakamura, M., Park, Y.S., Motoyama, Y., Hironaka, Y., Nishimura, F., Nakagawa, I., Yamada, S., Matsuda, R., Tamura, K., et al. (2014). Cytotoxic human peripheral blood-derived γδT cells kill glioblastoma cell lines: Implications for cell-based immunotherapy for patients with glioblastoma. J. Neurooncol. *116*, 31–39.

Nhung, H.T.M., Anh, B.V., Huyen, T.L., Hiep, D.T., Thao, C.T., Lam, P.N., and Liem, N.T. (2018). Ex vivo expansion of human peripheral blood natural killer cells and cytotoxic T lymphocytes from lung cancer patients. Oncol. Lett. *15*, 5730–5738.

Nicol, A., Tokuyama, H., Mattarollo, S., Hagi, T., Suzuki, K., Yokokawa, K., and Nieda, M. (2011). Clinical evaluation of autologous gamma delta T cell-based immunotherapy for metastatic solid tumours. Br. J. Cancer *105*, 778–786.

Noguchi, A., Kaneko, T., Kamigaki, T., Fujimoto, K., Ozawa, M., Saito, M., Ariyoshi, N., and Goto, S. (2011). Zoledronate-activated Vγ9γδ T cell-based immunotherapy is feasible and restores the impairment of γδ T cells in patients with solid tumors. Cytotherapy *13*, 92–97.

North, J., Bakhsh, I., Marden, C., Pittman, H., Addison, E., Navarrete, C., Anderson, R., and Lowdell, M.W. (2007). Tumor-Primed Human Natural Killer Cells Lyse NK-Resistant Tumor Targets: Evidence of a Two-Stage Process in Resting NK Cell Activation. J. Immunol. *178*, 85–94.

O'Leary, J.G., Goodarzi, M., Drayton, D.L., and von Andrian, U.H. (2006). T cell- and B cell-independent adaptive immunity mediated by natural killer cells. Nat. Immunol. *7*, 507–516.

Orsini, D.L.M., Res, P.C.M., Van Laar, J.M., Muller, L.M., Soprano, A.E.L., Kooy, Y.M.C., Tak, P.P., and Koning, F. (1993). A Subset of Vδ1+ T Cells Proliferates in Response to Epstein-Barr Virus-

Transformed B Cell Lines In Vitro. Scand. J. Immunol. *38*, 335–340.

Pandya, A.D., Al-Jaderi, Z., Høglund, R.A., Holmøy, T., Harbo, H.F., Norgauer, J., and Maghazachi, A.A. (2011). Identification of human NK17/NK1 cells. PLoS One *6*, e26780.

Pardoll, D.M. (2012). The blockade of immune checkpoints in cancer immunotherapy. Nat. Rev. Cancer *12*, 252–264.

Parkhurst, M.R., Riley, J.P., Dudley, M.E., and Rosenberg, S.A. (2011). Adoptive transfer of autologous natural killer cells leads to high levels of circulating natural killer cells but does not mediate tumor regression. Clin. Cancer Res. *17*, 6287–6297.

Pegram, H.J., Haynes, N.M., Smyth, M.J., Kershaw, M.H., and Darcy, P.K. (2010). Characterizing the anti-tumor function of adoptively transferred NK cells in vivo. Cancer Immunol. Immunother. *59*, 1235–1246.

Pende, D., Parolini, S., Pessino, A., Sivori, S., Augugliaro, R., Morelli, L., Marcenaro, E., Accame, L., Malaspina, A., Biassoni, R., et al. (1999). Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells. J. Exp. Med. *190*, 1505–1516.

Peng, D., Kryczek, I., Nagarsheth, N., Zhao, L., Wei, S., Wang, W., Sun, Y., Zhao, E., Vatan, L., Szeliga, W., et al. (2015). Epigenetic silencing of TH1-type chemokines shapes tumour immunity and immunotherapy. Nature *527*, 249–253.

Peragine, N., Torelli, G.F., Mariglia, P., Pauselli, S., Vitale, A., Guarini, A., and Foà, R. (2015). Immunophenotypic and functional characterization of ex vivo expanded natural killer cells for clinical use in acute lymphoblastic leukemia patients. Cancer Immunol. Immunother. *64*, 201–211.

Peritt, D., Robertson, S., Gri, G., Showe, L., Aste-Amezaga, M., and Trinchieri, G. (1998). Differentiation of human NK cells into NK1 and NK2 subsets. J. Immunol. *161*, 5821–5824.

Perko, R., Kang, G., Sunkara, A., Leung, W., Thomas, P.G., and Dallas, M.H. (2015). Gamma Delta T cell Reconstitution is Associated with Fewer Infections and Improved Event Free Survival following Hematopoietic Stem Cell Transplantation for Pediatric Leukemia Gamma Delta T Cells after HSCT. Biol Blood Marrow Transpl. *21*, 130–136.

Peruzzi, M., Wagtmann, N., and Long, E.O. (1996). A p70 killer cell inhibitory receptor specific for several HLA-B allotypes discriminates among peptides bound to HLA-B*2705. J. Exp. Med. *184*, 1585–1590.

Phillips, J.H., and Lanier, L.L. (1986). Dissection of the lymphokine-activated killer phenomenon Relative Contribution of Peripheral Blood Natural Killer Cells and T Lymphocytes to Cytolysis. J. Exp. Med. *164*, 814–825.

Piali, L., Fichtel, A., Terpe, H.-J., Imhof, B.A., and Gisler, R.H. (1995). Endothelial Vascular Cell Adhesion Molecule Expression Is Suppressed by Melanoma and Carcinoma. J. Exp. Med. *181*, 811–816.

Platonova, S., Cherfils-Vicini, J., Damotte, D., Crozet, L., Vieillard, V., Validire, P., André, P., Dieu-Nosjean, M.C., Alifano, M., Régnard, J.F., et al. (2011). Profound coordinated alterations of intratumoral NK cell phenotype and function in lung carcinoma. Cancer Res. *71*, 5412–5422.

Poggi, A., Venturino, C., Catellani, S., Clavio, M., Miglino, M., Gobbi, M., Steinle, A., Ghia, P., Stella,

S., Caligaris-Cappio, F., et al. (2004). Vδ1 T Lymphocytes from B-CLL Patients Recognize ULBP3 Expressed on Leukemic B Cells and Up-Regulated byTrans-Retinoic Acid. Cancer Res. *64*, 9172–9179.

Poznanski, S.M., Nham, T., Chew, M. V., Lee, A.J., Hammill, J.A., Fan, I.Y., Butcher, M., Bramson, J.L., Lee, D.A., Hirte, H.W., et al. (2018). Expanded CD56superbrightCD16+ NK cells from ovarian cancer patients are cytotoxic against autologous tumor in a patient-derived xenograft murine model. Cancer Immunol. Res. *6*, 1174–1185.

Pressey, J.G., Adams, J., Harkins, L., Kelly, D., You, Z., and Lamb, L.S. (2016). In vivo expansion and activation of γδ T cells as immunotherapy for refractory neuroblastoma A phase 1 study. Med. (Baltimore) *95*, e4909.

Lo Presti, E., Toia, F., Oieni, S., Buccheri, S., Turdo, A., Mangiapane, L.R., Campisi, G., Caputo, V., Todaro, M., Stassi, G., et al. (2017). Squamous cell tumors recruit γδ T cells producing either IL17 or IFNγ depending on the tumor stage. Cancer Immunol. Res. *5*, 397–407.

Qin, G., Mao, H., Zheng, J., Sia, S.F., Liu, Y., Chan, P., Lam, K., Peiris, J.S.M., Lau, Y., and Tu, W. (2009). Phosphoantigen-Expanded Human γδ T Cells Display Potent Cytotoxicity against Monocyte-Derived Macrophages Infected with Human and Avian Influenza Viruses. J. Infect. Dis. *200*, 858–865.

R, C.T. (2020). R: A language and environment for statistical computing.

Rabinowich, H., Sedlmayr, P., Herberman, R.B., and Whiteside, T.L. (1991). Increased proliferation, lytic activity, and purity of human natural killer cells cocultured with mitogen-activated feeder cells. Cell. Immunol. *135*, 454–470.

Rei, M., Gonçalves-Sousa, N., Lança, T., Thompson, R.G., Mensurado, S., Balkwill, F.R., Kulbe, H., Pennington, D.J., and Silva-Santos, B. (2014). Murine CD27(-) Vγ6(+) γδ T cells producing IL-17A promote ovarian cancer growth via mobilization of protumor small peritoneal macrophages. Proc. Natl. Acad. Sci. U. S. A. *111*, E3562-3570.

Ribot, J.C., Ribeiro, S.T., Correia, D. V., Sousa, A.E., and Silva-Santos, B. (2014). Human γδ Thymocytes Are Functionally Immature and Differentiate into Cytotoxic Type 1 Effector T Cells upon IL-2/IL-15 Signaling. J. Immunol. *192*, 2237–2243.

Robertson, M.J., Cameron, C., Lazo, S., Cochran, K.J., Voss, S.D., and Ritz, J. (1997). Costimulation of human natural killer cell proliferation: Role of accessory cytokines and cell contact-dependent signals. Nat. Immun. *15*, 213–226.

Rohaan, M.W., Wilgenhof, S., and Haanen, J.B.A.G. (2019). Adoptive cellular therapies: the current landscape. Virchows Arch. *474*, 449–461.

Romee, R., Schneider, S.E., Leong, J.W., Chase, J.M., Keppel, C.R., Sullivan, R.P., Cooper, M.A., and Fehniger, T.A. (2012). Cytokine activation induces human memory-like NK cells. Blood *120*, 4751–4760.

Romee, R., Rosario, M., Berrien-elliott, M.M., Wagner, J.A., Jewell, B.A., Schappe, T., Leong, J.W., Abdel-latif, S., Schneider, S.E., Willey, S., et al. (2016). Cytokine-induced memory-like natural killer cells exhibit enhanced responses against myeloid leukemia. Sci Transl Med. *8*, 357ra123.

Rong, L., Li, K., Li, R., Liu, H.M., Sun, R., and Liu, X.Y. (2016). Analysis of tumor-infiltrating gamma delta T cells in rectal cancer. World J. Gastroenterol. *22*, 3573–3580.

Rosenberg, S.A., and Restifo, N.P. (2015). Adoptive cell transfer as personalized immunotherapy for

human cancer. Science. *348*, 62–68.

Rosenberg, S.A., Lotze, M.T., Muul, L.M., Leitman, S., Chang, A.E., Ettinghausen, S.E., Matory, Y.L., Skibber, J.M., Shiloni, E., Vetto, J.T., et al. (1985). Observations on the Systemic Administration of Autologous Lymphokine-Activated Killer Cells and Recombinant Interleukin-2 to Patients with Metastatic Cancer. N. Engl. J. Med. *313*, 1485–1492.

Rosenstein, M., Yron, I., Kaufmann, Y., and Rosenberg, S.A. (1984). Lymphokine-activated Killer Cells: Lysis of Fresh Syngeneic Natural Killer-resistant Murine Tumor Cells by Lymphocytes Cultured in Interleukin 2. Cancer Res. *44*, 1946–1953.

Rubnitz, J.E., Inaba, H., Ribeiro, R.C., Pounds, S., Rooney, B., Bell, T., Pui, C.H., and Leung, W. (2010). NKAML: A pilot study to determine the safety and feasibility of haploidentical natural killer cell transplantation in childhood acute myeloid leukemia. J. Clin. Oncol. *28*, 955–959.

Ruggeri, L., Capanni, M., Urbani, E., Perruccio, K., Shlomchik, W.D., Tosti, A., Posati, S., Rogaia, D., Frassoni, F., Aversa, F., et al. (2002). Effectiveness of Donor Natural Killer Cell Alloreactivity in Mismatched Hematopoietic Transplants. Science. *295*, 2097–2100.

Sabry, M., and Lowdell, M.W. (2013). Tumor-primed NK cells: Waiting for the green light. Front. Immunol. *4*, 408.

Sabry, M., Zubiak, A., Hood, S.P., Simmonds, P., Arellano-Ballestero, H., Cournoyer, E., Mashar, M., Graham Pockley, A., and Lowdell, M.W. (2019). Tumor- And cytokine-primed human natural killer cells exhibit distinct phenotypic and transcriptional signatures. PLoS One *14*, e0218674.

Sakamoto, M., Nakajima, J., Murakawa, T., Fukami, T., Yoshida, Y., Murayama, T., Takamoto, S., Matsushita, H., and Kakimi, K. (2011). Adoptive immunotherapy for advanced non-small cell lung cancer using zoledronate-expanded γδ T cells: A Phase I clinical study. J. Immunother. *34*, 202–211.

Sakamoto, N., Ishikawa, T., Kokura, S., Okayama, T., Oka, K., Ideno, M., Sakai, F., Kato, A., Tanabe, M., Enoki, T., et al. (2015). Phase I clinical trial of autologous NK cell therapy using novel expansion method in patients with advanced digestive cancer. J. Transl. Med. *13*, 277.

Salot, S., Laplace, C., Saïagh, S., Bercegeay, S., Tenaud, I., Cassidanius, A., Romagne, F., Dreno, B., and Tiollier, J. (2007). Large scale expansion of γ9δ2 T lymphocytes: Innacell γδ™ cell therapy product. J. Immunol. Methods *326*, 63–75.

Schilbach, K., Frommer, K., Meier, S., Handgretinger, R., and Eyrich, M. (2008). Immune response of human propagated ɣδ-T-cells to neuroblastoma recommend the Vδ1+ subset for ɣδ-T-cell-based immunotherapy. J. Immunother. *31*, 896–905.

Schild, H., Mavaddat, N., Litzenberger, C., Ehrich, E.W., Davis, M.M., Bluestone, J.A., Matis, L., Draper, R.K., and Chien, Y. hsiu (1994). The nature of major histocompatibility complex recognition by γδ T cells. Cell *76*, 29–37.

Schleypen, J.S., Von Geldern, M., Weiß, E.H., Kotzias, N., Rohrmann, K., Schendel, D.J., Falk, C.S., and Pohla, H. (2003). Renal cell carcinoma-infiltrating natural killer cells express differential repertoires of activating and inhibitory receptors and are inhibited by specific HLA class I allotypes. Int. J. Cancer *106*, 905–912.

Schleypen, J.S., Baur, N., Kammerer, R., Nelson, P.J., Rohrmann, K., Gröne, E.F., Hohenfellner, M., Haferkamp, A., Pohla, H., Schendel, D.J., et al. (2006). Cytotoxic markers and frequency predict

functional capacity of natural killer cells infiltrating renal cell carcinoma. Clin. Cancer Res. *12*, 718–725.

Sciammas, R., Johnson, R.M., Sperling, A.I., Brady, W., Linsley, P.S., Spear, P.G., Fitch, F.W., and Bluestone, J.A. (1994). Unique antigen recognition by a herpesvirus-specific TCR-gamma delta cell. J. Immunol. *152*, 5392–5397.

Scotet, E., Martinez, L.O., Grant, E., Barbaras, R., Jenö, P., Guiraud, M., Monsarrat, B., Saulquin, X., Maillet, S., Estève, J.P., et al. (2005). Tumor recognition following Vγ9Vδ2 T cell receptor interactions with a surface F1-ATPase-related structure and apolipoprotein A-I. Immunity *22*, 71–80.

Selin, L.K., Stewart, S., Shen, C., Mao, H.Q., and Wilkins, J.A. (1992). Reactivity of γδ T Cells Induced by the Tumour Cell Line RPMI 8226: Functional Heterogeneity of Clonal Populations and Role of GroEL Heat Shock Proteins. Scand. J. Immunol. *36*, 107–117.

Shibuya, A., Campbell, D., Hannum, C., Yssel, H., Franz-Bacon, K., McClanashan, T., Kitamura, T., Nicholl, J., Sutherland, G.R., Lanier, L.L., et al. (1996). DNAM-1, a novel adhesion molecule involved in the cytolytic function of T lymphocytes. Immunity *4*, 573–581.

Siegers, G.M., Dhamko, H., Wang, X.H., Mathieson, A.M., Kosaka, Y., Felizardo, T.C., Medin, J.A., Tohda, S., Schueler, J., Fisch, P., et al. (2011). Human Vδ1 γδ T cells expanded from peripheral blood exhibit specific cytotoxicity against B-cell chronic lymphocytic leukemia-derived cells. Cytotherapy *13*, 753–764.

Siegers, G.M., Ribot, E.J., Keating, A., and Foster, P.J. (2013). Extensive expansion of primary human gamma delta T cells generates cytotoxic effector memory cells that can be labeled with Feraheme for cellular MRI. Cancer Immunol. Immunother. *62*, 571–583.

Siegler, U., Meyer-Monard, S., Jörger, S., Stern, M., Tichelli, A., Gratwohl, A., Wodnar-Filipowicz, A., and Kalberer, C.P. (2010). Good manufacturing practice-compliant cell sorting and large-scale expansion of single KIR-positive alloreactive human natural killer cells for multiple infusions to leukemia patients. Cytotherapy *12*, 750–763.

Sivori, S., Vitale, M., Morelli, L., Sanseverino, L., Augugliaro, R., Bottino, C., Moretta, L., and Moretta, A. (1997). P46, a Novel Natural Killer Cell-Specific Surface Molecule That Mediates Cell Activation. J. Exp. Med. *186*, 1129–1136.

Sivori, S., Pende, D., Bottino, C., Marcenaro, E., Pessino, A., Biassoni, R., Moretta, L., and Moretta, A. (1999). NKp46 is the major triggering receptor involved in the natural cytotoxicity of fresh or cultured human NK cells. Correlation between surface density of NKp46 and natural cytotoxicity against autologous, allogeneic or xenogeneic target cells. Eur. J. Immunol. *29*, 1656–1666.

Sivori, S., Parolini, S., Falco, M., Marcenaro, E., Biassoni, R., Bottino, C., Moretta, L., and Moretta, A. (2000). 2B4 functions as a co-receptor in human NK cell activation. Eur. J. Immunol. *30*, 787–793.

Spanholtz, J., Preijers, F., Tordoir, M., Trilsbeek, C., Paardekooper, J., de Witte, T., Schaap, N., and Dolstra, H. (2011). Clinical-grade generation of active NK cells from cord blood hematopoietic progenitor cells for immunotherapy using a closed-system culture process. PLoS One *6*, e20740.

Spranger, S., Bao, R., and Gajewski, T.F. (2015). Melanoma-intrinsic β-catenin signalling prevents anti-tumour immunity. Nature *523*, 231–235.

Steinle, A., Li, P., Morris, D.L., Groh, V., Lanier, L.L., Strong, R.K., and Spies, T. (2001). Interactions of human NKG2D with its ligands MICA, MICB, and homologs of the mouse RAE-1 protein family.

Immunogenetics *53*, 279–287.

Straetemans, T., Kierkels, G.J.J., Doorn, R., Jansen, K., Heijhuurs, S., dos Santos, J.M., van Muyden, A.D.D., Vie, H., Clemenceau, B., Raymakers, R., et al. (2018). GMP-grade manufacturing of T cells engineered to express a defined γδTCR. Front. Immunol. *9*, 1062.

Sugie, T., Murata-Hirai, K., Iwasaki, M., Morita, C.T., Li, W., Okamura, H., Minato, N., Toi, M., and Tanaka, Y. (2013). Zoledronic acid-induced expansion of γδ T cells from early-stage breast cancer patients: Effect of IL-18 on helper NK cells. Cancer Immunol. Immunother. *62*, 677–687.

Sun, J.C., Beilke, J.N., and Lanier, L.L. (2009). Adaptive immune features of natural killer cells. Nature *457*, 557–561.

Tada, T., Ohzeki, S., Utsumi, K., Takiuchi, H., Muramatsu, M., Li, X.F., Shimizu, J., Fujiwara, H., and Hamaoka, T. (1991). Transforming growth factor-beta-induced inhibition of T cell function. Susceptibility difference in T cells of various phenotypes and functions and its relevance to immunosuppression in the tumor-bearing state. J. Immunol. *146*, 1077–1082.

Takahashi, E., Kuranaga, N., Satoh, K., Habu, Y., Shinomiya, N., Asano, T., Seki, S., and Hayakawa, M. (2007). Induction of CD16+ CD56bright NK cells with antitumour cytotoxicity not only from CD16-CD56bright NK cells but also from CD16- CD56dim NK cells. Scand. J. Immunol. *65*, 126–138.

Tallerico, R., Todaro, M., Di Franco, S., Maccalli, C., Garofalo, C., Sottile, R., Palmieri, C., Tirinato, L., Pangigadde, P.N., La Rocca, R., et al. (2013). Human NK Cells Selective Targeting of Colon Cancer–Initiating Cells: A Role for Natural Cytotoxicity Receptors and MHC Class I Molecules. J. Immunol. *190*, 2381–2390.

Tanaka, Y., Morita, C.T., Tanaka, Y., Nieves, E., Brenner, M.B., and Bloom, B.R. (1995). Natural and synthetic non-peptide antigens recognized by human γδ T cells. Nature *375*, 155–158.

Tang, X., Yang, L., Li, Z., Nalin, A.P., Dai, H., Xu, T., Yin, J., You, F., Zhu, M., Shen, W., et al. (2018). First-in-man clinical trial of CAR NK-92 cells: safety test of CD33-CAR NK-92 cells in patients with relapsed and refractory acute myeloid leukemia. Am. J. Cancer Res. *8*, 1083–1089.

Tauriello, D.V.F., Palomo-Ponce, S., Stork, D., Berenguer-Llergo, A., Badia-Ramentol, J., Iglesias, M., Sevillano, M., Ibiza, S., Cañellas, A., Hernando-Momblona, X., et al. (2018). TGFβ drives immune evasion in genetically reconstituted colon cancer metastasis. Nature *554*, 538–543.

Tawfik, D., Groth, C., Gundlach, J.P., Peipp, M., Kabelitz, D., Becker, T., Oberg, H.H., Trauzold, A., and Wesch, D. (2019). TRAIL-receptor 4 modulates γδ T cell-cytotoxicity toward cancer cells. Front. Immunol. *10*, 2044.

Textor, S., Bossler, F., Henrich, K.O., Gartlgruber, M., Pollmann, J., Fiegler, N., Arnold, A., Westermann, F., Waldburger, N., Breuhahn, K., et al. (2016). The proto-oncogene Myc drives expression of the NK cell-activating NKp30 ligand B7-H6 in tumor cells. Oncoimmunology *5*, e1116674.

Thomas, M.L., Badwe, R.A., Deshpande, R.K., Samant, U.C., and Chiplunkar, S. V. (2001). Role of adhesion molecules in recruitment of Vδ1 T cells from the peripheral blood to the tumor tissue of esophageal cancer patients. Cancer Immunol. Immunother. *50*, 218–225.

Todaro, M., D'Asaro, M., Caccamo, N., Iovino, F., Francipane, M.G., Meraviglia, S., Orlando, V., La Mendola, C., Gulotta, G., Salerno, A., et al. (2009). Efficient Killing of Human Colon Cancer Stem Cells by γδ T Lymphocytes. J. Immunol. *182*, 7287–7296.

Tomasello, E., Yessaad, N., Gregoire, E., Hudspeth, K., Luci, C., Mavilio, D., Hardwigsen, J., and Vivier, E. (2012). Mapping of NKp46+ cells in healthy human lymphoid and non-lymphoid tissues. Front. Immunol. *3*, 344.

Torelli, G.F., Rozera, C., Santodonato, L., Peragine, N., D'Agostino, G., Montefiore, E., Napolitano, M.R., Monque, D.M., Carlei, D., Mariglia, P., et al. (2015). A good manufacturing practice method to ex vivo expand natural killer cells for clinical use. Blood Transfus. *13*, 464–471.

Tremblay-Mclean, A., Coenraads, S., Kiani, Z., Dupuy, F.P., and Bernard, N.F. (2019). Expression of ligands for activating natural killer cell receptors on cell lines commonly used to assess natural killer cell function. BMC Immunol. *20*, 8.

Turin, I., Delfanti, S., Ferulli, F., Brugnatelli, S., Tanzi, M., Maestri, M., Cobianchi, L., Lisini, D., Luinetti, O., Paulli, M., et al. (2018). In Vitro Killing of Colorectal Carcinoma Cells by Autologous Activated NK Cells is Boosted by Anti-Epidermal Growth Factor Receptor-induced ADCC Regardless of RAS Mutation Status. J. Immunother. *41*, 190–200.

Uldrich, A.P., Le Nours, J., Pellicci, D.G., Gherardin, N.A., Mcpherson, K.G., Lim, R.T., Patel, O., Beddoe, T., Gras, S., Rossjohn, J., et al. (2013). CD1d-lipid antigen recognition by the γδ TCR. Nat. Immunol. *14*, 1137–1145.

Upadhaya, S., Hubbard-Lucey, V.M., and Yu, J.X. (2020). Immuno-oncology drug development forges on despite COVID-19. Nat. Rev. Drug Discov. *19*, 751–752.

Viey, E., Fromont, G., Escudier, B., Morel, Y., Da Rocha, S., Chouaib, S., and Caignard, A. (2005). Phosphostim-Activated γδ T Cells Kill Autologous Metastatic Renal Cell Carcinoma. J. Immunol. *174*, 1338–1347.

Vitale, M., Bottino, C., Sivori, S., Sanseverino, L., Castriconi, R., Marcenaro, E., Augugliaro, R., Moretta, L., and Moretta, A. (1998). NKp44, a novel triggering surface molecule specifically expressed by activated natural killer cells, is involved in non-major histocompatibility complex-restricted tumor cell lysis. J. Exp. Med. *187*, 2065–2072.

Vitale, M., Falco, M., Castriconi, R., Parolini, S., Zambello, R., Semenzato, G., Biassoni, R., Bottino, C., Moretta, L., and Moretta, A. (2001). Identification of NKp80, a novel triggering molecule expressed by human NK cells. Eur. J. Immunol. *31*, 233–242.

Voron, T., Colussi, O., Marcheteau, E., Pernot, S., Nizard, M., Pointet, A.L., Latreche, S., Bergaya, S., Benhamouda, N., Tanchot, C., et al. (2015). VEGF-A modulates expression of inhibitory checkpoints on CD8+ T cells in tumors. J. Exp. Med. *212*, 139–148.

Voskens, C.J., Watanabe, R., Rollins, S., Campana, D., Hasumi, K., and Mann, D.L. (2010). *Ex-vivo* expanded human NK cells express activating receptors that mediate cytotoxicity of allogeneic and autologous cancer cell lines by direct recognition and antibody directed cellular cytotoxicity. J. Exp. Clin. Cancer Res. *29*, 134.

Wada, I., Matsushita, H., Noji, S., Mori, K., Yamashita, H., Nomura, S., Shimizu, N., Seto, Y., and Kakimi, K. (2014). Intraperitoneal injection of in vitro expanded Vγ9Vδ2 T cells together with zoledronate for the treatment of malignant ascites due to gastric cancer. Cancer Med. *3*, 362–375.

Wang, Y., Wang, C., Xiao, H., Niu, C., Wu, H., Jin, H., Yao, C., He, H., Tian, H., Han, F., et al. (2017). Adjuvant treatment combining cellular immunotherapy with chemotherapy improves the clinical outcome

of patients with stage II/III gastric cancer. Cancer Med. *6*, 45–53.

Wendel, M., Galani, I.E., Suri-Payer, E., and Cerwenka, A. (2008). Natural killer cell accumulation in tumors is dependent on IFN-γ and CXCR3 ligands. Cancer Res. *68*, 8437–8445.

West, E., Scott, K., Jennings, V., and Melcher, A. (2011). Immune activation by combination human lymphokine-activated killer and dendritic cell therapy. Br. J. Cancer *105*, 787–795.

WHO. 2018. Cancer. Retrieved 20th October 2018, from https://www.who.int/cancer/en/.

WHO. 2020. Who Report on Cancer: Setting Priorities, Investing Wisely and Providing Care for all. Retrieved 23rd November 2020, from https://www.who.int/news-room/fact-sheets/detail/cancer.

Wilhelm, M., Kunzmann, V., Eckstein, S., Reimer, P., Weissinger, F., Ruediger, T., and Tony, H.P. (2003). γδ T cells for immune therapy of patients with lymphoid malignancies. Blood *102*, 200–206.

Wilhelm, M., Smetak, M., Schaefer-Eckart, K., Kimmel, B., Birkmann, J., Einsele, H., and Kunzmann, V. (2014). Successful adoptive transfer and in vivo expansion of haploidentical γδ T cells. J. Transl. Med. *12*, 45.

Willcox, C.R., Pitard, V., Netzer, S., Couzi, L., Salim, M., Silberzahn, T., Moreau, J.F., Hayday, A.C., Willcox, B.E., and Déchanet-Merville, J. (2012). Cytomegalovirus and tumor stress surveillance by binding of a human γδ T cell antigen receptor to endothelial protein C receptor. Nat. Immunol. *13*, 872–879.

Wrobel, P., Shojaei, H., Schittek, B., Gieseler, F., Wollenberg, B., Kalthoff, H., Kabelitz, D., and Wesch, D. (2007). Lysis of a broad range of epithelial tumour cells by human γδ T cells: Involvement of NKG2D ligands and T-cell receptor- versus NKG2D-dependent recognition. Scand. J. Immunol. *66*, 320–328.

Wu, J., Groh, V., and Spies, T. (2002). T Cell Antigen Receptor Engagement and Specificity in the Recognition of Stress-Inducible MHC Class I-Related Chains by Human Epithelial γδ T Cells. J. Immunol. *169*, 1236–1240.

Wu, P., Wu, D., Ni, C., Ye, J., Chen, W., Hu, G., Wang, Z., Wang, C., Zhang, Z., Xia, W., et al. (2014). γδT17 cells promote the accumulation and expansion of myeloid-derived suppressor cells in human colorectal cancer. Immunity *40*, 785–800.

Wu, Y., Kyle-cezar, F., Woolf, R.T., Naceur-lombardelli, C., Owen, J., Biswas, D., Lorenc, A., Vantourout, P., Gazinska, P., Grigoriadis, A., et al. (2019). An innate-like Vδ1+ γδ T cell compartment in the human breast is asociated with remission in triple-negative breast cancer. Sci. Transl. Med. *11*, eaax9364.

Xia, H.L., Li, C.J., Hou, X.F., Zhang, H., Wu, Z.H., and Wang, J. (2017). Interferon-γ affects leukemia cell apoptosis through regulating Fas/FasL signaling pathway. Eur. Rev. Med. Pharmacol. Sci. *21*, 2244–2248.

Xu, Y., Xiang, Z., Alnaggar, M., Kouakanou, L., Li, J., He, J., Yang, J., Hu, Y., Chen, Y., Lin, L., et al. (2020). Allogeneic Vγ9Vδ2 T-cell immunotherapy exhibits promising clinical safety and prolongs the survival of patients with late-stage lung or liver cancer. Cell. Mol. Immunol. *18*, 427–439.

Yamaguchi, T., Fujimiya, Y., Suzuki, Y., Katakura, R., and Ebina, T. (1997). A simple method for the propagation and purification of γδT cells from the peripheral blood of glioblastoma patients using solid-phase anti-CD3 antibody and soluble IL-2. J. Immunol. Methods *205*, 19–28.

Yamasaki, A., Onishi, H., Morisaki, T., and Katano, M. (2011). Induction of cytotoxic T lymphocytes by CEA peptide-pulsed γδ T-cells isolated from patients with advanced cancer. Anticancer Res. *31*, 2419–2424.

Ye, J., Ma, C., Wang, F., Hsueh, E.C., Toth, K., Huang, Y., Mo, W., Liu, S., Han, B., Varvares, M.A., et al. (2013). Specific recruitment of γδ regulatory T cells in human breast cancer. Cancer Res. *73*, 6137–6148.

Yin, T., Wang, G., He, S., Liu, Q., Sun, J., and Wang, Y. (2016). Human cancer cells with stem cell-like phenotype exhibit enhanced sensitivity to the cytotoxicity of IL-2 and IL-15 activated natural killer cells. Cell. Immunol. *300*, 41–45.

Yron, I., Wood, T.A., Spiess, P.J., and Rosenberg, S.A. (1980). In vitro growth of murine T cells. V. The isolation and growth of lymphoid cells infiltrating syngeneic solid tumors. J. Immunol. *125*, 238–245.

Zappacosta, F., Borrego, F., Brooks, A.G., Parker, K.C., and Coligan, J.E. (1997). Peptides isolated from HLA-Cw*0304 confer different degrees of protection from natural killer cell-mediated lysis. Proc. Natl. Acad. Sci. U. S. A. *94*, 6313–6318.

Zhang, S., Liu, W., Hu, B., Wang, P., Lv, X., Chen, S., and Shao, Z. (2020). Prognostic Significance of Tumor-Infiltrating Natural Killer Cells in Solid Tumors: A Systematic Review and Meta-Analysis. Front. Immunol. *11*, 1242.

Zocchi, M.R., Costa, D., Venè, R., Tosetti, F., Ferrari, N., Minghelli, S., Benelli, R., Scabini, S., Romairone, E., Catellani, S., et al. (2017). Zoledronate can induce colorectal cancer microenvironment expressing BTN3A1 to stimulate effector γδ T cells with antitumor activity. Oncoimmunology *6*, e1278099.

8. Supplementary material

8.1. Supplementary methods

8.1.1. Optimization of NK cells expansion conditions

Peripheral blood samples from six healthy donors were provided by the Transfusion Medicine Department of the Champalimaud Foundation and PBMCs were isolated after separating whole venous blood over a Ficoll-Hypaque gradient (GE Healthcare, Uppsala, Sweden). Cells were then washed twice with PBS (Corning Life Sciences), resuspended in culture medium and incubated overnight in a T-flask 25 cm^2 (Thermo Fisher Scientific) at 37ºC in a humidified atmosphere containing 5% CO_2. Next day, the cell populations of interest were isolated from PBMCs through FACS (**section 8.1.2**) and then cultured under different conditions.

PBMCs from the first three healthy donors received (donor G, donor H and donor I) were used to evaluate the best conditions to expand NK cells, namely: a) the cytokine combination, b) the use or not of feeder cell stimulation and c) the time point to isolate NK cells (summarized in **Figure 9.1**). Four different cytokine combinations were tested: a) IL-2 (1000 IU/mL, Miltenyi Biotec), IL-15 (180 IU/mL, Miltenyi Biotec) and IL-21 (1 IU/mL, Miltenyi Biotec); b) IL-2 alone (1000 IU/mL, Miltenyi Biotec); c) IL-2 (1000 IU/mL, Miltenyi Biotec) and IL-15 (180 IU/mL, Miltenyi Biotec) and d) IL-15 alone (180 IU/mL, Miltenyi Biotec). To investigate the effect of feeder cell stimulation in NK cell expansion, NK cells were co-cultured with the MHC-I negative leukemic cell line K-562. K-562 cells were irradiated at 55 Gy before being co-cultured with NK cells or the $CD3^-$ population isolated from the PBMCs. The immune cell:target cell ratio used was 1:10 and the stimulation was performed every 10 days. NK cells were isolated through FACS (**section 8.1.2**) in three different time points: a) at day 0, b) after expanding the $CD3^-$ population for 7 days or c) following the expansion of the $CD3^-$ population for 14 days. NK cells obtained following the expansion of the $CD3^-$ population were maintained in culture after their positive sorting for at least more 7 days, while NK cells obtained at day 0 were expanded at least for 14 days. Independently of the condition being tested, at day 0, cells were cultured in a 24-well plate (Corning Life Sciences) at a cell density of $0.20x10^6$–$0.25x10^6$ cells/well. After the second sorting, cells were maintained in the same culture conditions, however if the cell number obtained was lower than $0.10x10^6$, cells were cultured in a 96-well plate (Corning Life Sciences). Total cell number, cell viability and NK cells' frequency were assessed at different time points. Results are shown as the mean of the three donors. Total cell number and NK cells' frequency-fold increases were calculated by dividing the values obtained at day 14 by the ones obtained at day 0.

PBMCs from the other three healthy donors (donor J, donor K and donor L) were used to determine: a) the optimal cell concentration to start NK cell expansion, b) the NK cell subsets expanded and c) the effector capacity of the expanded cells. NK cells were obtained from PBMCs through a positive sorting (**section 8.1.2**) at day 0 and cultured in a 24-well plate (Corning Life Sciences) at three different cell densities: a) $0.5x10^6$, b) $1x10^6$ and c) $2x10^6$ cells/mL/well. Cells were expanded in culture medium supplemented with IL-2 (1000 IU/mL, Miltenyi Biotec) and IL-15 (180 IU/mL, Miltenyi Biotec) and stimulated in the beginning of the culture with K-562 cells irradiated at 55 Gy (at a 10:1 immune cell:target cell ratio). Total cell number and cell viability were determined at different time points and

results are shown as the mean of the three donors. The frequency of NK cells and respective subsets were evaluated by flow cytometry at the beginning and end of the expansion. After a 14-day culture, the effector potential of the expanded cells was evaluated in a degranulation assay (**section 2.8.1**) and/or an IFN-γ production assay (**section 2.8.2**). In both assays, different effector cell:target cell ratios were assessed, when the final number of NK cells was sufficient.

8.1.2. FACS to isolate NK cells

Three different time points to isolate NK cells by FACS were tested: a) at day 0, b) after expanding the $CD3^-$ population for 7 days and c) following the expansion of the $CD3^-$ population for 14 days. For strategy b) and c), PBMCs at day 0 were stained with the LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (Thermo Fisher Scientific) and the anti-human CD3 PE-Cy™7 (clone UCHT1, BD Biosciences), and the $CD3^-$ population was isolated through a negative sorting. To isolate NK cells directly from PBMCs at day 0 - strategy a) - or after the expansion of the $CD3^-$ population - strategies b) and c) –, cells were stained with the LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (Thermo Fisher Scientific), the anti-human CD3 PE-Cy™7 (clone UCHT1, BD Biosciences), the anti-human CD56 PE (clone NCAM16.2, BD Biosciences) and the anti-human CD16 BV785 (clone B73.1, BioLegend), and a positive sorting of the NK cells was performed. Briefly, cells were washed with PBS (Corning Life Sciences) and incubated for 20 min with a master mix containing the viability dye and the respective antibodies (according to each strategy). After the staining, cells were washed with FACS buffer, resuspended in 200 µL of FACS buffer and sorted by FACS (FACSAria™ Fusion, BD Biosciences). The gating strategy used to sort NK cells is presented in **Figure 9.2 A in Supplementary data**.

8.2. Supplementary figures

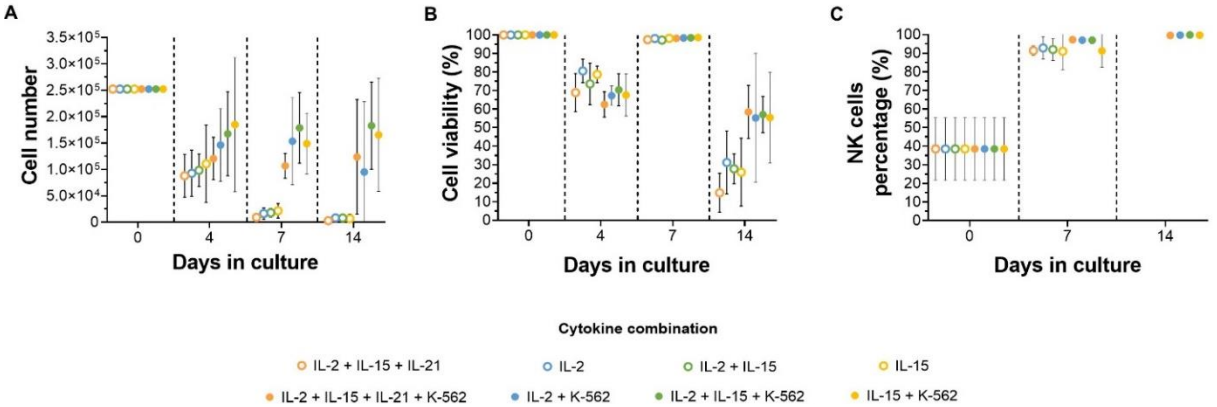


Figure 8.1 – Expansion protocols using K-562 cell stimulation allows the *in vitro* growth of NK cells. Four different cytokine combinations (IL-2+IL-15+IL-21, IL-2, IL-2+IL-15 and IL-15) in the presence or absence of K-562 cell stimulation were used to determine the best protocol to expand NK cells *in vitro*. PBMCs from three healthy donors (G, H and I) were used to obtain the $CD3^-$ population, which was cultured with the different expansion protocols and after 7 days, NK cells were positively sorted and cultured for more 7 days in the same culture conditions. The total cell number **(A)**, cell viability **(B)** and NK cells' frequency **(C)** were assessed at different time points. Graphs represent the mean of the three donors ± standard deviation.

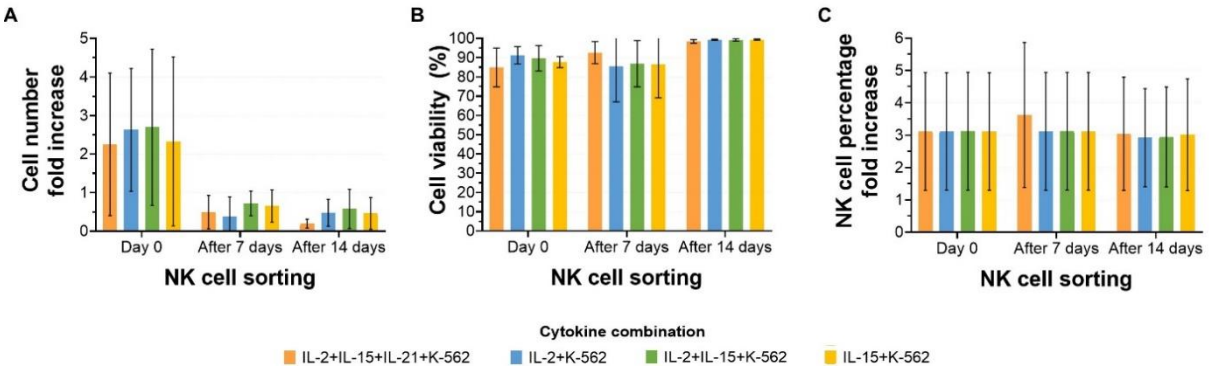


Figure 8.2 – The sorting of NK cells before *in vitro* culture allows to yield a higher total cell number-fold increase. PBMCs of three healthy donors (G, H and I) were used to isolate NK cells in three different time points. In the first strategy, NK cells were positively sorted from the PBMCs at day 0 and expanded for 14 days. The second strategy consisted in the isolation of the $CD3^-$ population from the PBMCs at day 0 through a negative sorting and its subsequent culture for 7 days, after which NK cells were isolated through a positive sorting and further cultured for more 7 days. The third strategy is similar to the second, however, the $CD3^-$ population was maintained in culture for 14 days and NK cells were isolated through a positive sorting at day 14. Independently of the strategy, cells were stimulated with irradiated K-562 cells (at a 1:10 effector cell:target cell ratio) and cultured with different cytokine combinations, namely IL-2+IL-15+IL-21, IL-2 alone, IL-2+IL-15 and IL-15 alone. The total cell number, cell viability and NK cells' frequency were evaluated at the beginning (day 0) and end of the culture (day 14). Graphs show the total cell number-fold increase **(A)**, cell viability at the end of the expansion **(B)** and NK cells' frequency-fold increase **(C)**. Graphs represent the mean of the three donors ± standard deviation.

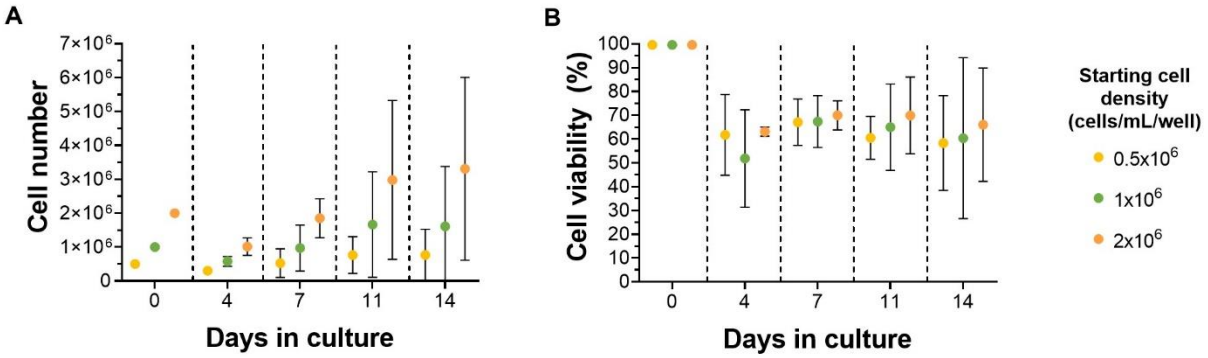


Figure 8.3 – *In vitro* expansion of NK cells is not affected by the initial cell density. NK cells were isolated from the PBMCs of three healthy donors (J, K and L) through a positive sorting and cultured at different cell densities ($0.5x10^6$, $1x10^6$ and $2x10^6$ cells/mL/well) with IL-2+IL-15 and stimulated with irradiated K-562 cells (at a 10:1 effector cell:target cell ratio). During the 14-day expansion, the total cell number **(A)** and cell viability **(B)** were assessed at different time points. Graphs represent the mean of the three donors ± standard deviation.

9. Appendix

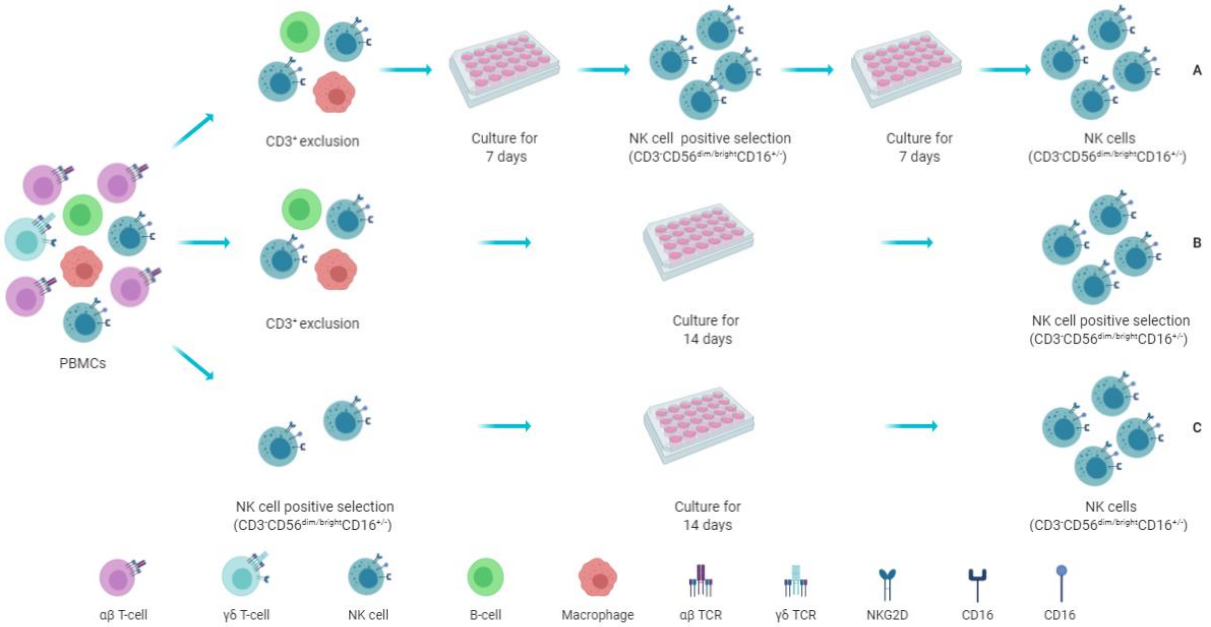
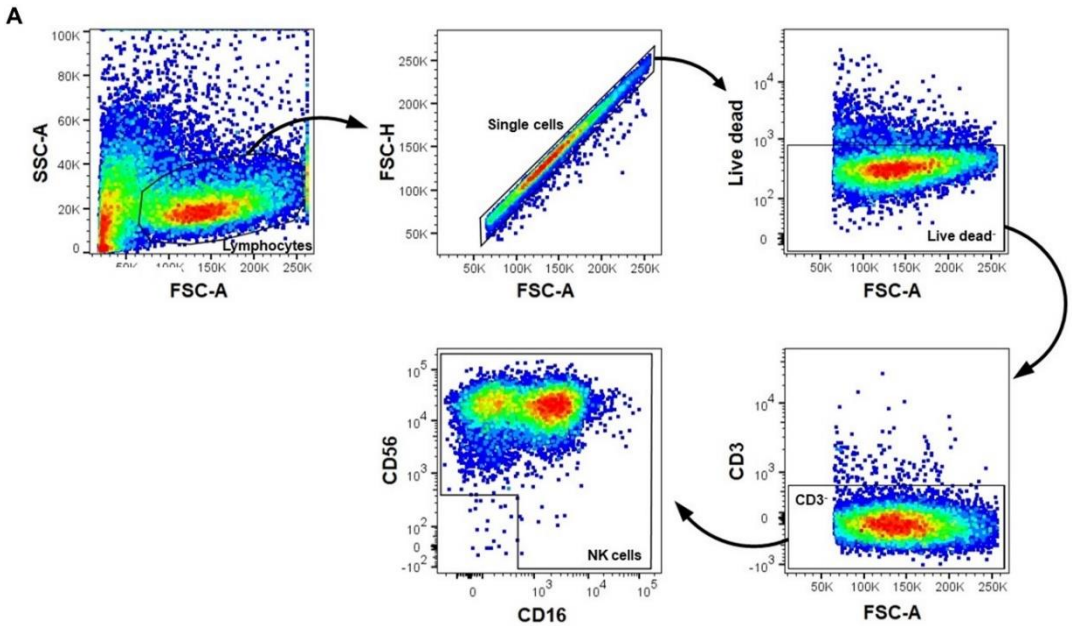


Figure 9.1– NK cell expansion conditions. PBMCs of three healthy donors (G, H and I) were used to evaluate the effect of different time points to isolate NK cells on the total cell number, cell viability and NK cells' frequency. NK cells were isolated from the PBMCs of the three healthy donors in three different time points: after a 7-day expansion of the $CD3^-$ population **(A)**, after a 14-day expansion of the $CD3^-$ population **(B)** and at day 0 **(C)**. The first isolation strategy consisted in the isolation of the $CD3^-$ population from the PBMCs at day 0 through a negative sorting and its subsequent culture for 7 days, after which NK cells were isolated through a positive sorting and further cultured for more 7 days **(A)**. The second isolation strategy is similar to the first one, however, the $CD3^-$ population was maintained in culture for 14 days and NK cells were isolated through a positive sorting at day 14 **(B)**. In the third NK cell istolation strategy, NK cells were positively sorted from the PBMCs at day 0 and expanded for 14 days **(C)**. Independently of the strategy, cells were stimulated with 55 Gy-irradiated K-562 cells (at a 1:10 effector cell:target cell ratio) and cultured with different cytokine combinations, namely IL-2+IL-15+IL-21, IL-2 alone, IL-2+IL-15 and IL-15 alone. Illustration created with BioRender.com.



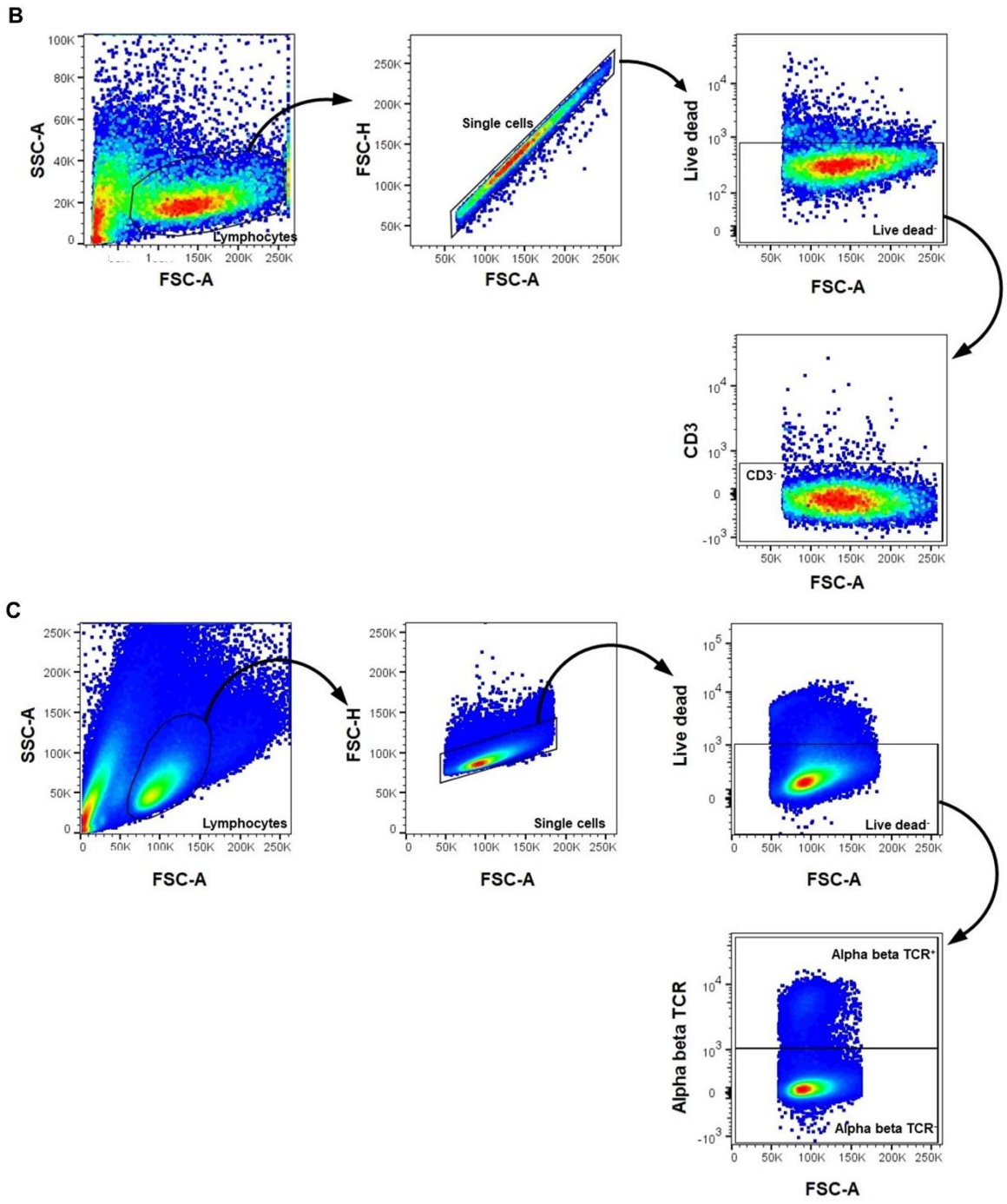


Figure 9.2 – Gating strategies to sort NK cells and γδ T-cells by FACS. Gating strategy to isolate NK cells from PBMCs of healthy donors, through a positive sorting, to determine the best expansion protocol to grow NK cells *in vitro* **(A)**. Gating strategy to isolate NK cells expanded from the tumour tissue of patients with cancer through a negative sorting **(B)**. Gating strategy to isolate γδ T-cells expanded from the tumour tissue of patients with cancer, through a negative sorting **(C)**. SSC-A: Side scatter area; FSC-A: Forward scatter area; FSC-H: Forward scatter height.

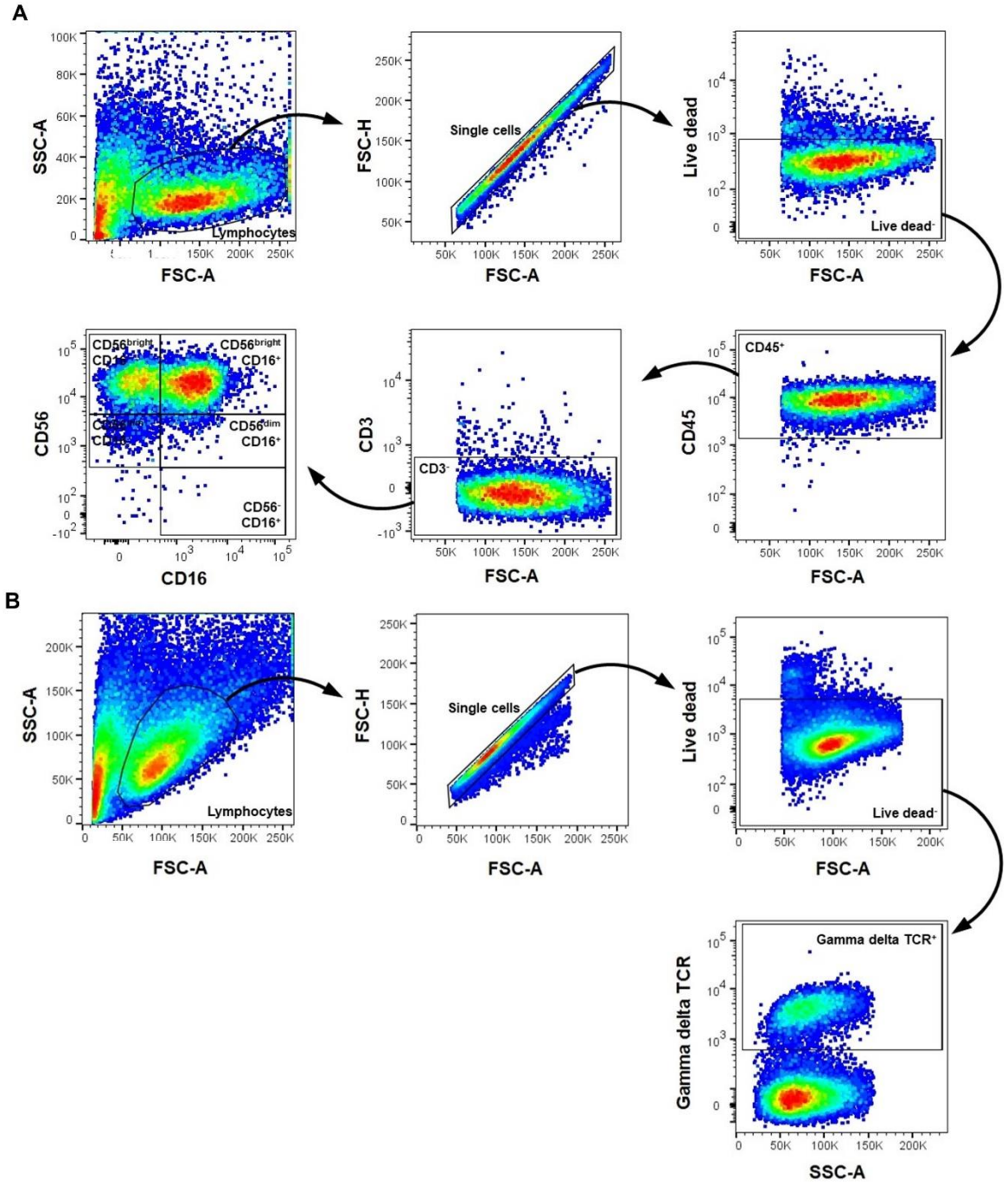


Figure 9.3 - Gating strategies to analyse NK cells (A) and γδ T-cell percentages (B) during their *in vitro* expansion.

Table 9.1 - Completed clinical trials using γδ T-cell therapies for cancer treatment.

Cancer type	Phase	Year	Treatment	Enrolled patients	Results	Clinical trial ID or reference
NSCLC	I/II	2019	Autologous γδ T-cells, DC-CIK or combination	40	N/A	NCT02425748
Advanced pancreatic cancer	I/II	2019	IRE surgery as monotherapy or combined with allogeneic γδ T-cells	60	- 132 patients with late-stage cancer, including lung, liver, pancreatic, breast or other types of tumours. - Only 18 patients with cancer (8 with liver cancer and 10 with lung cancer) received more than 5 infusions and were followed. - Therapy prolonged survival in 7 of the 8 patients with liver cancer and in 9 of the 10 patients with lung cancer to at least 10 months. - 3 liver cancer and 2 lung cancer patients were alive between 30 and 35 months after ACT. - No significant adverse effects. (Xu *et al*., 2020)	NCT03180437
Breast cancer	I/II	2019	Cryosurgery/IRE surgery/surgery, autologous γδ T-cells or combination	100		NCT03183206
Liver cancer	I/II	2019	Cryosurgery/IRE surgery, autologous γδ T-cells or combination	30		NCT03183219
Lung cancer	I/II	2019	Cryosurgery/IRE surgery, autologous γδ T-cells or combination	30		NCT03183232
Melanoma, NSCLC, RCC	II	2018	Autologous γδ T-cells	8	N/A	NCT02459067
Pancreatic cancer, invasive intraductal papillary-mucinous carcinoma (IPMC)	I	2017	Gemcitabine as monotherapy or combined with autologous γδ T-cells	28/48	- No severe adverse effects were obviously related to the γδ T-cell therapy. - No significant differences in recurrence-free survival or overall survival were detected between gemcitabine as monotherapy or combined with autologous γδ T-cell therapy.	(Aoki *et al*., 2017)
Gastric cancer (stage II/III)	II	2017	Chemotherapy as monotherapy or combined with autologous NK, CIK and γδ T-cells	169	- The combination of chemotherapy with cellular immunotherapy was well-tolerated. - The combination of chemotherapy with cellular immunotherapy significantly improved the clinical outcome (3-year disease-free survival and overall survival rates) of patients with stage II/II gastric cancer when compared with chemotherapy alone.	(Wang *et al*., 2017)
Ovarian cancer, Tubal carcinoma, Primary peritoneal cancer	I/II	2017	Autologous γδ T-cells and zoledronate	7	N/A	UMIN000015233

Hepatocellular Liver cancer	I/II	2016	Autologous γδ T-cells, DC-CIK or combination	40	- A case report of a patient with recurrent mediastinal lymph node metastasis after liver transplantation due to cholangiocarcinoma (stage IV) involved in this clinical trial demonstrated the safety (no adverse effects were observed) and efficacy (peritoneal lymph node metastasis was depleted) of allogeneic γδ T-cell treatment. (Alnaggar *et al*., 2019)	NCT02425735
Breast cancer	I/II	2016	Autologous γδ T-cells, DC-CIK or combination	40	N/A	NCT02418481
Gastric cancer	Pilot study	2014	Autologous $Vγ9^{+}Vδ2^{+}$ T-cells and zoledronate	7	- Adoptively transferred $Vγ9^{+}Vδ2^{+}$ T-cells recognize tumour cells and exert anti-tumour effector activity *in vivo* when they access to the tumour cells. - Therapy had acceptable tolerability. - Therapy demonstrated a clinical benefit for local control of malignant ascites caused by peritoneal dissemination of gastric cancer.	(Wada *et al*., 2014)
Advanced haematological malignancies	Pilot study	2014	Lymphodepleting chemotherapy (fludarabine and cyclophosphamide) followed by haploidentical γδ T-cell infusion, zoledronate and IL-2	4	- 3 of the 4 patients achieved complete remission, which lasted between 2 and 8 months. - No signs of GVHD.	(Wilhelm *et al*., 2014)
CRC	I	2013	Autologous Vγ9Vδ2 T-cells	6/12	- γδ T-cells maintained their effector phenotype and function *in vivo*.	(Izumi *et al*., 2013)
Advanced RCC	I/II	2011	Autologous γδ T-cells, zoledronic acid and IL-2	11	- 1 patient exhibited a complete response, 5 presented stable disease and the remaining 5 experienced disease progression. - 10 out of the 11 patients developed adverse reactions, although most symptoms reverted to normal during treatment.	(Kobayashi *et al*., 2011)

Advanced or recurrent NSCLC refractory to or intolerant of current conventional treatments	I	2011	Autologous γδ T-cells	15	- No severe adverse effects were detected. - No objective responses were observed, 6 patients had stable disease and 6 patients experienced disease progression 4 weeks after the sixth transfer of autologous γδ T-cells. - Median survival of 586 days. - Median progression-free survival of 126 days.	(Sakamoto *et al*., 2011)
Metastatic solid tumours	I	2011	Autologous Vγ9Vδ2 T-cells and zoledronate	18	- Of the 15 patients treated with autologous Vγ9Vδ2 T-cells and zoledronate, most experienced disease progression while 3 presented stable disease. - Of the 3 patients treated with autologous Vγ9Vδ2 T-cells and zoledronate combined with other therapies, 2 experienced partial remission and 1 had complete remission.	(Nicol *et al*., 2011)
Solid tumours	I	2011	Autologous γδ T-cells	25/57	- No severe toxicity was observed. - In 4 of the 5 patients treated only with autologous, 2 patients presented stable disease for 12 weeks, while the other 2 experienced disease progression. - In 10 of the 20 patients treated with combined therapy, 3 patients had a partial response, 1 presented stable disease and 6 experienced disease progression.	(Noguchi *et al*., 2011)
Recurrent NSCLC	I	2010	Autologous γδ T-cells	10	- Neither complete nor partial response was achieved in any patient. - Four weeks after six consecutive injections of autologous γδ T-cells, 3 patients presented stale disease and 5 experienced disease progression.	(Nakajima *et al*., 2010)
Multiple myeloma	Pilot study	2009	Autologous γδ T-cells	6	- No severe treatment-related adverse effects. - No clear-cut correlation between the total amount of γδ T-cells infused and clinical outcomes.	(Abe *et al*., 2009)
Hepatocellular carcinoma	I	2009	Autologous γδ T-cells	2	N/A	NCT00562666

Metastatic RCC	I	2008	Autologous $V\gamma9^{+}V\delta2^{+}$ T-cells and low-dose IL-2	10	- Therapy was well-tolerated. - - 6 patients exhibited stable disease and 4 patients experienced disease progression. - - Time to progression was 25.7 weeks.	(Bennouna *et al*., 2008)
Advanced RCC	Pilot study	2007	Autologous γδ T-cells and IL-2	7	- No severe adverse events were observed. - Therapy was well-tolerated. - Of the 5 patients showed an increase in the number of γδ T-cells in peripheral blood and a prolongation of tumour doubling time.	(Kobayashi *et al*., 2007)
WT1 positive tumours	N/A	2017	PepTivator(R) WT1-added autologous γδ T-cells	5	N/A	UMIN000015410
Advanced hepatocellular carcinoma	N/A	2016	Autologous γδ T-cells and zoledronic acid	15	N/A	UMIN000011184

Table 9.2 - Completed clinical trials for cancer treatment using stimulants for γδ T-cell expansion *in vivo*.

Cancer type	Phase	Year	Treatment	Enrolled patients	Results	Clinical trial ID or reference
Refractory neuroblastoma	I	2016	Zometa and IL-2	4/29	- Therapy is well-tolerated. - Therapy restored γδ T-cell counts to the range observed in healthy volunteers and promoted a moderate expansion of NK cells.	(Pressey *et al*., 2016)
Breast cancer	II	2014	Neoadjuvant chemotherapy as monotherapy or combined with Zometa	53	- Zometa administration demonstrated a higher breast conservation rate when compared to chemotherapy, but no significant pathologic response or effect on circulating tumour cells.	NCT01367288; (Lelièvre *et al*., 2018)
Neuroblastoma	I	2014	Zoledronic acid and IL-2	4	N/A	NCT01404702

Advanced Prostate cancer	II	2013	Gonadotropin-releasing hormone analogue, androgen ablation therapy and Zometa	44	- After one week of zoledronic acid administration, no patient showed a decreased in prostate-specific antigen. - One month after zoledronic acid administration, no patient had changes in γδ T-cell frequencies or function.	NCT00582556
RCC, malignant melanoma and AML	I/II	2012	Zoledronic acid and low-dose IL-2	21	- Therapy was well-tolerated. - Therapy promoted activation and expansion of γδ T-cells in vivo. - No objective responses were observed in patients with solid tumours. 2 partial remissions were detected in patients with acute myeloid leukaemia.	(Kunzmann *et al*., 2012)
Metastatic RCC	Pilot study	2011	Zoledronic acid and low-dose IL-2	12	- No objective clinical responses were observed, however, 2 patients experienced prolonged stable disease. - 4 out of the 9 patients that received at least one cycle of therapy exhibited a modest increase in Vγ9Vδ2 T-cell frequency at day 8 of therapy. - Repeated administration of IL-2 and zoledronic acid resulted in an in vivo decrease of the percentage of Vγ9Vδ2 T-cells. -	(Lang *et al*., 2011)
Advanced metastatic breast cancer	I	2010	Zoledronate and low-dose IL-2	10	- 2 patients exhibited stable disease and 1 experienced partial remission. -	(Meraviglia et al., 2010)
Solid tumours	I	2010	BrHPP (IPH1101) and IL-2	28	- IPH1101 combined with low-dose IL-2 demonstrated to be safe and well-tolerated. - IPH1101 combined with low-dose IL-2 induced γδ T-cell expansion. - After three cycles of treatment, 12 patients had stable disease and 16 experienced disease progression. -	(Bennouna *et al*., 2010)
Metastatic RCC	II	2008	Zoledronic acid and IL-2	12	- 5 of the 8 patients presented an anti-tumour response. - No patient presented changes in the CD3 or γδ T-cell populations. -	NCT00582790

					- None of the treatments showed appreciable toxicity.	
Metastatic hormone-refractory prostate cancer	I	2007	Zoledronate alone or in combination with low-dose IL-2	9/18	- 6 of the 9 patients treated with zoledronate combined with IL-2 exhibited favourable clinical responses: 2 patients had a partial remission and 4 presented stable disease for 14 to 16 months. - Only 2 of the 9 patients treated with zoledronate alone showed a clinical response (stable disease over 14 months and a partial remission).	(Dieli *et al*., 2007)

Table 9.3 - Suspended or not yet recruiting clinical trials using γδ T-cell therapies for cancer treatment.

Cancer type	Phase	Year	Treatment	Enrolled patients	Recruitment status	Clinical trial ID or reference
Hepatocellular carcinoma	I	2019	Autologous γδ T-cells	20	Not yet recruiting	NCT04032392
Relapsed or refractory solid tumours (CRC, triple negative breast cancer, sarcoma, nasopharyngeal carcinoma, prostate cancer, gastric cancer)	I	2019	Haploidentical NKG2DL-targeting CAR-γδ T-cells	10	Not yet recruiting	NCT04107142
AML	I	2018	Chemotherapy (fludarabine and cyclophosphamide) and allogeneic γδ T-cells	9	Suspended	NCT03790072
Leukaemia and lymphoma	I	2017	Lymphodepleting chemotherapy and allogeneic CD19 CAR-γδ T-cells	48	Unknown	NCT02656147

Table 9.4 – Ongoing clinical trials for cancer treatment using stimulants for γδ T-cell expansion *in vivo.*

Cancer type	Phase	Year	Treatment	Enrolled patients	Recruitment status	Clinical trial ID or reference
Advanced solid or hematopoietic tumours	I	2020	Humanized anti-BTN3A monoclonal antibody ICT01 as monotherapy or in combination with a checkpoint inhibitor	150	Recruiting	NCT04243499
Breast cancer	I	2016	Alendronate	6	Suspended	NCT02781805

Table 9.5 – NK cell inhibitory receptors and ligands.

	Inhibitory receptors	Inhibitory ligands
MHC-specific receptor	NKG2A/KLRD1	HLA-E
	KIR2DL1	HLA-C2
	KIR2DL2/3	HLA-C1 / HLA-B[b]
	KIR2DL4	HLA-G
	KIR2DL5	?
	KIR3DL1	MHC-I / HLA-A-Bw4 / HLA-B-Bw4
	KIR3DL2	HLA-A*03 / HLA-A*11
	ILT2/LIR-1	Different MHC-I alleles
	LAG-3	MHC-II
	KLRB1/CD161/NK1.1	MHC-I
	LLT1 LILRB1 LILRB2	MHC-I
	LFA1	HLA-I
	NKG2A	HLA-E
	TIM3	MHC-II
MHC-I non-related receptor	Siglec-7	Ganglioside DSGb5
	IRP60	α-Herpes virus Pseudorabide virus Phosphatidylserine Phosphatidylethnolamine
	Tactile	PVR
	IL1R8	IL-37
	KLRG1	E-cadherin N-cadherin R-cadherin
	Siglec-9	Sialic acid
	2B4/CD244	CD48
	LAIR1	Ep-CAM
	CD96	CD155
	CD73	Antibodies
	PD-1	PD-L1/2
	TIGIT	PVR
	NKp44	PCNA NID1
	CD39 A2AR	Adenosine
	TIM3	Galectin 9 Phosphatidylserine HMGB1 CEACAM1
	TGFβR II	TGFβ